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# Université de Paris

École doctorale Bio Sorbonne Paris Cité 562

Institut Cochin (U1016) / Équipe Signalisation de l'insuline et du glucose,  
et glucotoxicité

## **O-GlcNAcylation : how does O-GlcNAc transferase regulate liver physiology ?**

**Paula ORTEGA-PRIETO**

Thèse de doctorat de Biologie Cellulaire et Moléculaire,  
Physiologie, Physiopathologie

Dirigée par **Catherine POSTIC**

Présentée et soutenue publiquement à Institut Cochin le 10 Décembre 2020

### **Composition du Jury :**

<b>Philippe GUAL</b> DR, Université Côte d'Azur	Rapporteur & examinateur
<b>Philippe LEFEBVRE</b> DR, Institut Pasteur de Lille et Université de Lille	Rapporteur & examinateur
<b>Christèle DESBOIS-MOUTHON</b> CR-HDR, Université de Paris	Examinatrice
<b>Anne-Sophie VERCOUTTER-EDOUART</b> CR-HDR, Université de Lille	Examinatrice
<b>Carles CANTÓ</b> LECTURER, Nestlé Institute of Health Laussane	Examineur
<b>Hélène GILGENKRANTZ</b> DR, Université de Paris	Présidente du jury
<b>Catherine POSTIC</b> DR, Université de Paris	Directrice de thèse
<b>Tarik ISSAD</b> DR, Université de Paris	Membre invité du jury





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# List of congress participations

## ORAL PRESENTATIONS

**Drivers of Type 2 Diabetes: From Genes to Environment | Seoul (South Korea) | October 2018**

**Oral presentation:** Ortega-Prieto et al. : *Importance of the OGT-ChREBP-PPARalpha Axis in Hepatic Glucose Sensing*

## POSTER PRESENTATIONS

**Génomique Fonctionnelle du Foie | Rennes (France) | February 2020**

**Poster:** Ortega-Prieto et al. : *Lack of O-GlcNAc transferase in the liver triggers the development of bile duct-derived fibrosis*

**Metabolism meets epigenetics | Heidelberg (Germany) | November 2019**

**Poster:** Ortega-Prieto et al. : *PPAR $\alpha$  regulates chromatin accessibility of ChREBP target genes in response to carbohydrate intake*

**Spetses Summer Campus | Spetses (Greece) | August 2018**

**Poster:** Ortega-Prieto et al. : *Importance of the OGT-ChREBP-PPARalpha Axis in Hepatic Glucose Sensing*

# International research stays

**Visiting PhD student | October 2018 – December 2018 | January 2020**

Biomedical Center, Ludwig-Maximilians-Universitat, Munich, Germany  
PI and supervisor: Carla Margulies

Topic: Influence of PPAR $\alpha$  in the regulation of ChREBP target genes

**Visiting PhD student | June 2018 – July 2018**

Biobam, Centro de Investigacion Principe Felipe, Valencia, Spain  
PI and supervisor: Ana Conesa

Topic: Influence of PPAR $\alpha$  in the regulation of ChREBP target genes

# List of publications

**Ortega-Prieto P, Postic C.** Carbohydrate Sensing Through the Transcription Factor ChREBP. *Front Genet* (2019) **10**: doi:10.3389/fgene.2019.00472

## Summary

**TITLE** : O-GlcNAcylation : how does O-GlcNAc transferase (OGT) regulate liver physiology ?

**ABSTRACT** :

O-GlcNAcylation is a dynamic and reversible post-translational modification mediated through the activity of only two enzymes. O-GlcNAc transferase (OGT) catalyzes the addition of a single N-acetylglucosamine (GlcNAc) from the molecule UDP-GlcNAc to serine or threonine residues on specific proteins modifying its stability, localization and transcriptional activation. O-GlcNAcase (OGA) controls the removal of GlcNAc residues from OGT target proteins. Disruption of physiological levels of O-GlcNAcylation is related to abnormal cellular functions and pathologies including glucotoxicity, metabolic syndrome and cancer. Our team previously reported that the carbohydrate responsive transcription factor ChREBP is regulated by O-GlcNAcylation through OGT. Considering the implications of ChREBP in the context of gluco-lipototoxicity we aimed to understand the role of OGT in the regulation of ChREBP, glucose metabolism and lipogenesis in the liver *in vivo*. My work first confirmed that ChREBP directly interacts with OGT and that O-GlcNAcylation of ChREBP increases its transcriptional activity. Using a catalytic mutant of OGT (OGT<sup>H498A</sup>) we demonstrated that interaction between ChREBP and OGT<sup>H498A</sup> was stronger than with OGT<sup>WT</sup>, O-GlcNAcylation levels of ChREBP were decreased as well as reporter activity of the *Lpk* promoter. Our study also reveals that, despite the identification of Ser839 as a potential O-GlcNAcylation site for ChREBP function, mutation of that residue did not impair ChREBP O-GlcNAcylation and/or transcriptional activity, suggesting that residues important for the control of ChREBP activity by O-GlcNAcylation still remain to be identified. Importantly, our study confirms *in vivo* and *in vitro* that the OGT-ChREBP axis is important for the regulation of lipogenic gene expression and triglyceride synthesis in the liver. To determine the metabolic consequences of a targeted deletion of the OGT enzyme in hepatocytes *in vivo*, we developed a mouse model with hepatocyte-specific ablation of OGT (OGT<sup>Δ</sup>) by crossing OGT<sup>lox/lox</sup> with Alb-Cre mice. While these mice did not exhibit major disruption of metabolic homeostasis they exhibited a severe liver phenotype with the presence of numerous regeneration nodules visible after weaning. Analysis of

proliferation markers demonstrated an exacerbated proliferative state in OGT<sup>Δ</sup> at 8 weeks. Histological analyzes revealed the presence of pro-inflammatory cells and signs of fibrosis in the spans surrounding the regeneration nodules associated with a significant increase in markers of inflammation. Interestingly, while OGT was significantly reduced in livers of young mice (4 week-old), we observed re-expression of OGT in livers of older mice (8 week-old) suggesting a counter selection against OGT deficient cells. Microarray analysis comparing 4 and 8 week-old mice (OGT<sup>F</sup> vs OGT<sup>Δ</sup>) suggested that loss of OGT at 4 weeks causes ER and oxidative stress and DNA damage. Follow up of these mice over 1 year revealed a sexual dimorphism with females developing several tumor-like structures. Altogether, our results suggest an essential role for OGT in liver physiology.

**KEYWORDS:**

OGT, O-GlcNAcylation, liver, inflammation, proliferation, DNA damage, ER stress, fibrosis

**TITRE:** O-GlcNAcylation: comment la O-GlcNAc transférase (OGT) contrôle la physiologie hépatique?

**RÉSUMÉ:**

La glucotoxicité est un phénomène cellulaire qui initie un cercle vicieux dans lequel une hyperglycémie élevée et chronique conduit au développement du diabète de type 2. Parmi les mécanismes impliqués, il est décrit qu'un excès de glucose stimule la voie de biosynthèse de l'hexosamine (HBP) et augmente ainsi la O-GlcNAcylation, une modification post-traductionnelle très dynamique. Seules deux enzymes régulent le cycle de l'O-GlcNAcylation, la glycosyltransférase OGT (O-GlcNAc transférase) et la glycoside hydrolase OGA (O-GlcNAcase), qui ajoutent et retirent respectivement la fraction GlcNAc vers et à partir des protéines accepteuses. Ces deux enzymes assurent la médiation du cycle dynamique de la O-GlcNAcylation sur une grande variété de protéines cytosoliques, nucléaires et mitochondriales de manière sensible aux nutriments et au stress. En conséquence, la O-GlcNAcylation a été proposée pour fonctionner comme un capteur de nutriments et de stress régulant les processus cellulaires allant de la transcription et de la traduction à la transduction du signal et au métabolisme. La perturbation de l'homéostasie O-GlcNAc a été impliquée dans la pathogenèse d'une pléthore de maladies humaines, dont le cancer, le diabète et la neurodégénérescence. Notre laboratoire a précédemment montré que la O-GlcNAcylation est parmi les principaux effecteurs du métabolisme et contribue de manière significative à la gluco-lipotoxicité dans le foie et dans les cellules  $\beta$  pancréatiques. Alors que les fonctions cellulaires de la O-GlcNAcylation émergent, on sait peu de choses sur les mécanismes précis dont l'enzyme OGT détecte les signaux environnementaux pour provoquer des changements moléculaires et physiologiques.

Notre équipe a précédemment montré que le facteur de transcription ChREBP (Carbohydrate Responsive Element Binding Protein) est régulé par O-GlcNAcylation *via* l'OGT. ChREBP, dont l'activité est induite par le métabolisme du glucose, est un acteur clé de l'induction des gènes intervenant dans la synthèse *de novo* d'acides gras (la lipogenèse) en réponse au glucose dans le foie. Des études précédentes chez la souris ont révélé qu'une lipogenèse active, *via* l'activation de ChREBP, était associée à une amélioration de la sensibilité à l'insuline dans le tissu adipeux et dans le foie. En particulier, ChREBP, en limitant la toxicité liée à l'accumulation d'acides gras délétères, serait un acteur majeur de la sensibilité à l'insuline hépatique. Compte tenu du

rôle de ChREBP dans les mécanismes de gluco-lipototoxicité, nous avons cherché dans un premier temps à mieux caractériser le rôle de l'OGT dans la régulation de ChREBP, du métabolisme du glucose et de la lipogenèse hépatique *in vivo*. Nous avons tout d'abord confirmé l'interaction directe entre ChREBP et OGT et nous avons montré que la O-GlcNAcylation de ChREBP augmente son activité transcriptionnelle *in vitro*. En utilisant un mutant catalytique de OGT (OGT<sup>H498A</sup>), nous avons démontré une interaction plus forte entre ChREBP et OGT<sup>H498A</sup>. Cependant, nous observé des niveaux d' O-GlcNAcylation de ChREBP diminués ainsi qu'une activité réduite du gène rapporteur de l'enzyme clef de la glycolyse, la L-Pyruvate kinase (*Lpk*) lorsque le mutant catalytique de l'OGT est exprimé. Notre étude révèle également que, malgré l'identification de la Ser839 comme site potentiel de O-GlcNAcylation de ChREBP, la mutation de ce résidu n'altère, ni la O-GlcNAcylation de ChREBP, ni son activité transcriptionnelle. Nos résultats suggèrent donc que les résidus importants pour le contrôle de l'activité ChREBP par O-GlcNAcylation restent à être identifiés. Notre étude confirme cependant *in vivo* et *in vitro* que l'axe OGT-ChREBP est important pour la régulation de l'expression des gènes lipogéniques et la synthèse des triglycérides dans le foie.

Dans un deuxième temps, afin de déterminer les conséquences métaboliques d'une inhibition ciblée de OGT dans le foie, nous avons développé un modèle murin de délétion hépatique (OGT<sup>Δ</sup>) en croisant des souris OGT<sup>lox/lox</sup> avec des souris Alb-Cre. De manière étonnante, les souris OGT<sup>Δ</sup> ne présentent pas de perturbations majeures de l'homéostasie métabolique (le poids corporel et les concentrations circulantes de glucose étaient similaires chez les souris OGT<sup>Δ</sup>) par rapport aux témoins, mais présentent un phénotype hépatique sévère avec la présence de nombreux nodules de régénération visibles après le sevrage. L'analyse des marqueurs de prolifération par qPCR et Western blot (expression des cyclines A2, B1 et D1, marquage Ki67) a révélé un état prolifératif exacerbé dès 8 semaines. Les analyses histologiques ont montré la présence de cellules pro-inflammatoires et des signes de fibrose (expression augmentée de TGFβ) dans les travées entourant les nodules de régénération associés à une augmentation significative des marqueurs de l'inflammation (TNFα, Mcp1 and Ccl5, en particulier). Nos données révèlent donc que la perte d'OGT hépatique entraîne une modification du cycle cellulaire hépatique conduisant à un phénotype hépatique sévère, associé à une inflammation et une fibrose. Fait intéressant, alors que l'OGT était significativement inhibée à 4 semaines, nous avons observé une ré-expression de l'OGT dans les hépatocytes de souris plus âgées (8 semaines) suggérant une sélection contre les cellules

déficiences en OGT. L'analyse bioinformatique des puces à ADN (Volcano plots, REACTOME) a permis de mettre en évidence des clusters de gènes dérégulés dans le foie de souris déficientes pour l'OGT. En particulier, ces analyses ont montré que la perte de l'OGT à 4 semaines provoque une réponse au stress de réticulum endoplasmique, un stress oxydant et des dommages à l'ADN. Enfin, le suivi de ces souris sur un an a révélé un dimorphisme sexuel, les femelles développant des structures hépatiques ressemblant à des tumeurs tandis que les mâles semblaient présenter un phénotype hépatique normal.

L'ensemble de résultats obtenus au cours de mon travail de thèse suggère un rôle essentiel de l'OGT dans la physiologie hépatique.

**MOT CLEFS** : OGT, O-GlcNAcylation, foie, inflammation, prolifération, dommage à l'ADN, stress du RE, fibrose

# List of abbreviations

3HB	(R)-3-hydroxybutyrate
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
6PGDH	6-phosphogluconate dehydrogenase
$\alpha$ -KG	$\alpha$ -ketoglutarate
$\alpha$ -SMA	alpha-smooth muscle actin

## A

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AcAc	Acetoacetate
ACC	Acetyl-CoA carboxylase
ACLY	ATP-citrate lyase
ADP	Adenosine diphosphate
AEA	N-arachidonyl ethanolamine
AF-1	Activation function 1
Ala	Alanine
ALDA	Aldolase A
AMPK	AMP-activated protein kinase
Angpt1	Angiopoietin-1
APC	Antigen-presenting cell
ARE	Antioxidant response element
ASH1	Absent, small, or homeotic discs 1
ASK1	Apoptosis signal-regulating kinase 1
Asn	Asparagine
Asp	Aspartate
ASXL1	Additional sex comb like-1
ATF	Activating transcription factor
ATF2	Activating transcription factor 2
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate

## B

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BAP1	BRCA1-associated protein 1
BAs	Bile acids
BAT	Brown adipose tissue
BEMAD	$\beta$ -elimination/Michael addition with DTT
BIP	Binding-immunoglobulin protein GRP-78
BMAL1	Brain and muscle ARNT-like 1
BMDM	Bone-marrow derived macrophages

## C

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C	C-terminal
CABIN1	Calcineurin-binding protein 1
CAC	Citric acid cycle
CACT	Carnitine acyl-carnitine translocase
CaMKII	Calcium/calmodulin-dependent protein kinase II

CBX7	Chromobox homolog 7
CCL4	Carbon tetrachloride
CD	Cluster of differentiation
CDCA	Chenodeocycholic acid
ChIP	Chromatin immunoprecipitation
Chk1	Checkpoint kinase 1
CHOP	C/EBP-homologous protein
ChoRE	Carbohydrate response element
ChREBP	Carbohydrate response element binding protein
CK1	Casein kinase 1
CLOCK	Circadian locomotor output cycles kaput
COMPASS	Complex proteins associated with Set1
CPT1	Carnitine palmitoyltransferase 1
CREB	Cyclic AMP-responsive element-binding protein
CRTC2	CREB regulated transcription coactivator 2
CRY	Cryptochrome
CS	Citrate synthase
CSC	Cancer stem cells
CTGF	Connective tissue growth factor
CTP	Citrate transport protein
CUL3	Cullin-3
CYP	Cytochrome P450

## **D**

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DBP	D site-binding protein
DLK1	Delta-like protein 1

## **E**

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ECH	Erythroid cell-derived protein with CNC homology
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eIF2a	Eukaryotic translation initiation factor-2 $\alpha$
eIF4E	Eukaryotic initiation factor 4E
ELOVL6	Elongation of long-chain fatty acids family member 6
EMT	Endothelial-mesenchymal transition
ENO	Enolase
EpCAM	Epithelial cellular adhesion molecule
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ES	Embryonic stem
ETC	Electron transport chain
EZH2	Enhancer of zeste 2

## **F**

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F6P	Fructose-6-phosphate
FADH2/FAD	Flavin adenine dinucleotide
FAO	Fatty acid $\beta$ -oxidation

FASN/FAS	Fatty acid synthase
FBPase	Fructose-1,6-biphosphatase
FBXL2	F-box and leucine rich repeat protein 2
FFA	Free fatty acid
FGF	Fibroblast growth factor
FOXM1	Forkhead box M1
FoxO1	Forkhead box protein O1
FSH	Follicle-stimulating hormone
Fuc	Fucose
FXR	Farnesoid X receptor

## **G**

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G6P	Glucose-6-phosphate
G6Pase	Glucose-6-phosphatase
G6PDH	Glucose-6-phosphate dehydrogenase
GADD34	DNA damage-inducible protein 34
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAT	Glutamine:fructose amidotransferase
GK	Glucokinase
GKRP	Glucokinase regulatory protein
GlcN	Glucosamine
GlcNAc	N-acetylglucosamine
GLUT	Glucose transporter
GNPNAT1	Glucosamine 6-phosphate N-acetyltransferase
GPI	Glycosylphosphatidylinositol
GRACE	Glucose-response activation conserved element
Grb2	Growth factor receptor-bound protein 2
GS	Glutamine synthetase
GSH	Glutathione
GSK3 $\beta$	Glycogen synthase kinase 3 beta
GTP	Guanosine diphosphate

## **H**

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H2B	Histone 2B
H3K9	Histone 3 Lysine 9
H4	Histone 4
HAT	Histone acetylase
HBP	Hexosamine biosynthesis pathway
HCA	Hyochoolic acids
HCC	Hepatocellular carcinoma
HCF-1	Host cell factor 1
HDAC	Histone deacetylase
HDCA	6 $\alpha$ -hydroxylated hyodeoxycholic
HFD	High-fat diet
HIRA	Histone cell cycle regulator A
HK	Hexokinase

HLF	Hepatic leukemia factor
HNF4 $\alpha$	Hepatocyte nuclear factor 4 $\alpha$
HSC	Hepatic stellate cell
Hsp	Heat shock protein
<b>I</b>	
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IEC	Intestinal epithelial cells
IGF-1	Insulin growth factor-1
IL	Interleukine
IR	Insulin receptor
IRE1	Inositol requiring enzyme 1 $\alpha/\beta$
IRS	Insulin receptor substrate
ISS	Intronic splicing silencer
<b>J</b>	
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Jab1	JAK-binding protein
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
<b>K</b>	
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KC	Kupffer cell
KDM2A	Lysine-specific demethylase 2A
KEAP1	Kelch-like ECH associated protein 1
<b>L</b>	
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L-PK	Liver-pyruvate kinase
LATS2	Large tumor suppressor kinase 2
LCA	Lithocholic acid
LD	Luminal domain
LID	Low glucose inhibitory domain
LPS	Lipopolysaccharide
LSD2	Lysine-specific demethylase 2
LSEC	Liver sinusoidal endothelial cell
LXRE	LXR-responsive elements
LXRs	Liver X receptors
Lys	Lysine
LYVE1	Lymphatic vessel endothelial hyaluronic acid receptor
<b>M</b>	
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Man	Mannose
MAN1A1	$\alpha$ 1,2-mannosidase IA
MAP3K	MAPK kinase kinase
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral-signaling protein
MCD	Choline-deficient methionine-supplemented
MEK	MAP kinase kinase
MEKK	MAPK/Erk kinase kinase
MKK	MAPK kinase
MLK	Mixed lineage kinase
MLKL	Mixed lineage kinase domain like pseudokinase

MLL5	Mixed lineage leukemia 5
MLX	Max like protein X
MMP-2	Matrix metalloproteinase-2
MOB1	Monopolar spindle-one-binder 1
MOF	Males absent on the first
mOGT	Mitochondrial OGT
mTOR	Mammalian target of rapamycin
MTS	Mitochondrial-targeting sequence
<b>N</b>	
<hr/>	
N	N-terminal
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
ncOGT	Nucleocytoplasmic OGT
NCor	Nuclear receptor corepressor
NOS2	Nitric oxide synthase 2
NRF2	Nuclear factor erythroid 2-related factor 2
NSL	Nonspecific lethal
NuRD	Nucleosome remodeling and deacetylation
<b>O</b>	
<hr/>	
OG	O-GlcNAcylation
OGA	O-GlcNAcase
OGA-L	OGA full length
OGA-S	OGA short
OGT	O-GlcNAc transferase
<b>P</b>	
<hr/>	
P1	Promoter 1
PB	Processing bodies
PC	Pyruvate carboxylase
PcG	Polycomb group
PCGF4	Polycomb group RING finger protein 4
PDGF	Platelet-derived growth factor
PDH	Pyruvate dehydrogenase
PDK1	3-phosphoinositide-dependent protein kinase 1
Pdx1	Pancreatic and duodenal homeobox 1
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
PER	Period
PERK	PKR-like ER kinase
PFK1	Phosphofructokinase 1
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases 3
PGC-1 $\alpha$	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PGI	Phosphoglucose isomerase
PGK	Phosphoglycerate kinase
PGM3	Phosphoacetylglucosamine mutase
PGM3	Phosphoglycerate mutase
Phf2	Plant homeodomain finger 2
PHx	Partial hepatectomy
PI3K	Phosphatidylinositol-3-kinase
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein kinase A
PKM2	Pyruvate kinase muscle isoform 2
PLIN1	Perilipin 1
PPAR	Peroxisome proliferator-activated receptors
PPO	Phosphoinositide-domain
PPP	Pentose phosphate pathway
PPRE	Peroxisome proliferator response element
PR-DUB	Polycomb Repressive DeUBiquitinase
PRC1	Polycomb repressive complex 1
PRC2	Polycomb repressive complex 2
PROX1	Prospero homeobox protein 1
PTM	Post-translational modification

## R

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RING1B	Ring Finger Protein 1B
RIPK3	Receptor interacting serine/threonine kinase 3
RNA pol II	RNA polymerase II
RNF2	Ring finger protein 2
RNF51	RING finger protein 51
ROR	Receptor tyrosine kinase-like orphan receptor
ROS	Reactive oxygen species
RPI	Ribose-5-phosphate isomerase
RuPE	Ribulose-5-phosphate-3-epimerase
RXR	Retinoid X receptor
RYBP	RING1 And YY1 Binding Protein

## S

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SCAP	SREBP-cleavage activating protein
SCD1	Stearoyl-CoA desaturase
Ser	Serine
SG	Stress granules
SHP	Small heterodimer partner
SILAC	Stable isotope labelling by amino acids
Skp2	S-phase kinase-associated protein 2
SMAD	Sma Mothers against decapentaplegic
sOGT	Short OGT
SOS	Son of sevenless
Sox2	Sex determining region Y-box 2

SOX9	SRY-related HMG box transcription factor 9
Sp1	Specificity protein 1
SREBP1	Sterol regulatory element binding protein 1
STAT	Signal transducer and activator of transcription
STZ	Streptozotocin

## T

---

TAK1	Transforming growth factor $\beta$ -activated kinase-1
TAL	Transaldolase
TAZ	Tafazzin
TCA	Tricarboxylic acid cycle
TEF	Thyrotroph embryonic factor
TET	Ten-eleven translocation
TG	Triglyceride
TGF	Transforming Growth Factor
TGF $\beta$ R1	TGF $\beta$ type I receptor
Thr	Threonine
TKT	Transketolase
TNF $\alpha$	Tumor necrosis factor alpha
TPI	Triosephosphate isomerase
TPL	TOPLESS
TPRs	Tetratricopeptide repeats
TRAF2	Tumor necrosis factor receptor-associated factor 2
TSR	Thrombospondin type 1 repeats
TSSs	Transcription start sites
TTFL	Transcription-translation feedback loop
Tyr	Tyrosine

## U

---

UAP1	UDP-N-acetylglucosamine pyrophosphorylase
UBN1	Ubinuclein 1
UCP1	Uncoupling protein 1
UDP	Uridine-diphosphate
UDP-GlcNAc	Uridine-diphosphate-N-acetylglucosamine
UPR	Unfolded protein response
USP2A	Ubiquitin-specific protease-2a
USP7	Ubiquitin-specific-processing protease 7
UTP	Uridine-triphosphate

## V

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VEGF	Vascular endothelial growth factor
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein

## W

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WAT	White adipose tissue
-----	----------------------

## X

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X5P	Xylulose-5-phosphate
XBPI	X-box-binding protein 1

Xi	Inactive X chromosome
XIC	X-inactivation center
Xm	Maternal X chromosome
Xp	Paternal X chromosome

**Y**

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YAP	Yes-associated protein
YB-1	Y-box binding protein 1

**Z**

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ZAK	Containing kinase AZK
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# Introduction

# Chapter 1: Introduction to glycosylation and O-GlcNAcylation

## 1. Introduction to glycosylation

Glycobiology is the study of the biology, structure and biosynthesis of glycans. Glycans are carbohydrate chains, which exist as free form or covalently assembled to proteins or lipids (glycoconjugates) modulating a great variety of functions both in physiological and pathophysiological states. Glycosylation usually occurs in the endoplasmic reticulum (ER) and Golgi apparatus, where most of the terminal processing occurs. This process is carried out by glycosyltransferases and glycosidases and controlled by substrate availability, enzyme activity and location but also their level of gene expression. In this context, better understanding the regulation of protein glycosylation has become very important since several diseases, such as diabetes (Reily *et al.*, 2019) and cancer (Pinho and Reis, 2015; Mereiter *et al.*, 2019; Peixoto *et al.*, 2019), have been associated to glycosyltransferases and/or glycosidases deregulation or abnormal glycosylation of one or more glycoproteins (Reily *et al.*, 2019). The most common types of glycoconjugates (**Figure 1**) are found on the cellular surface and secreted macromolecules. However, Torres and Hart (1984) described a unique type of O-glycosylation (O-GlcNAcylation) occurring in the nucleus and cytoplasm.

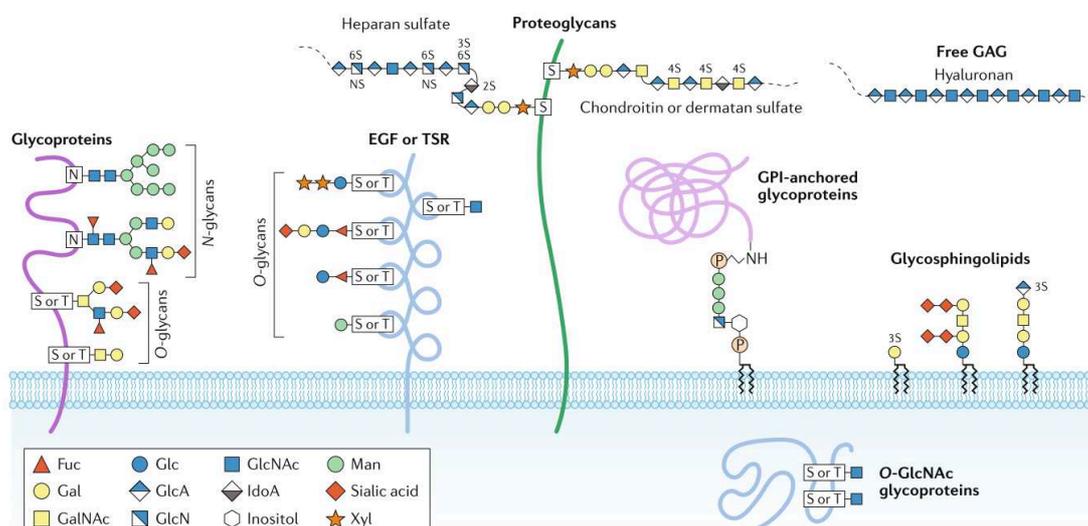


Figure 1 Principal glycosylation types in humans.

Glycoproteins are glycans linked to oxygen (O-glycans) or nitrogen (N-glycans) atoms of amino acid residues. N-glycans consist of a core glycan containing two N-acetylglucosamine (GlcNAc) and three mannose (Man) residues. O-glycans are the most diverse forms of protein glycosylation and consist in the attachment of glycans to the oxygen atom of serine (Ser) or threonine (Thr) residues. The different types of O-glycans are named based on the initial sugar binding to the protein residue and some of them as O-linked fucose (Fuc) or O-linked Man are

commonly associated to specific proteins such as epidermal growth factor (EGF) repeats or thrombospondin type 1 repeats (TSR). O-GlcNAc glycoproteins are a unique type of O-linked glycan proteins located in the cytoplasm and the nucleus. Proteoglycans are a class of glycoproteins, attached to the extracellular matrix, constituted by long glycosaminoglycan (GAG) chains binding through O-linked glycosylation to proteins through a specific tetrasaccharide core (GlcA-Gal-Gal-Xyl). These proteoglycans can be classified according to the composition of the glycosaminoglycan chain. Glycosylphosphatidylinositol (GPI)-anchored glycoproteins is a glycoconjugate containing a soluble protein attached to phosphoethanolamine via a phosphodiester linkage, binding to a Man3-GlcN core which is embedded in the cell membrane through a link between the GlcN residue to phosphatidylinositol. Glycosphingolipids are sphingolipids associated to the cellular membrane in which a glycan is attached to a ceramide. Adapted from Reily *et al.*, 2019.

## 2. O-GlcNAcylation

O-GlcNAcylation was discovered in 1984 when Torres and Hart used bovine milk galactosyltransferase and UDP-[<sup>3</sup>H]Gal (tritiated uridine-5'-diphosphogalactose) to probe cell surfaces of murine immune system for terminal GlcNAc moieties (Torres and Hart, 1984). Surprisingly, most of those moieties were enriched within the nucleus and the cytoplasm (Holt and Hart, 1986). In fact, O-GlcNAcylation is a dynamic post-translational modification (PTM) where a single GlcNAc moiety is  $\beta$ -O-linked to Serine (Ser) and Threonine (Thr) amino acids on nuclear, cytoplasmic and mitochondrial polypeptides. Unlike traditional PTMs such as phosphorylation or ubiquitination, O-GlcNAcylation is regulated by only two enzymes: O-GlcNAc transferase (OGT) is the protein in charge of linking the moieties GlcNAc from the UDP-GlcNAc metabolite to

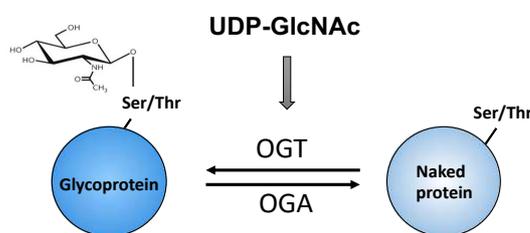


Figure 2. General mechanism of O-GlcNAcylation.

OGT modifies proteins by addition of GlcNAc moieties coming from UDP-GlcNAc to its residues Ser/Thr. OGA carries out the reversible reaction by hydrolysis of GlcNAc residues.

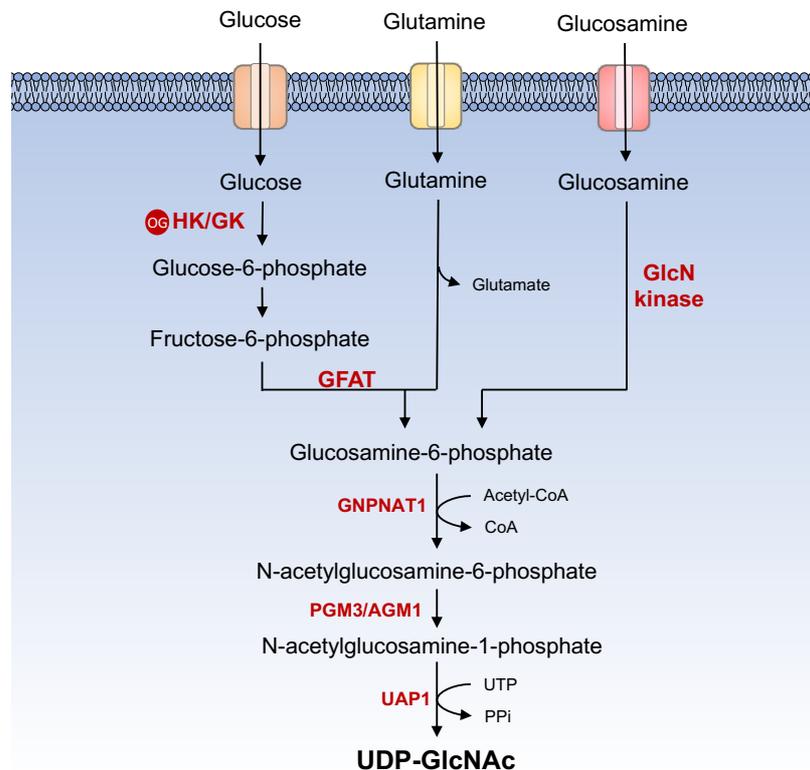
the Ser/Thr residues of target proteins; O-GlcNAcase (OGA) has the opposite role hydrolyzing the GlcNAc moieties (**Figure 2**). These two enzymes mediate the dynamic cycling of the commonly called O-GlcNAcylation on a wide variety of cytosolic, nuclear and mitochondrial

proteins in a nutrient- and stress-responsive manner. In fact, Decourcelle *et al.* (2020) recently gave a closer insight in the regulation of the OGT-OGA dynamic in colonic cells, showing that i) the modulation of O-GlcNAcylation levels leads to compensatory regulation of OGT and OGA expression to recover basal levels of O-GlcNAcylation, and ii) OGT regulation seems to occur mostly through PTMs whereas the regulation of OGA is predominantly transcriptional (Decourcelle *et al.*, 2020). As a

result, O-GlcNAcylation was proposed to function as a nutrient and stress sensor regulating cellular processes ranging from transcription and translation to signal transduction and metabolism.

## I. UDP-GlcNAc and role of O-GlcNAc in metabolism

UDP-GlcNAc, for uridine-diphosphate-N-acetylglucosamine, the donor substrate for OGT, is an acetylated amino sugar nucleotide produced *via* the activation of the hexosamine biosynthesis pathway (HBP). The first rate-limiting step in the HBP is driven by the glutamine:fructose amidotransferase (GFAT) enzyme, transferring an amino group from glutamine to fructose-6-phosphate (F6P) generating glucosamine-6-phosphate (**Figure 3**). In fact, HBP is often referred as ‘nutrient-sensing’ pathway since it requires glucose, glutamine and/or glucosamine, as well as a source of acetyl-CoA, such as fatty acids, and high energy phosphates, such as ATP or UTP, for the synthesis of UDP-GlcNAc. Furthermore, O-GlcNAcylation regulates enzymes of glucose uptake, insulin signaling, gluconeogenesis, lipogenesis, glycogen biosynthesis and mitochondrial function (Bond and Hanover, 2015; Hart, 2019) highlighting the importance of O-GlcNAcylation as a nutrient sensor.



*Figure 3. Scheme of the hexosamine biosynthesis pathway (HBP). Glucose enters the cell as simple glucose where is rapidly phosphorylated by the hexokinase/glucokinase (HK/GK) enzymes to glucose-6-phosphate and transformed to fructose-6-phosphate. Fructose-6-phosphate is metabolized to glucosamine-6-phosphate with addition of an amino group coming from glutamine by the enzyme L-glutamine-D-fructose-6-phosphate*

*aminotransferase (GFAT). Glucosamine can also enter the cell and be directly phosphorylated by glucosamine kinase (GlcN kinase) for the generation of glucosamine-6-P. Glucosamine-6-P is subsequently metabolized by glucosamine 6-phosphate N-acetyltransferase (GNPNAT1) to N-acetylglucosamine-6-P, through the addition of a group acetyl. The phosphoacetylglucosamine mutase (PGM3 also called AGMI) converts N-acetylglucosamine-6-P in N-acetylglucosamine-1-phosphate, which finally, by the addition of a molecule of uridine-diphosphate (UDP) via the UDP-N-acetylglucosamine pyrophosphorylase (UAPI), generates the metabolite uridine-diphosphate-N-acetylglucosamine (UDP-GlcNAc) essential for the modification of proteins by OGT.*

## II. O-GlcNAc transferase (OGT)

O-GlcNAc transferase or OGT is a protein encoded by a single gene located in the region Xq13.1 of the human X chromosome and the region between the markers DXMit41 and DXMit95 in the mouse X chromosome (Shafi *et al.*, 2000). *Ogt* is a very conserved gene between eukaryotes and present high homology in vertebrates. Formed by 23 exons and 21 introns, *Ogt* was hypothesized to contain two alternative promoters, shown in **Figure 5A** (Hanover *et al.*, 2003). Hanover *et al.* (2003) described that *Ogt* fourth intron, normally removed to produce a transcript of 6.5 kb, can be also used as “exon 5”, producing a transcript longer than 8 kb later described to be part of the mitochondrial OGT isoform (Love *et al.*, 2003). In addition, a new important sequence in OGT mRNA called intronic splicing silencer (ISS) was recently described. Located between exons 4 and 5, it has been suggested to dynamically regulate OGT expression under O-GlcNAcylation variation levels. The authors suggested that OGT containing the ISS sequence could function as a nuclear reservoir of pre-mRNA that can quickly be spliced to produce fully-spliced OGT mRNA (**Figure 4**) (Park *et al.*, 2017). Indeed, Tan *et al.* 2020 recently validated this hypothesis and suggested, using RNA-seq data of different cells, that O-GlcNAcylation itself could be a master regulator of ISS splicing (Tan *et al.*, 2020). The authors show that decrease in the global levels of O-GlcNAcylation favors the splicing of detained introns in a wide range of genes. However, they were not able to identify whether this effect is due to the O-GlcNAcylation of different proteins involved in the regulation of intron splicing or if the modified factor is involved in chromatin modification, nucleosome remodeling or any other transcriptional machinery known to be modulated by OGT (Tan *et al.*, 2020).

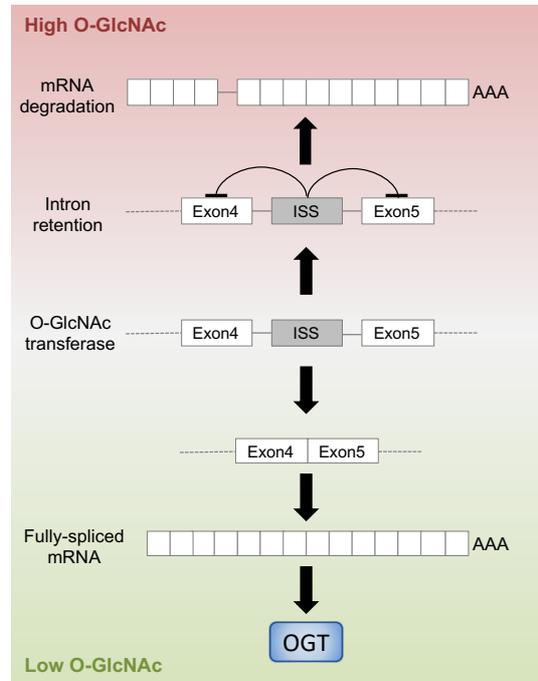


Figure 4. Intronic splicing silencer (ISS) regulation of OGT. Under physiological conditions, cells produce ISS-containing OGT mRNAs suggested to act as reservoir for rapid response to changes in O-GlcNAcylation. Low levels of O-GlcNAcylated proteins activate the full splicing of OGT mRNA for protein expression. In contrast, high levels of O-GlcNAc activate the retention of ISS and nuclear degradation. Adapted from Tan et al., 2020.

#### a. OGT protein structure and isoforms

Full-length human OGT contains 1036 amino acids, corresponding to a molecular weight of 110 kDa. Interestingly, the *OGT* gene encodes for three different isoforms produced by complex splicing of its mRNA and different start codons: the nucleocytoplasmic form (ncOGT, 110 kDa), the mitochondrial-tagged isoform (mOGT, 103 kDa) and a short isoform (sOGT, 70 kDa). The carboxy-terminal region is common to all the isoforms containing three catalytic domains (N-Cat, Int-D and C-Cat) and a phosphoinositide-domain (PPO). However, the N-terminal region of the protein differs by the number of tetratricopeptide repeats (TPRs), shown to mediate substrate interaction (Iyer and Hart, 2003), with 13.5, 9 and 2.5 repetitions in ncOGT, mOGT and sOGT respectively (Jínek *et al.*, 2004) (**Figure 5B**). Furthermore, the OGT isoforms were reported to also differ in their subcellular localization: ncOGT is located in the cytoplasm and nucleus, sOGT is predominantly located in the cytoplasm, whereas the mOGT isoform is thought to be located largely in the mitochondria (Kreppel, Blomberg and Hart, 1997; Lubas *et al.*, 1997; Hanover *et al.*, 2003; Love *et al.*, 2003). A recent study reported low levels of mOGT in several human cell lines and mouse tissues while elevated levels of ncOGT were sufficient to increase the levels of mitochondrial protein O-GlcNAcylation suggesting a non-essential function of mOGT or a redundancy

between mOGT and ncOGT (Trapannone *et al.*, 2016). These findings highlight the importance of better characterizing the function of these “less described” OGT isoforms.

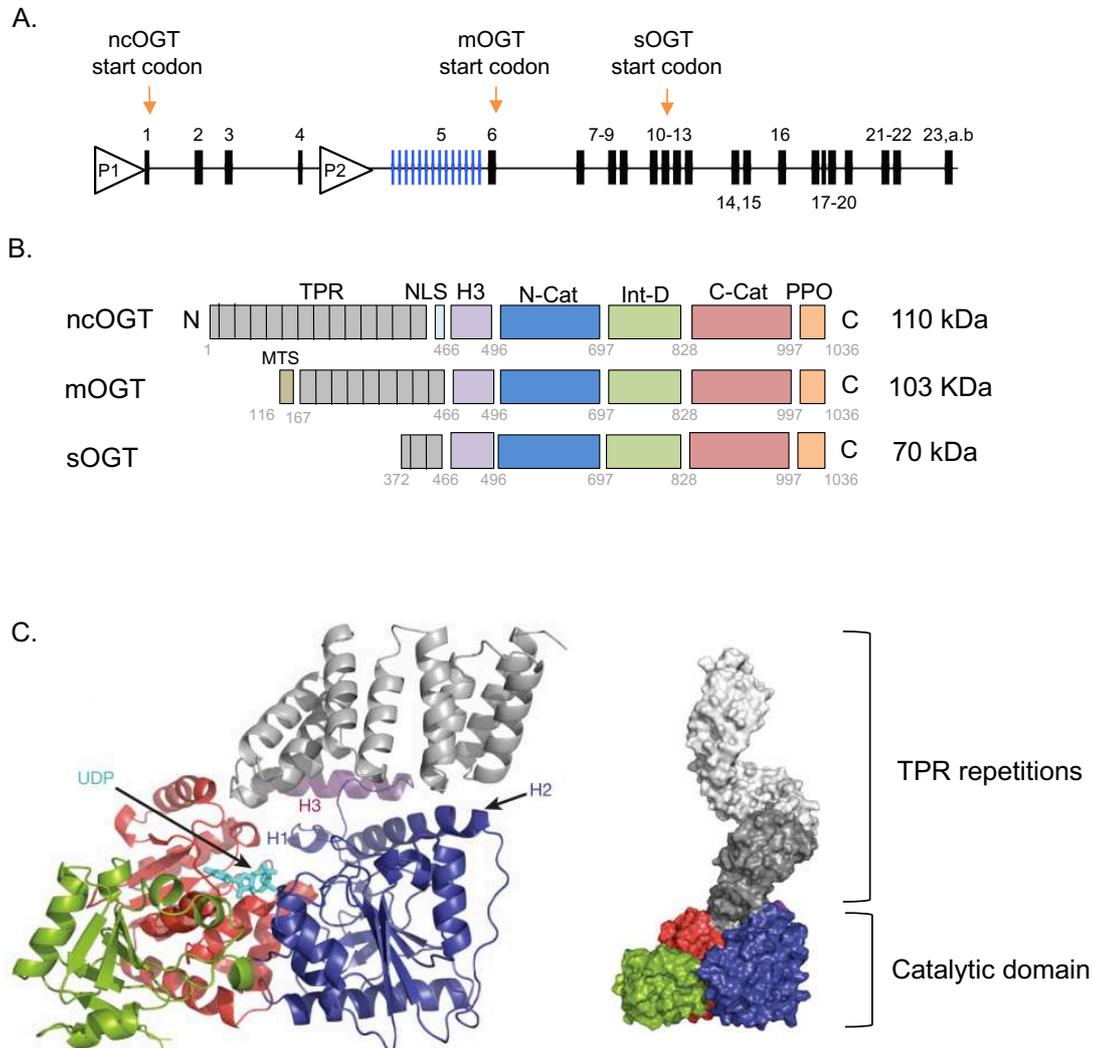


Figure 5. O-GlcNAc transferase (OGT) gene and protein structure.

A. The mammalian *Ogt* locus proposed by Hanover *et al.* (2003) is composed of 23 exons and 21 introns. ncOGT is produced from promoter 1 (P1) by splicing from the exon 4 to 6 generating a 6.5 kb product. mOGT is generated from an alternative promoter 2 (P2) and uses exon 5 to originate the N-terminal mitochondrial-tagged region. sOGT is thought to originate from a third alternative codon. Adapted from Hanover *et al.*, 2003. B. Protein structure scheme of the different isoforms of OGT. The C-terminal region (C) is conserved between isoforms containing three catalytic domains: N-Cat, formed by two helices called H1 and H2, and C-Cat, linked by a region called intervening domain (Int-D); together with a phosphoinositide-domain (PPO), proposed to be used by OGT to bind to the cellular membrane in response to insulin signaling (X. Yang *et al.*, 2008). However, the amino terminal region (N) differs in number of TPR and, in the case of mOGT, it contains a mitochondrial-targeting sequence (MTS) which will lead to its translocation to the mitochondria. A transitional helix, called H3, binds the TPR repeats to the catalytic domains. C. Crystal structure of OGT binding to the metabolite UDP, published by Lazarus *et al.* (2011).

Structural insight into the OGT architecture was provided for the first time by Jínek *et al.* (2004), which proportionated the crystal structure of the human TPR

domain. The TPRs create a super helical structure, similar to proteins such as importin- $\alpha$  or E-cadherin, allowing an OGT-protein interaction through asparagine (Asn) residues independent of the target polypeptide amino acid sequence (Jínek *et al.*, 2004). However, it was not until 2011, when Lazarus *et al.* identified the crystal structure of human OGT binding to a peptide substrate and UDP, that a model structure for the full-length OGT protein was proposed (**Figure 5C**). Altogether, this model for OGT allows the authors to define several mutants of OGT with highly reduced activity, such as H498A and H558A, located both in the N-Cat domain suggested to be involved in the interaction with target peptides (Lazarus *et al.*, 2011).

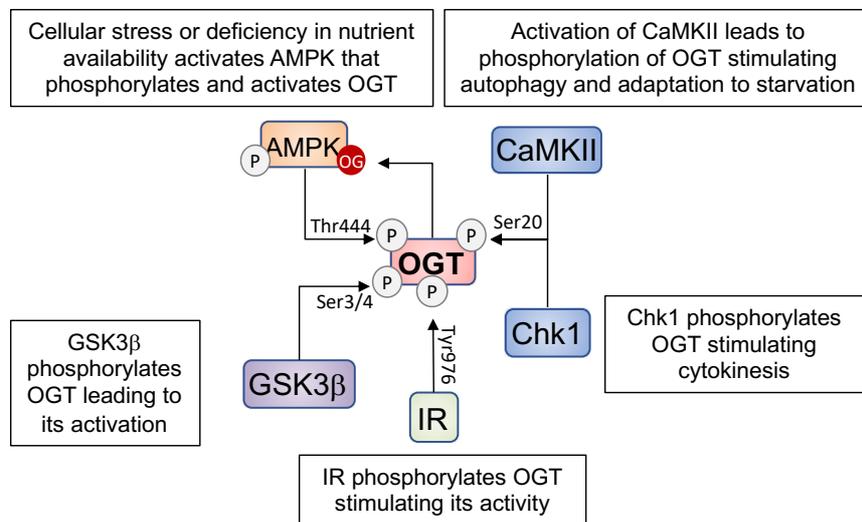
### **b) Regulation of OGT activity via phosphorylation**

OGT can be modified by several PTMs, including phosphorylation, O-GlcNAcylation (Lubas and Hanover, 2000), acetylation (Lundby *et al.*, 2012), ubiquitination (Wagner *et al.*, 2011) and sumoylation (Lumpkin *et al.*, 2017) occurring in the protein under specific physiological changes. However, most of these PTMs were discovered by mass spectrometry and the number of PTMs studied by other methods is limited to only some specific phosphorylated residues. Other methods such as point mutation of the discovered residues could help understand the regulation of OGT activity and stability. OGT PTM appears to depend on the protein-protein interaction through the TPR region of OGT (**Figure 5B**) (Kreppel, Blomberg and Hart, 1997; Kreppel and Hart, 1999).

Under circumstances of cellular stress or deficiency in nutrient availability, AMP-activated protein kinase (AMPK) acts as an energy sensor to maintain cellular energy levels (Foretz, Even and Viollet, 2018). Upon activation *in vitro* in C2C12 differentiated myotubes, AMPK phosphorylation of OGT on Thr444 occurs resulting in stimulation of OGT nuclear localization, nuclear O-GlcNAcylation and acetylation of the lysine (Lys) 9 of histone 3 (H3K9), potentially *via* nuclear exportation of HDAC5 (Bullen *et al.*, 2014), while decreasing O-GlcNAcylation of the histone H2B (Whelan, Lane and Hart, 2008). Analysis of substrate selectivity in HEK293T cells treated with a specific activator of AMPK (A-769662), reported that OGT phosphorylated on Thr444 presents high affinity for AMPK O-GlcNAcylation which further potentiates its activity (Bullen *et al.*, 2014). The authors further validated that acute inhibition of O-GlcNAc cycling *via* physiological or pharmacological stimuli abolishes activation of AMPK (Bullen *et al.*, 2014) (**Figure 6**). These results reveal an important cross-talk between OGT-AMPK which synergistically participates in the regulation of cellular metabolism, growth,

proliferation or tissue function but also reveal a role for AMPK in the regulation of O-GlcNAc mediated epigenetic modulation (Cork, Thompson and Slawson, 2018). Moreover, calcium/calmodulin-dependent protein kinase II (CaMKII), an upstream kinase that phosphorylates AMPK, also phosphorylates OGT on Ser20 which induces its activation thereby stimulating autophagy and metabolic adaptation to starvation (Ruan *et al.*, 2017) (**Figure 6**). The residue Ser20 of OGT is also phosphorylated by the Checkpoint kinase 1 (Chk1) protein in HeLa cells, stabilizing OGT which is required for cytokinesis (Li *et al.*, 2017). The protein Glycogen synthase kinase 3 beta (GSK3 $\beta$ ), constitutionally active and inhibited *via* activation of Wnt and insulin signaling pathways, was shown to phosphorylate OGT at Ser3 and Ser4 in mouse brain. While the authors suggest that other phosphorylation sites could be needed for the complete activation of OGT by GSK3 $\beta$ , phosphorylation on Ser3 and Ser4 enhanced OGT activity (Kaasik *et al.*, 2013) (**Figure 6**).

The C-terminus of OGT can also be the target of PTMs. Indeed, in response to insulin in 3T3-L1 adipocytes, activation of the insulin receptor (IR) allows phosphorylation of OGT at Tyrosine 976 (Tyr976), which stimulates its activity (Kreppel, Blomberg and Hart, 1997; Whelan, Lane and Hart, 2008).



*Figure 6. Regulation of OGT via phosphorylation. In conditions of cellular stress or deficiency in nutrient availability, AMPK is activated by phosphorylation leading to phosphorylation of OGT at Thr444 causing a positive feedback where OGT modifies and potentiates the activity of AMPK. CaMKII phosphorylates OGT on Ser20 stimulating autophagy and adaptation to starvation. The same residue is modified by Chk1 causing activation of cytokinesis. IR stimulates OGT activity via phosphorylation on Tyr976. GSK3 $\beta$  activates OGT by phosphorylating Ser3 and/or Ser4.*

### c) OGT-targeted sequence

While numerous proteins were described to be post-translationally modified by OGT, the substrate specificity of OGT is lacking a well-defined consensus sequence. One of the major challenges in molecular biology is to unravel the complex PTM code. The ‘Ying-Yang’ model between phosphorylation and O-GlcNAcylation hypothesizes that O-GlcNAcylation and phosphorylation regulate protein functions, at least in part, by competing and blocking each other occupancy at given sites (Wells, Vosseller and Hart, 2001). In 2014, based on this hypothesis, Leney and colleagues used mass spectrometry-based assay to identify a frequently occurring and very specific phosphorylation/O-GlcNAcylation interplay motif, (pS/pT)P(V/A/T)(gS/gT). This motif was found to be enriched in the human phosphoproteome, allowing the prediction of hundreds of potential O-GlcNAc modified proteins (Leney *et al.*, 2017). The following year, another group published a preferred human OGT sequence defined as [TS][PT][VT]S/T[RLV][ASY], using a peptide library to screen substrates (Pathak *et al.*, 2015). However, the attempt of several groups to develop computational methods to accurately predict O-GlcNAcylation sites have still been unsatisfactory due to the recognition sites ambiguity (Wang *et al.*, 2011; Jia, Liu and Wang, 2013; Kao *et al.*, 2015; Jia, Zuo and Zou, 2018). Regardless, target specificity was recently shown to depend on the aspartate (Asp) residues of the TPR region since mutation to alanine (Ala) changed substrate specificity and increased rates of glycosylation in proteins not recognized by wild-type OGT (Joiner *et al.*, 2019).

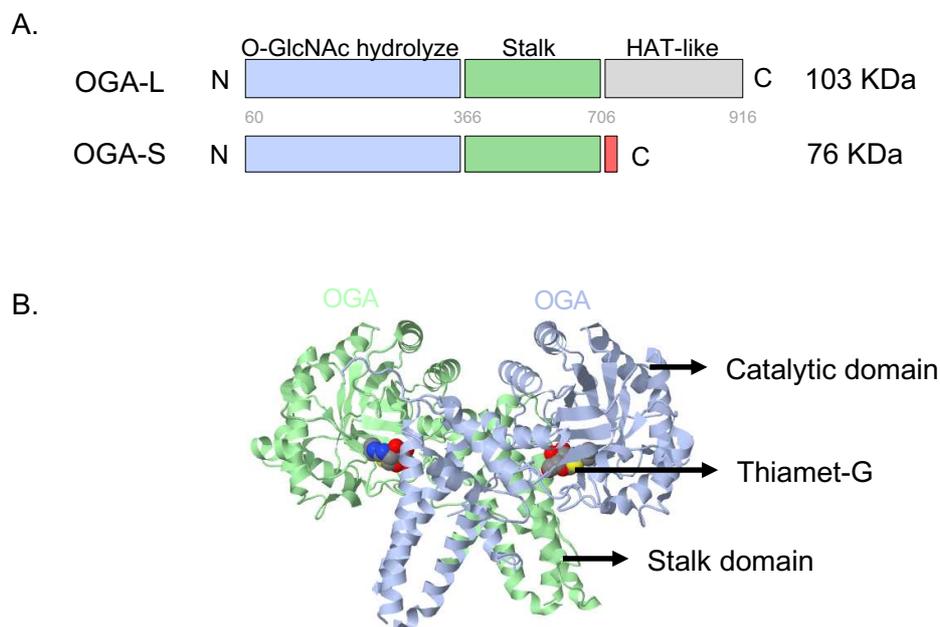
## III. O-GlcNAcase (OGA)

O-GlcNAcase or OGA is the opposite enzyme to OGT, which main role is the hydrolyzation of O-GlcNAc residues from OGT’s target proteins. This protein, encoded by a single gene of 16 exons, is called *MGEA5* for meningioma-expressed antigen 5 since it was identified as an antigen expressed in meningioma patients. OGA is located in the position 10q24.32 of the human genome (Heckel *et al.*, 1998).

### a) OGA protein structure and isoforms

OGA is a 916 amino acid protein (103 kDa) present as two isoforms in vertebrates: a full-length form and a short form (Comtesse, Maldener and Meese, 2001). The full-length form (OGA-L, apparent molecular weight of 120-130 kDa) is located predominantly in the cytoplasm and the nucleus (Gao *et al.*, 2001; Wells *et al.*, 2002; Toleman *et al.*, 2004) and contains a N-terminal O-GlcNAc hydrolyze catalytic domain

(also called GH84), a disordered domain (Stalk) containing a caspase-3 cleavage site at residue 413 (Butkinaree *et al.*, 2008), and a HAT-like C-terminal domain. The short form (OGA-S, apparent molecular weight of 75 kDa) lacks the C-terminal domain but contains 15 unique amino acids derived from the incomplete splicing of intron 10 (Macauley and Vocadlo, 2009). OGA-S is located predominantly at the nucleus (Comtesse, Maldener and Meese, 2001) and at lipid droplets surfaces (Keembiyehetty *et al.*, 2011). Although the enzymatic activity evaluation of OGA *in vitro* revealed a Km 20-fold lower for OGA-S than the one of OGA-L (E. J. Kim *et al.*, 2006; Macauley and Vocadlo, 2009), the relative contribution of the OGA isoforms *in vivo* is still unclear and would need further investigation (**Figure 7A**).



FirstGlance in Jmol

*Figure 7. O-GlcNAcase (OGA) protein structure.*

*A. Full-length OGA (OGA-L) contains in the N-terminus the O-GlcNAc hydrolyze catalytic domain, a disordered helical domain (Stalk) and a functionally unknown HAT-like domain in the C-terminus. OGA-S lacks the HAT-like domain and contains a unique 15 amino acids peptide. B. Structure of OGA binding to Thiamet-G using the crystal structure from Li *et al.* (2017) obtained by FirstGlance in Jmol (PBD:5UN9). (FirstGlance in Jmol: a simple tool for macromolecular visualization, available on-line at [firstglance.jmol.org](http://firstglance.jmol.org) v. 2.5.1 (free software, Creative Commons License), 2006)*

Despite the difficulties in identifying the crystal structure of eukaryotic OGA protein, three independent groups recently solved the crystal structure of human OGA, revealing its substrate recognition and function (Li *et al.*, 2017; Elsen *et al.*, 2017; Roth *et al.*, 2017). The authors reported that OGA acts as an homodimer where the active site is located between the catalytic domain of one monomer and the C-terminal helical bundle of the second one (Roth *et al.*, 2017). Li and colleagues (2017) co-crystallized OGA with Thiamet-G, a potent inhibitor of OGA generally used in the “O-

GlcNAcylation world” to study biological consequences of increased O-GlcNAcylation levels (Li *et al.*, 2017) (**Figure 7B**). However, the specific peptide recognized by OGA, like it is also the case for OGT, remains challenging due to the wide range of proteins modulated by O-GlcNAcylation.

### **b) Regulation of OGA activity by PTMs**

As previously mentioned in **Part 1.2**, the regulation of OGT and OGA balance is a complex and dynamic process where a compensatory mechanism is elicited with changes in O-GlcNAcylation levels. Decourcelle *et al.* (2020) recently gave closer insight into the regulation of OGA suggesting it is mostly transcriptional, contrary to OGT which is predominantly regulated by PTMs (Decourcelle *et al.*, 2020).

In addition, OGA interacts and can be a direct target of OGT (Whisenhunt *et al.*, 2006). Nonetheless, the study of the highly dynamic relationship between OGT-OGA has been challenging. The Ser405 residue of OGA has been shown to be O-GlcNAcylated as well as highly conserved among vertebrates. A recent paper demonstrated that OGA O-GlcNAcylation at Ser405 decreases its stability (Gorelik *et al.*, 2019). However, the identification of specific residues modified by O-GlcNAcylation *in vivo* is nearly impossible without affecting the whole O-GlcNAc proteome.

OGA was predicted to be also modified by phosphorylation, acetylation and ubiquitination at several residues (Hornbeck *et al.*, 2015). Since these sites were identified by mass spectrometry, their functional relevance should be evaluated with approaches such as site direct mutagenesis.

## **IV. Cross-talk between O-GlcNAcylation and other PTMs**

PTMs occur in almost all the proteins and the way these modifications combine to generate complex biological outcomes is only beginning to be understood (Lothrop, Torres and Fuchs, 2013; Yang and Qian, 2017). Here I will recapitulate the importance of the cross-talk between O-GlcNAcylation and phosphorylation/ubiquitination.

### **a) Interplay with phosphorylation**

The relationship between O-GlcNAc modification and phosphorylation has been well studied (see review Laarse, Leney and Heck, 2018). O-GlcNAcylation is often compared to phosphorylation since it is a reversible and dynamic process occurring in Ser and Thr residues of targeted proteins. However, unlike phosphorylation carried out

by over 600 phosphatases and 200 kinases in mammals (Smoly *et al.*, 2017), O-GlcNAcylation is carried out, as mentioned above, by only two enzymes, OGT and OGA. In fact, there are several ways in which O-GlcNAcylation and phosphorylation can be related: a) competing for the same residue, b) competing sequentially with phosphorylation on neighboring residues, c) O-GlcNAcylation and/or d) interaction with phosphatases or kinases.

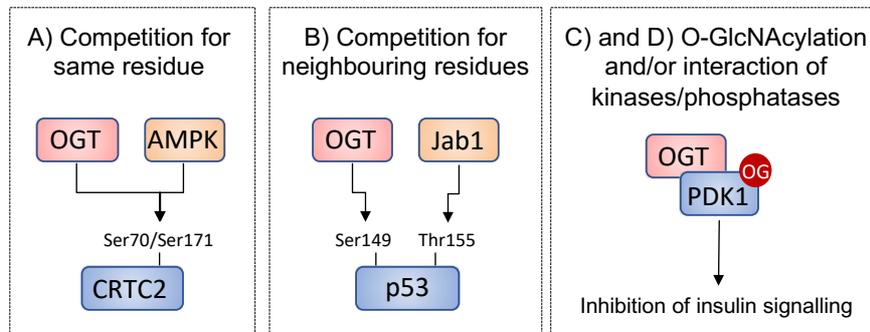


Figure 8. Cross-talk between O-GlcNAcylation and phosphorylation.

A) Example of competition between OGT and kinase (AMPK) for the residues Ser70 and Ser171 on CRTC2. B) Example of sequentially influence between O-GlcNAcylation and Jab1-mediated phosphorylation on residues Ser149 and Thr155 of the tumor protein p53. C) and D) Example of O-GlcNAcylation and interaction of OGT with 3-phosphoinositide-dependent protein kinase 1 (PDK1).

The transcription factor c-Myc was the first demonstration of phosphorylation and O-GlcNAcylation competition: phosphorylation of c-Myc on Thr58 leads to its degradation by the proteasomal machinery, while O-GlcNAc modification on the same residue increases its stability and activity (Kamemura *et al.*, 2002). In addition, CREB regulated transcription coactivator 2 (CRTC2), which is retained into the cytoplasm *via* AMPK phosphorylation of residues Ser70 and Ser171, was shown in primary mouse hepatocytes to be O-GlcNAcylated on these residues by competition with phosphorylation (**Figure 8**). These O-GlcNAc modifications translated in CRTC2 recruitment to the nucleus activating the transcription of gluconeogenic target genes (Dentin *et al.*, 2012).

Influence of phosphorylation and O-GlcNAcylation in neighboring residues is exemplified with the tumor protein p53 for which O-GlcNAcylation at Ser149 is associated with its stability and correlates with a decrease in JAK-binding protein (Jab1)-mediated phosphorylation of residue Thr155 (Yang *et al.*, 2006; Lee *et al.*, 2017) (**Figure 8**).

An example of O-GlcNAcylation as a regulator of phosphatase or kinase activity is the situation of prolonged insulin release. Under these conditions, OGT translocates

to the plasma membrane where it is activated *via* phosphorylation (Whelan, Lane and Hart, 2008). O-GlcNAcylation of key insulin signaling proteins such as 3-phosphoinositide-dependent protein kinase 1 (PDK1) (X. Yang *et al.*, 2008) leads to their inactivation thereby terminating the insulin signal (Zhang, Yin and Yang, 2014) (**Figure 8**). This mechanism appears as a mean to negatively control insulin signaling.

#### **b) Interplay with ubiquitination**

Ubiquitination, a PTM occurring on lysine (Lys) residues, targets proteins for proteasomal degradation. The interplay between O-GlcNAcylation and ubiquitination has been investigated in the past years (see review: Ruan, Nie and Yang, 2013).

For example, the expression of p53 is tightly controlled by ubiquitination-mediated proteasomal degradation. Under conditions of O-GlcNAcylation stimulation through Thiamet-G treatment, OGA silencing or OGT overexpression, O-GlcNAcylation of p53, promotes p300-dependent acetylation of p53 at Lys382 which impairs its ubiquitination and subsequent degradation (De Queiroz *et al.*, 2016) (**Figure 9**).

In specific examples, O-GlcNAcylation of proteins is also associated with the recruitment of deubiquitinases. This is the case for the transcriptional co-activator Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ). The protein host cell factor 1 (HCF-1) recruits OGT to PGC-1 $\alpha$  for its PTM. O-GlcNAcylation facilitates the recruitment of the deubiquitinase BRCA1-associated protein 1 (BAP1) protecting PGC-1 $\alpha$  from degradation (Ruan *et al.*, 2012; Lane *et al.*, 2019) (**Figure 9**).

Another example is the E3 ubiquitin ligase F-Box And Leucine Rich Repeat Protein 2 (FBXL2). Recently, O-GlcNAcylation was shown to promote poly-ubiquitination and proteasomal degradation of FBXL2 avoiding the ubiquitination and degradation of important proteins as the oncogenic transcription factor Forkhead box M1 (FOXO1), highly expressed in cancer cells (Inoue *et al.*, 2018; Ueda *et al.*, 2020) (**Figure 9**).

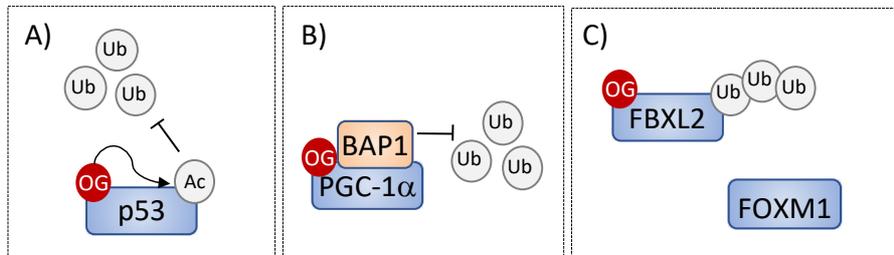


Figure 9. Interplay between O-GlcNAcylation and ubiquitination.

A) O-GlcNAcylation of p53 prevents its ubiquitination by promoting its acetylation and stability. B) PGC-1 $\alpha$  O-GlcNAcylation promotes the interaction with the deubiquitinase BAP1 leading to protein stability. C) FBXL2 O-GlcNAcylation promotes poly-ubiquitination and protein degradation preventing FOXM1 proteasomal degradation.

### 3. Role of OGT in transcriptional regulation and epigenetic modifications

As mentioned above, OGT is not only present in cytoplasm but also in the nucleus (Torres and Hart, 1984). Within the nucleus, OGT modifies a wide range of transcription factors and complexes modulating gene expression. As we will describe below, the role of OGT in transcriptional regulation can occur through many different molecular mechanisms both indirectly or directly. In addition, the role of OGT in the nucleus also involves the regulation of several epigenetic complexes (Leturcq, Lefebvre and Vercoutter-Edouart, 2017). Some examples are described below.

Nearly all RNA polymerase II (RNA pol II) proteins were shown to be O-GlcNAcylated (Krause *et al.*, 2018). The outcome of this modification depends on the parallel modification of specific transcription factors and modulated sites by OGT. As an example, assembly of the pre-initiation complex previous to transcriptional initiation requires the O-GlcNAcylation of RNA pol II at the C-terminal domain (Lewis, Burlingame and Myers, 2016). This O-GlcNAc moiety needs to be removed by OGA to allow the phosphorylation of RNA pol II with the consequential elongation initiation (Resto *et al.*, 2016).

The role of OGT in chromatin remodeling, a dynamic and highly regulated process during transcriptional activation, has been explored over the past 10 years. The well-established “histone code”, referring to the covalent and reversible modification of histones, responds to cellular stimuli or metabolism. O-GlcNAc modifications in histones have been reported but the specific function of only some of them was demonstrated so far. **Table 1** summarizes the best characterized histone O-GlcNAc modifications.

Histones O-GlcNAcylation			
Histone	Residue	Function	Reference
H2A	Ser40	Associated to viviparity	Hirosawa <i>et al.</i> , 2016
	Thr101	Alters dimerization of H2A and H2B resulting in open chromatin state and enhanced transcription	Lercher <i>et al.</i> , 2015
H2B	Ser36	Potential role in histone tail dynamics	Sakabe, Wang and Hart, 2010
	Ser112	Facilitates ubiquitination at Lys120 leading to transcriptional activation. Associated to DNA damage response.	Fujiki <i>et al.</i> , 2011; Chen <i>et al.</i> , 2013
H2AX	Ser139	Occurs in response to DNA damage, prevents phosphorylation in the same residue and suppresses amplification of phosphorylation signal events on chromatin.	Chen and Yu, 2016
H3	Ser10	Competes with phosphorylation on the same residue. Phosphorylation of H3S10 is tightly associated to cell cycle progression.	Zhang <i>et al.</i> , 2011
	Thr32	Prevents phosphorylation at H3S10 and H3S28	Fong <i>et al.</i> , 2012
H4	Ser47	Potential role in histone tail dynamics	Sakabe, Wang and Hart, 2010

Table 1. Histone O-GlcNAc modifications, function and references.

## I. OGT interacts with the HIRA histone chaperone complex

The histone cell cycle regulator A (HIRA) complex is composed by the HIRA protein, Ubinuclein 1 (UBN1) and calcineurin-binding protein 1 (CABIN1). The role of this complex is to mediate the incorporation of the variant H3.3 into the nucleosome (Loppin *et al.*, 2005). H3.3 is enriched on actively transcribed genes (transcription start sites (TSSs), gene bodies and enhancers) (Pchelintsev *et al.*, 2013) and was suggested to destabilize nucleosomes to facilitate nucleosome dynamics during transcriptional activation (Jin and Felsenfeld, 2007). In 2016, OGT was shown to be recruited into the HIRA complex through its interaction with UBN1 (**Figure 10**), facilitating the association of the HIRA complex with H3.3 (Lee and Zhang, 2016). O-GlcNAcylation sites have been identified on UBN1 and HIRA but only the sites on UBN1 were demonstrated to be required for the formation of HIRA-H3.3 complex and H3.3 nucleosome assembly (Lee and Zhang, 2016).

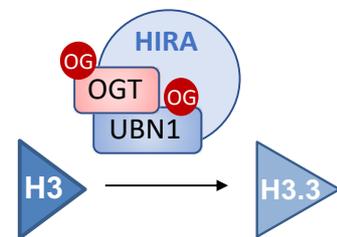


Figure 10. Regulation of HIRA complex via UBN1 O-GlcNAcylation.

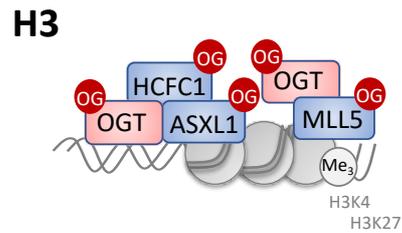
## II. OGT regulates the methylases MLL5 and ASXL1

Mixed lineage leukemia 5 (or MLL5) is a methyltransferase which catalyzes the trimethylation of Lys4 on H3 (H3K4me3). This epigenetic modification is necessary on the promoter region of target genes to initiate transcription (Tessarz and Kouzarides, 2014; Wu and Zhang, 2014). MLL5 is involved in the regulation of cell cycle progression, spermatogenesis and hematopoiesis. In 2013, OGT was shown to interact with MLL5 (Zhou *et al.*,

2013). Ding and colleagues later demonstrated in HEK293T cells that O-GlcNAcylation of MLL5 promotes protein stability by Ubiquitin-specific-processing protease 7 (USP7)-mediated deubiquitination (Ding *et al.*, 2015). In 2018, MLL5 was shown to activate gene transcription through the ASXL1-OGT axis. Additional sex comb like-1 (ASXL1) regulates gene expression through methylation of H3K27 (repressive mark), as part of the Polycomb repressive complex 2 (PRC2) or H3K4 (active mark). The authors also demonstrated that ASXL1 is part of a protein complex with HCF-1 and OGT, and that ASXL1 is stabilized by O-GlcNAcylation (**Figure 11**). Disruption of this complex inhibits myeloid differentiation, H3K4 methylation and O-GlcNAcylation of H2B, since ASXL1 is also associated to the Polycomb Repressive DeUbiquitinase (PR-DUB) complex (Inoue *et al.*, 2018).

## III. OGT regulates polycomb group (PcG) proteins

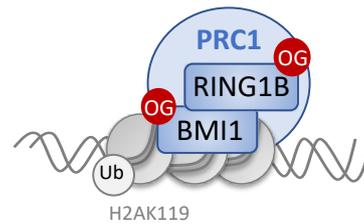
Polycomb group (PcG) proteins are master regulators of embryogenesis. Interestingly, PcGs also regulate genes involved in cellular processes such as X chromosome inactivation, genomic imprinting, cell cycle or stem cell biology (Schuettengruber *et al.*, 2017). PcG proteins are grouped in three different repressive complexes: PRC1, PRC2 and PR-DUB, which modify and remodel chromatin *via* modification of histone acetylation, methylation and ubiquitination. Interactome analysis of OGT recently revealed its interaction with several PcG proteins which will be discussed in the following sections (Gao *et al.*, 2018) (**Figure 14**).



*Figure 11. OGT regulation of MLL5 catalyzes the trimethylation of H3K4. MLL5 was suggested to activate gene transcription through the axis OGT-ASXL1 which is part of a complex with HCF-1. This complex modulates H3K4 and H3K27 methylation.*

### a) Regulation of Polycomb repressive complex 1 (PRC1) by OGT

Polycomb repressive complex 1 or PRC1 is formed by several proteins, including BMI1 and RING1B, shown to be O-GlcNAcylated. BMI1, also known as polycomb group RING finger protein 4 (PCGF4) or RING finger protein 51 (RNF51), is a protein O-GlcNAcylated at Ser255 in prostate cancer. O-GlcNAcylation on this site prevents BMI1 degradation and promotes its oncogenic activity (Li *et al.*, 2017). The catalytic subunit of PRC1 is called Ring Finger Protein 1B (RING1B) or Ring finger protein 2 (RNF2). RING1B is a E3 ubiquitin-protein described to monoubiquitylate Lys119 of H2A (H2K119Ub) promoting strong transcriptional repression (Vidal, 2009). In 2015, RING1B was reported to be O-GlcNAcylated in human embryonic stem (ES) cells, on at least two residues, Thr250/Ser251 and Ser278 (Maury *et al.*, 2015). These residues are located in the binding region to CBX7 and RYBP, two proteins targeting and repressing genes involved in early-lineage commitment of ES cells, and regulation of metabolism and cell-cycle progression respectively (Wang *et al.*, 2010; Morey *et al.*, 2013). Using mass spectrometry, O-GlcNAcylation of RING1B was shown to decrease during differentiation. Indeed, O-GlcNAcylated RING1B binds preferentially near genes related to differentiation while non-O-GlcNAcylated RING1B binds to genes related to metabolism and cell cycle processes. The authors then proposed that the regulation of RING1B by O-GlcNAcylation primarily impacts the specific interaction and recruitment with PRC1 subunits which modulates PRC1 DNA-binding and specific gene repression depending on cell fate (Maury *et al.*, 2015) (**Figure 12**).

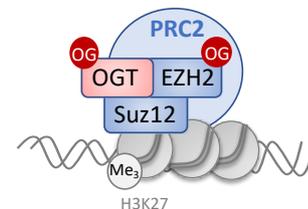


*Figure 12. Regulation of PRC1 by OGT.*

*OGT modifies two components of the PRC1 complex, BMI1 and RING1B which ubiquitination function modifies H2A at Lys119.*

### b) OGT regulates the component EZH2 of Polycomb repressive complex 2

Polycomb repressive complex 2 (PRC2) is a protein complex involved in the repression of target genes *via* the trimethylation of H3 at Lys27 (H3K27me<sub>3</sub>), catalyzed by the protein Enhancer Of Zeste 2 (EZH2) (Blackledge, Rose and Klose, 2015). OGT was reported, in MCF7 breast cancer cells, to O-GlcNAcylate EZH2 at Ser75, leading to its stability (Chu *et al.*, 2014) (**Figure 13**). A recent study identified several new O-GlcNAcylation sites in the EZH2



*Figure 13. Scheme of PRC2 regulation by OGT. OGT modulates the activity and stability of EZH2 via O-GlcNAcylation which methylates H3 at Lys27.*

protein in HEK293T cells. The authors concluded that N-terminal O-GlcNAc residues stabilizes EZH2 whereas the methyltransferase activity of EZH2 is controlled by O-GlcNAcylation on Ser279 (Lo *et al.*, 2018). The functional link between OGT-EZH2 appears to be cell and/or tissue specific-dependent since not significant effect was observed on EZH2 activity when OGT was knockdown in mouse ES cells (Myers, Panning and Burlingame, 2011). The OGT-EZH2 complex however, negatively regulates the expression of potential tumor suppressor genes in breast cancer cells (MCF7) (Chu *et al.*, 2014). These findings suggest a potential role for the OGT-EZH2 axis in the development of tumorigenesis *via* chromatin remodeling.

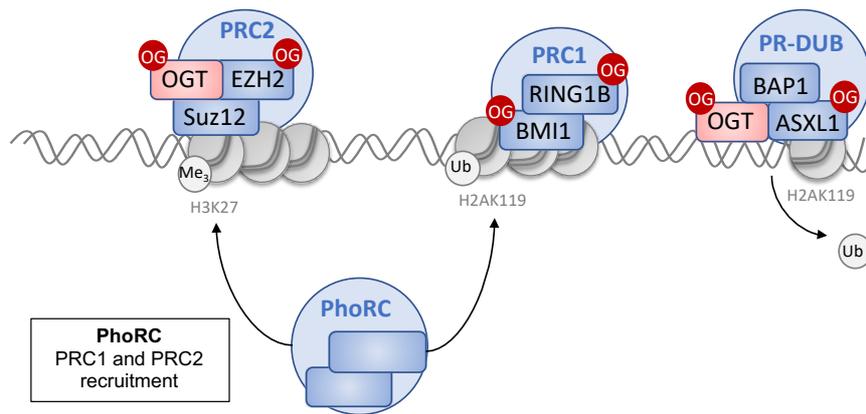


Figure 14. PcG protein complexes and function.

PRC1 is composed by BMI1 and RING1B, a protein that ubiquitinates H2A at Lys119. The deubiquitylation of H2ALys119 is carried out by the PR-DUB complex formed by ASXL1 and BAP1 deubiquitinase. PRC2 is formed by several proteins (RbAp46/48, EED, SUZ12 and EZH2). EZH2 methylates the Lys27 of H3 translating in gene repression. The PhoRC complex is involved in the recruitment of PRC1 and PRC2 to the chromatin. Adapted from Decourcelle, Leprince and Dehennaut, 2019.

#### IV. Lysine-specific demethylase 2 (LSD2) regulates OGT stability

Lysine-specific demethylase 2 (LSD2) is a protein traditionally known for its histone demethylase function. LSD2, associated to actively transcribed genes, regulates H3K4me2 demethylation (Fang *et al.*, 2010). In 2015, a E3 ubiquitin ligase activity was described for LSD2, mostly related to cancer cell growth inhibition (Yang *et al.*, 2015). LSD2 was reported to ubiquitylate OGT and to promote its proteasomal-mediated degradation. Since LSD2 is down-regulated in different cancers and cancer cell lines, Yang and collaborators proposed that an increase in OGT activity and a decrease in LSD2 expression could be required for cancer cell growth and proliferation (Yang *et al.*, 2015).

## V. Cross-regulation between OGT and TET proteins

The ten-eleven translocation (TET) proteins belong to a family of dioxygenase enzymes (TET1, TET2, TET3) involved in DNA demethylation through the conversion of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC). Proper addition or removal of this mark is necessary for vertebrate development and deregulation of DNA methylation is a feature in many diseases (Guibert and Weber, 2013). Indeed, these modifications play a role not only in DNA demethylation (Guo *et al.*, 2011) but also as a stable epigenetic mark (Bachman *et al.*, 2015) suggested to recruit specific chromatin-binding proteins to modulate chromatin structure and gene expression (Leturcq, Lefebvre and Vercoutter-Edouart, 2017; Li *et al.*, 2019). TET proteins which interact with OGT (Hrit *et al.*, 2018) are O-GlcNAcylated on several residues. O-GlcNAcylation of TET proteins correlates with changes in phosphorylation patterns, suggesting a competition between phosphorylation and O-GlcNAcylation on these proteins (Bauer *et al.*, 2015). Indeed, analysis of genome-wide distribution of OGT by Chromatin Immunoprecipitation (ChIP) overlaps significantly with TETs (Deplus *et al.*, 2013; Vella *et al.*, 2013).

### a) Interaction of OGT with TET1

TET1 plays an important role in the regulation of ES cell pluripotency and is normally downregulated during cell differentiation. TET1 presence on the promoter of genes leads to gene repression (Williams *et al.*, 2011; Delatte and Fuks, 2013; Shi *et al.*, 2013). Two studies in mouse ES cells suggested a close interaction and regulation between TET1 and OGT since depletion of either of them decreases chromatin association of the other thereby affecting its target gene expression (Deplus *et al.*, 2013; Vella *et al.*, 2013). In addition, TET1 is associated and co-localized with the corepressor mSin3A in ES cells (Williams *et al.*, 2011). Considering that OGT interacts with mSin3A (Yang, Zhang and Kudlow, 2002) it would be interesting to elucidate whether OGT-TET1-mSin3A could form a complex to repress genes in a nutrient-dependent manner. Finally, in mouse ES cells TET1 contributes to the recruitment of EZH2 to PRC2-binding sites (Wu *et al.*, 2011). Given the relationship between EZH2 and OGT, investigating the possibility that OGT acts as a nexus between EZH2 and TET1 to control substrate availability and stem cell fate decision would be of great interest.

## b) Interaction of OGT with TET2/3

TET2, a member of the TET family associated to transcriptional activation (Pastor, Aravind and Rao, 2013), is described to play an important role in tumor suppression (Cimmino *et al.*, 2017; Mouly *et al.*, 2018). In ES cells, TET2 can recruit OGT to chromatin thereby increasing H2B O-GlcNAcylation at Ser112 on the TSS region of promoters (Chen *et al.*, 2013). This modification leads to H2BK120ub (Chen *et al.*, 2013) (**Table 1**) and trimethylation of H3K4 and H3K79 by the SET1/Complex Proteins Associated with Set1 (COMPASS) complex, associated with active transcription (Bonnet, Devys and Tora, 2014).

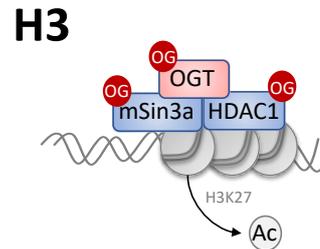
Although little is known about the relationship between TET3 and OGT, TET3 was also shown to interact with OGT and be modulated by O-GlcNAcylation. In HEK293T and HeLa cells expressing OGT and TET3 ectopically, Zhang and colleagues demonstrated that O-GlcNAcylation of TET3 regulates its nuclear export thereby decreasing its demethylase activity (Zhang *et al.*, 2014).

## VI. Role of OGT in the mSin3A-HDAC1 complex

The mSin3A-HDAC1 complex represses the expression of its target genes by deacetylation of histones and modulation of chromatin accessibility (Adams, Chandru and Cowley, 2018). However, a recent study highlighted its role as an important

transcriptional co-activator complex for the regulation of ES cells pluripotency (Saunders *et al.*, 2017). OGT binds to the mSin3A-HDAC1 complex and O-GlcNAcylates both proteins (Yang, Zhang

and Kudlow, 2002) (**Figure 15**). *In vitro* pull-down assays demonstrated a more efficient binding of OGT and mSin3A in the presence of OGA (Whisenhunt *et al.*, 2006). mSin3A is also part of the Nuclear Receptor Corepressor (NCoR)/mSin3A complex. Several of the proteins involved in mSin3A-mediated repression have been identified to be O-GlcNAcyated in ES cells including the Sex determining region Y-box 2 (SOX2), HCF-1, NCoR1 and NCoR2. These data suggest a complex relationship between OGT and mSin3A repressor complexes (Myers, Panning and Burlingame, 2011). HDAC1 O-GlcNAcylation is correlated with increased deacetylase activity with the subsequent repression of the cell cycle inhibitor p21 in hepatocellular carcinoma



*Figure 15. Scheme of OGT influence on mSin3A-HDAC1 complex.*

*OGT modifies both mSin3A and HDAC1 proteins leading to deacetylation of H3K27.*

(HCC) promoting cancer progression (Zhu *et al.*, 2016). Moreover, O-GlcNAcylated HDAC1 is increased in cancer cell lines and HCC biopsies compared to non-tumoral tissue and OGT was also described to play a role in HCC development as we will discuss in **Chapter 3** (Rikimaru *et al.*, 2007; Zhu *et al.*, 2012).

## VII. OGT interacts with the repressor complex NuRD

The Nucleosome Remodeling and Deacetylation (NuRD) complex is a gene transcription repressive complex which modulates chromatin accessibility. The NuRD complex contains 14 proteins and increased expression of 9 of them was recently identified in HCC biopsies compared to normal liver samples (Shao *et al.*, 2020). Co-immunoprecipitation experiments revealed that OGT interacts with the NuRD complex *via* binding to HDAC1 and HDAC2. The authors also showed that OGT promotes endothelial-mesenchymal transition (EMT) and invasion of cervical cancer cells (Gao *et al.*, 2018). However, the physiological relevance of the interaction between OGT and the complex NuRD needs to be further investigated.

## VIII. OGT regulates the MOF-NSL complex

The human Males absent on the first (MOF)-containing HAT nonspecific lethal (NSL) complex is formed by 9 proteins including OGT. OGT regulates the NSL complex *via* O-GlcNAcylation and stabilization of the subunit NSL3 that leads to the acetylation of H4 Lys5, Lys8, Lys16 (Wu *et al.*, 2017) (**Figure 16**). This complex plays an important role in dosage compensation, ES cell renewal, DNA damage and repair, cell survival and gene expression regulation (Yi Chen, Costa and Sun, 2015). In addition, MOF-NSL dysregulation has been implicated in several human cancers such as HCC (Zhang *et al.*, 2014) or colorectal cancer (Cao *et al.*, 2014).

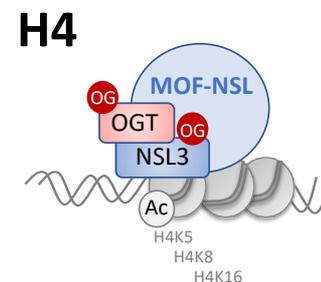


Figure 16. Scheme of OGT regulation of MOF-NSL complex. O-GlcNAcylation of NSL3 leads to acetylation of H4K5, H4K8, H4K16.

The following figure recapitulates the role of OGT in the regulation of epigenetics marks and complexes mentioned above.

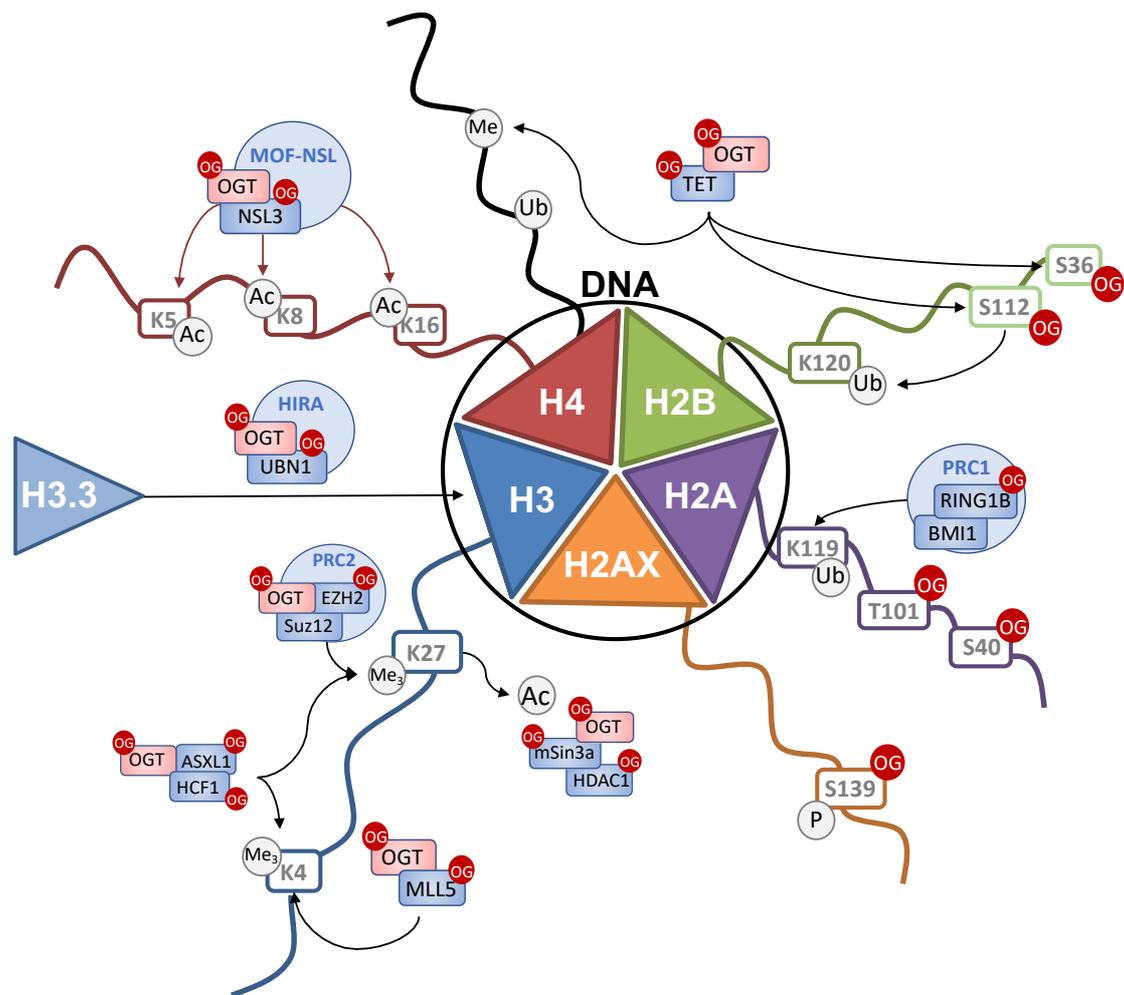


Figure 17. Implications of OGT in epigenetic modulations.

OGT interacts and modulates the activity of proteins involved in several complexes such as MOF-NSL, HIRA and the Polycomb repressor complexes: PRC1 and PRC2 and PR-DUB. Recapitulation of the epigenetic marks regulated by these complexes together with the O-GlcNAcylated residues described up to date. OG indicates proteins or residues shown to be O-GlcNAcylated.

## CHAPTER 1: RELEVANT POINTS

1. OGT is a glycosyl transferase which modifies Ser and Thr residues of target proteins located in the cytoplasm, nucleus and mitochondria
2. OGA is the enzyme that hydrolyzes O-GlcNAcylation of OGT target proteins
3. OGT and OGA dynamics need to be kept at equilibrium, disruption of this balance leads to pathological conditions such as metabolic syndrome or cancer
4. O-GlcNAcylation presents an important crosstalk with other PTMs such as phosphorylation, ubiquitination, acetylation and methylation
5. OGT plays an important role in the regulation of gene transcription and epigenetics

# Chapter 2: Role of OGT in metabolic and signaling pathways

## 1. Role of OGT in glucose metabolism

Under feeding conditions, carbohydrates are processed and digested in the digestive tract. The resulting monosaccharides, mainly glucose, are transported to different organs, including the liver, to generate energy in the form of ATP. In mammalian tissues, glycolysis is the pathway involved in the catabolism of glucose into pyruvate. Pyruvate is transported to the mitochondrial matrix to enter the citric acid cycle (also called Krebs cycle or TCA cycle) where a series of metabolic reactions will generate energy in form of GTP, as well as NADH and FADH<sub>2</sub>, both important electron carriers for the electron transport chain (ETC) to generate ATP. In some cases, such as ischemic conditions, pyruvate is used in a process called anaerobic glycolysis where pyruvate is converted into lactate to regenerate NAD<sup>+</sup> necessary for ATP generation. Excessive carbohydrates in the liver are primarily stored as glycogen *via* glycogenesis. However, continuous glucose uptake by the liver can also be converted into fatty acids *via de novo* lipogenesis using acetyl-CoA to generate triglycerides (TGs). TGs can be incorporated into very low-density lipoproteins (VLDL) to be stored as lipid droplets, transported and stored in white adipose tissue (WAT) or metabolized through the  $\beta$ -oxidation.

Fasting conditions stimulate the liberation of glucagon by the pancreatic  $\alpha$ -cells promoting generation of glucose by the liver to be used as a fuel for peripheral tissues. Overnight fasting causes glycogen breakdown *via* glycogenolysis to maintain glucose homeostasis. However, longer term fasting or starvation requires *de novo* glucose synthesis through gluconeogenesis (**Figure 18**).

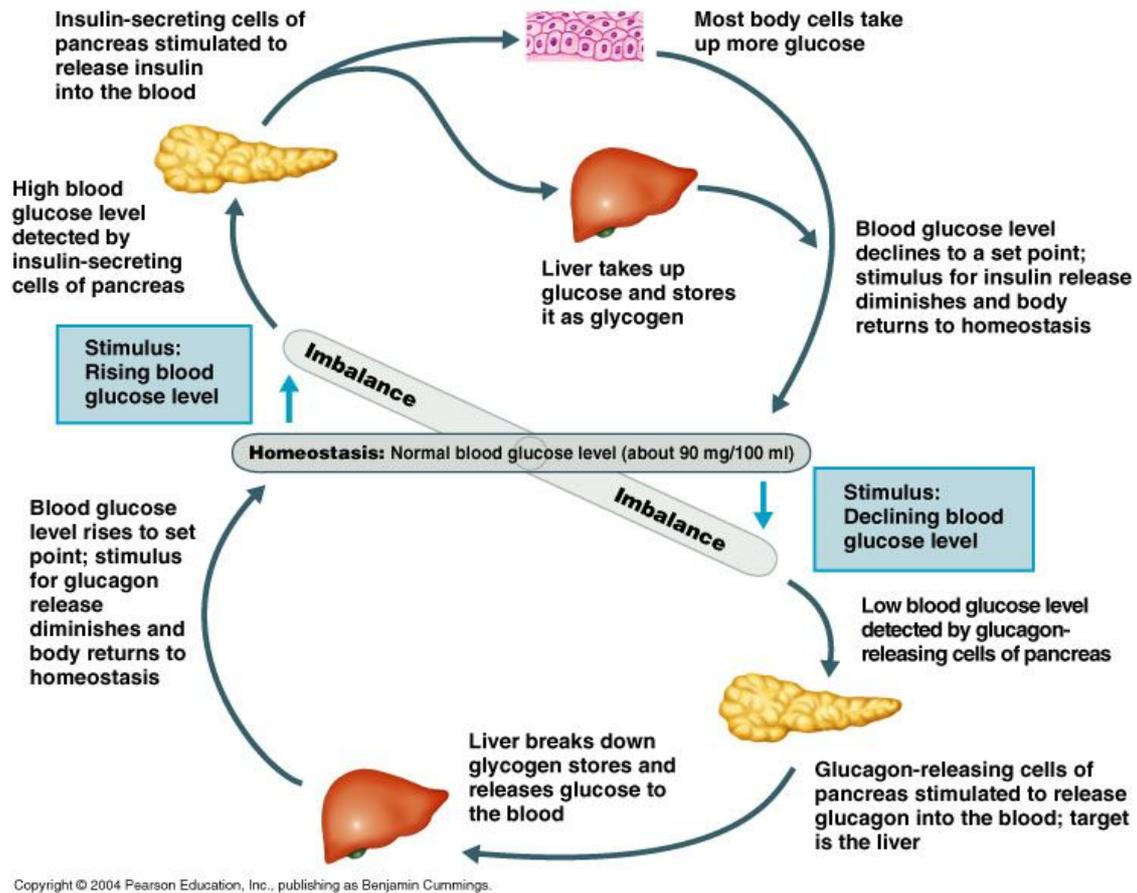


Figure 18. Schematic representation of glucose metabolism regulation. Decreased blood glucose during fasting promotes glucagon release by the pancreatic  $\alpha$ -cells. Glucagon secretion stimulates glycogen breakdown and glucose release to the blood returning to a homeostatic stage. Upon glucose uptake, blood glucose increases generating a stimulus in pancreatic  $\beta$ -cells which liberate insulin. Insulin release triggers glucose uptake by peripheral insulin-sensitive tissues and glucose storage in the form of glycogen in the liver. From Campbell and Reece, 2005.

## I. Glycolysis

Glycolysis is the first critical pathway for glucose metabolism in most cells for energy generation. This pathway, occurring in the cytosol, consists in two phases: the preparatory phase, called like this for the necessity to invest 2 ATPs; and the pay-off phase, since it generates 4 ATPs and 2 NADH per molecule of glucose entering the pathway (Nelson and Cox, 2017) (**Figure 19**). The glycolysis pathway has three key enzymes: Glucokinase (GK, also called HK), Phosphofruktokinase 1 (PFK1) and Liver-pyruvate kinase (L-PK). Two of these enzymes are O-GlcNAcylated (Yi *et al.*, 2012; Baldini *et al.*, 2016); however, these are not the only glycolytic enzymes regulated by OGT. Here, we will discuss the regulation of glycolysis by O-GlcNAcylation and OGT.

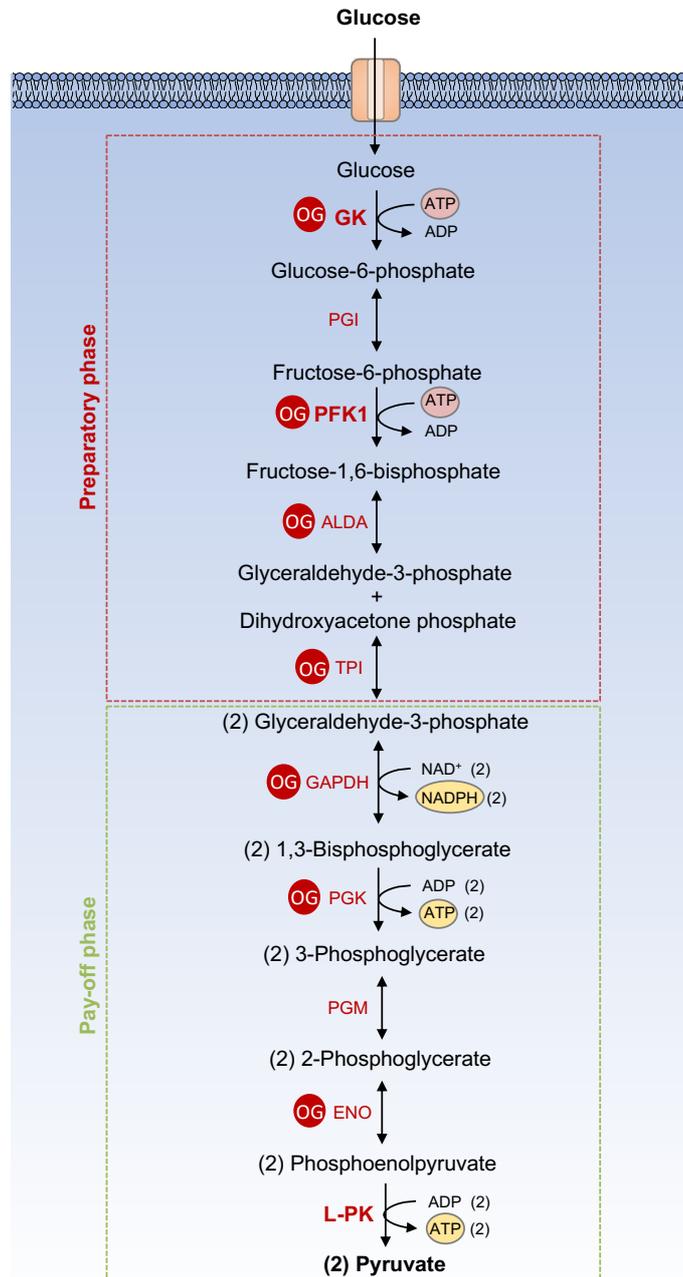


Figure 19. Scheme of the glycolysis pathway

Preparatory phase: Glucose is phosphorylated by glucokinase (GK/HK) to glucose-6-phosphate. Transformation to fructose-6-phosphate is carried out by Phosphoglucose isomerase (PGI). PFK1 catalyzes the glycolysis commitment reaction of phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate with the consumption of a second ATP molecule. Finally, Aldolase A (ALDA) breaks down fructose-1,6-bisphosphate to a molecule of glyceraldehyde-3-phosphate and a molecule of dihydroxyacetone phosphate. Triosephosphate isomerase (TPI) converts dihydroxyacetone phosphate to glyceraldehyde-3-phosphate to enter the pay-off phase. Pay-off phase: Starting with the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate by the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) with the production of NADPH. Phosphoglycerate kinase (PGK) dephosphorylates 1,3-bisphosphoglycerate to 3-phosphoglycerate generating a molecule of ATP for each molecule of glyceraldehyde-3-phosphate. 3-phosphoglycerate is converted to 2-phosphoglycerate by Phosphoglycerate mutase (PGM). Finally, Enolase (ENO) transforms 2-phosphoglycerate to phosphoenolpyruvate which is converted into pyruvate by the Liver-pyruvate kinase (L-PK) enzyme that generates the last two molecules of ATP. OG indicates O-GlcNAcylated proteins.

### a) Glucokinase: from glucose to glucose-6-phosphate

The GK hexokinase phosphorylates glucose to glucose-6-phosphate (G6P). GK activity is inhibited by the presence of F6P and the enzyme is sequestered in the nucleus *via* its interaction with the protein glucokinase regulatory protein (GKRP) (**Figure 20**). Increase in glucose concentration promotes competition of glucose with F6P for the GK-GKRP complex binding. It promotes GK nuclear export and release from GKRP (Van Schaftingen, Detheux and Da Cunha, 1994; Brown *et al.*, 1997; Han *et al.*, 2016; Matschinsky and Wilson, 2019). GK is transcriptionally regulated by the transcription factor Sterol regulatory element binding protein 1 (SREBP1). SREBP1 presents two isoforms generated from the same gene *Srebp1*: SREBP1a, mainly expressed in the intestine and spleen, and SREBP1c, highly expressed in liver, muscle and adipose tissue. SREBP1 proteins are synthesized as precursors bound to the ER membrane by interaction with SREBP-cleavage activating protein (SCAP) (Dorotea, Koya and Ha, 2020). Upon glucose consumption, insulin secretion stimulates SREBP1c translocation to the Golgi apparatus for maturation and activation *via* cleavage of the catalytic N-terminal domain. Mature SREBP1c translocates to the nucleus where it binds to the SRE binding sequence of the *Gk* promoter and several other genes involved in *de novo* lipogenesis (Foretz *et al.*, 1999; Dorotea, Koya and Ha, 2020). Interestingly, GK is also post-translationally modified by OGT. Glucose uptake favors the O-GlcNAcylation of GK increasing its stability and activity (Baldini *et al.*, 2016). In addition, OGT was shown to interact with the nuclear receptor REV-ERB $\alpha$  promoting OGT stability and function. In conditions of low insulin levels, the REV-ERB $\alpha$ /OGT complex interacts with TET proteins at the level of *Srebp1* promoter favoring the methylation and control of SREBP1c expression (Berthier *et al.*, 2018).

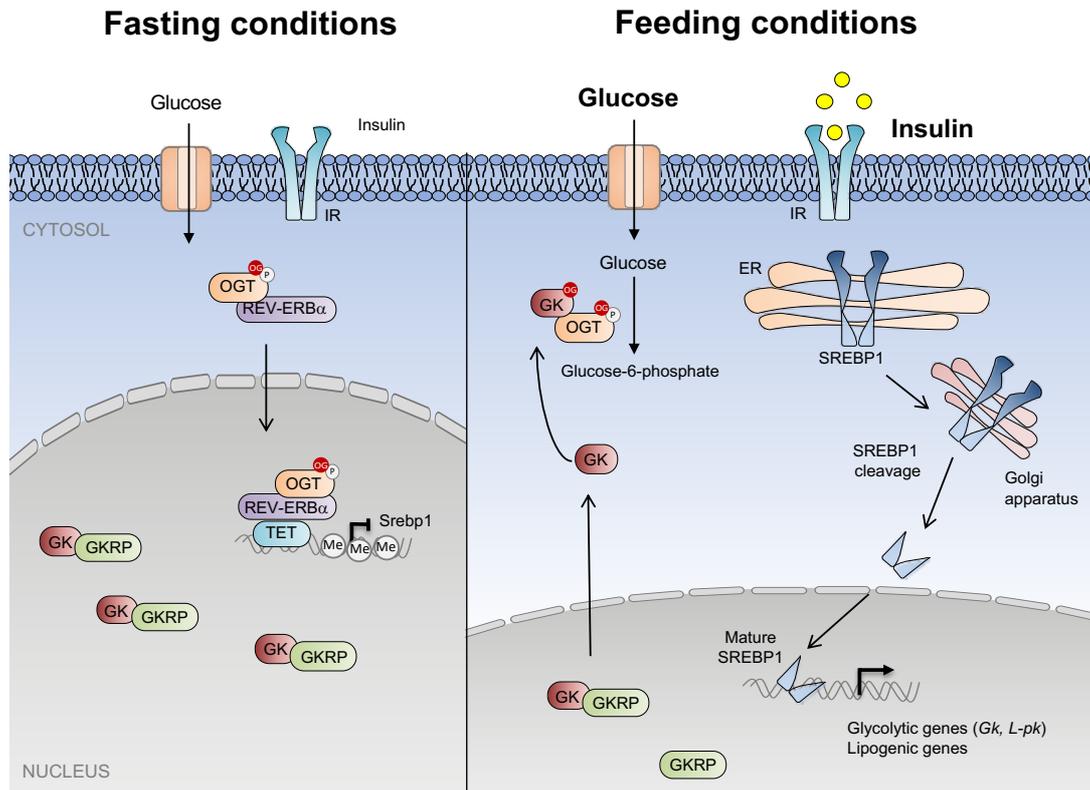


Figure 20. Glucokinase (GK) regulation by OGT. During fasting, GK is retained in the nucleus by interaction with the glucokinase regulatory protein (GKRP). OGT binding to REV-ERB $\alpha$  translocates into the nucleus and, via interaction and activation of TET proteins, represses the expression of Srebp1. Upon glucose and insulin stimulation, GK is liberated from the GKRP protein, exported from the nucleus and activated by O-GlcNAcylation. At the same time, SREBP1 translocates to the Golgi apparatus for cleavage and activation. Activation of SREBP1 promotes its translocation to the nucleus where it activates the expression of Gk. OG indicates O-GlcNAcylated proteins.

### b) Phosphofructokinase 1 (PFK1): second ATP consuming step

PFK1 catalyzes an irreversible reaction committing glucose to glycolysis. PFK1 is allosterically inhibited by ATP and citrate, which are considered signals of energy abundance; and is allosterically activated by fructose-2,6-bisphosphate, a non-glycolytic metabolite which regulates glucose metabolism in the liver. The product of this enzyme fructose-1,6-bisphosphate is particularly important since it positively regulates the last key regulatory step of glycolysis carried out by L-PK (Han *et al.*, 2016). PFK1 is modified at Ser529 through O-GlcNAcylation in response to hypoxia. PFK1 O-GlcNAcylation inhibits its activity and directs glucose to the pentose phosphate pathway (PPP) used by cancer cells to sustain proliferation. Blocking PFK-1 O-GlcNAcylation on Ser529 reduces cancer cell proliferation *in vitro* and tumor formation *in vivo* (Wen Yi *et al.*, 2012). Furthermore, HIF1 $\alpha$  and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases 3 (PFKFB3), both proteins involved in the

regulation of PFK1 are also O-GlcNAcylated. The O-GlcNAc modification in both cases causes activation and stability of HIF1 $\alpha$  and PFKFB3 proteins reducing ER stress-mediated apoptosis and cell cycle progression respectively. Induction of HIF1 $\alpha$  and PFKFB3 O-GlcNAcylation is correlated to proliferation and survival of cancer cells (Ferrer *et al.*, 2014; Lei *et al.*, 2020).

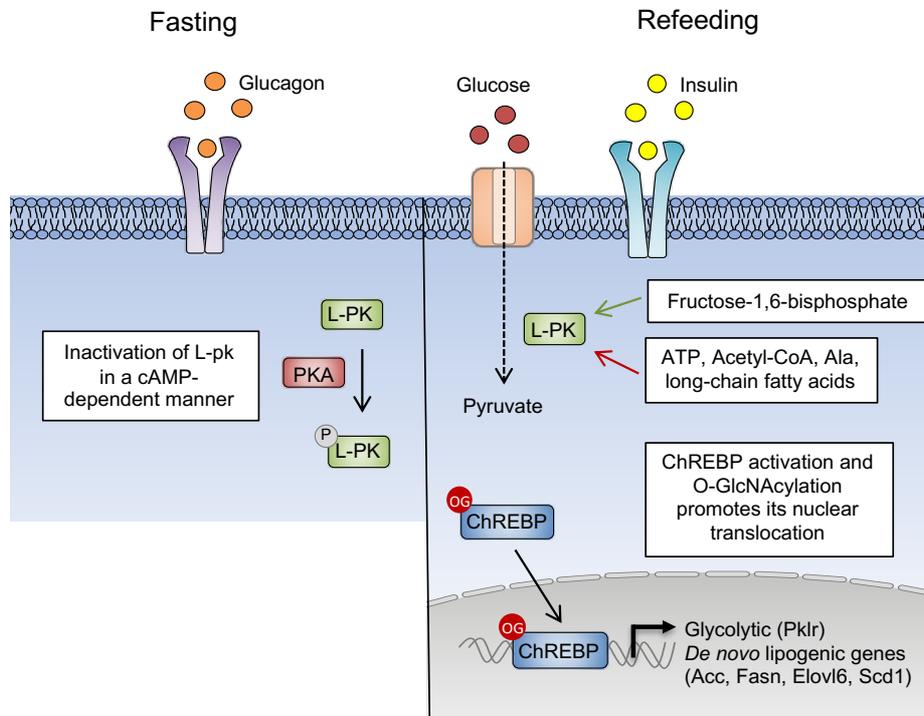
### c) Liver-pyruvate kinase (L-PK): final step in the generation of pyruvate

L-PK, which is encoded by the *Pklr* gene, catalyzes the last reaction of glycolysis for the generation of pyruvate. Its activity is allosterically regulated by fructose-1,6-bisphosphate and inhibited by signaling molecules of energy abundance such as ATP, acetyl-CoA or long-chain fatty acids. The amino acid Alanine can also act as an inhibitor of L-PK activity since it can be converted to pyruvate. L-PK is post-translationally regulated. During fasting and upon glucagon release, protein kinase A (PKA) phosphorylates L-PK in a cAMP-dependent manner (**Figure 21**). Under feeding conditions, L-PK is activated by dephosphorylation (Han *et al.*, 2016).

L-PK is also transcriptionally regulated by the glucose-sensing transcription factor carbohydrate response element binding protein (ChREBP) (Yamashita *et al.*, 2001). ChREBP contains a low glucose inhibitory domain (LID) and a glucose-response activation conserved element (GRACE) located in the N-terminus. Several years ago, a new constitutively activated isoform of ChREBP, called ChREBP $\beta$ , was identified to generate from an alternative first exon promoter, called exon 1 $\beta$ , lacking the LID domain (M. a Herman *et al.*, 2012). ChREBP $\beta$  regulatory mechanism is still being elucidated; however, it is thought to be transcriptionally activated by, the renamed, ChREBP $\alpha$  isoform since it contains ChREBP $\alpha$  consensus sequence carbohydrate response element (ChoRE) on its exon promoter 1 $\beta$  (M. a Herman *et al.*, 2012). Upon carbohydrate consumption, the GRACE domain of ChREBP is activated by glucose metabolites such as G6P or xylulose-5-phosphate (X5P), provoking an allosteric conformational change (McFerrin and Atchley, 2012), which allows the binding of activators and cofactors such as Max like protein X (Mlx), obligatory partner of ChREBP, the nuclear receptor Hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ) and, the recently identified partners, HCF-1 $\alpha$  and histone demethylase plant homeodomain finger 2 (Phf2) (Meng *et al.*, 2016; Bricambert *et al.*, 2018; Lane *et al.*, 2019). In 2011, ChREBP was described to be modified *via* O-GlcNAcylation (Guinez *et al.*, 2011). Recently, the modification of ChREBP was described to be depend on O-GlcNAcylation of HCF-1 $\alpha$

that leads to recruitment of OGT to ChREBP and ChREBP O-GlcNAcylation (Lane *et al.*, 2019). ChREBP O-GlcNAcylation was reported to increase its transcriptional activity and DNA binding (Guinez *et al.*, 2011; Yang *et al.*, 2017). Interestingly, several ChREBP residues modified by O-GlcNAcylation were recently identified by mass spectrometry (Yang *et al.*, 2017). In particular, O-GlcNAcylation at Thr517 and Ser839 were described to be essential for the nuclear importation of ChREBP and heterodimerization with Mlx (Yang *et al.*, 2017). Within the nucleus, ChREBP binds to its consensus binding sequence, ChoRE, on the *Pklr* promoter and several genes involved in *de novo* lipogenesis pathway (Abdul-Wahed, Guilmeau and Postic, 2017; Ortega-Prieto and Postic, 2019) (**Figure 21**).

### Regulation of L-PK



*Figure 21. Regulation of liver-pyruvate kinase (L-PK) by the nutritional status. During fasting, activation of protein kinase A (PKA) phosphorylates and inactivates LPK. Upon refeeding, glucose and insulin signaling promote the activation of L-PK via fructose-1,6-bisphosphate. Glucose metabolites induce allosteric conformational changes in the transcription factor ChREBP favoring its activation. ChREBP O-GlcNAcylation is essential for its translocation to the nucleus and transcriptional activation of L-PK. OG indicates O-GlcNAcylation of proteins.*

Pyruvate kinase muscle isoform 2 (PKM2) is an equivalent enzyme to L-PK since it catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate when present as a tetramer in the cytoplasm. However, this enzyme, when present in the nucleus in its dimer form, has also a nonmetabolic function as transcriptional factor of genes involved in proliferation and cell growth (Z. Zhang *et al.*, 2019). PKM2 is highly expressed in

proliferating and cancer cells, especially in HCC (Christofk *et al.*, 2008; Lv *et al.*, 2018). PKM2 was identified to be a target of OGT and O-GlcNAcylation several years ago (Chaiyawat *et al.*, 2015). A recent study reported that OGA-mediated PKM2 acetylation promotes its O-GlcNAcylation at Ser362 and Thr365. Because PKM2 regulation is controlled by its tetrameric status, O-GlcNAcylation of PKM2 inhibits its pyruvate kinase activity *via* blocking its tetramerization and increases cell proliferation and tumor growth in cancer HeLa cell (Singh *et al.*, 2020).

#### **d) Phosphoglycerate kinase (PGK)**

Phosphoglycerate kinase or PGK, the first ATP producing enzyme of glycolysis, was recently demonstrated to be O-GlcNAcyated on Thr255 (Nie *et al.*, 2020). This modification stimulates PGK activity enhancing lactate production and inducing PGK mitochondrial translocation. In the mitochondria, PGK acts as a kinase inhibiting the activity of the Pyruvate dehydrogenase (PDH) complex reducing oxidative phosphorylation. Furthermore, PGK O-GlcNAcylation levels are increased in human colon cancers. The authors demonstrated that inhibition of PGK O-GlcNAcylation at Thr255 decreases proliferation of colonic cancer cells, suppresses glycolysis, enhances TCA cycle and inhibits tumor growth in xenograft models (Nie *et al.*, 2020).

Although several studies reported O-GlcNAcylation of glycolytic enzymes such as Aldolase A (ALDA), Triosephosphate isomerase (TPI), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or Enolase (ENO), the contribution and functional role of these modifications have not been investigated yet (Champattanachai *et al.*, 2013; Lambert, Bastide and Cieniewski-Bernard, 2018).

## **II. Citric acid cycle**

The TCA cycle is a central hub for energy metabolism, synthesis of macromolecules and redox balance. Fuel sources for the TCA cycle include glucose, glutamine and fatty acids. The TCA cycle carries out a series of reactions which generate intermediate molecules used in the synthesis of macromolecules, as well as energy, in the form of ATP or GTP, and energy acceptor (NADH and FADH<sub>2</sub>) used, posteriorly, in the ETC.

In non-proliferative cells, most of the fuel source used in the TCA cycle is glucose, which enters the cycle in form of pyruvate. Pyruvate is primarily oxidized to acetyl-CoA by the PDH complex. In addition to glucose, cells can also use amino acids as fuel. Amino acids enter the TCA cycle after converting to acetyl-CoA or  $\alpha$ -keto acid

intermediates such as pyruvate, oxaloacetate or succinyl-CoA (Nelson and Cox, 2017). Glutamine is the most abundant amino acid in the human body and it enters the TCA cycle in the form of  $\alpha$ -ketoglutarate ( $\alpha$ -KG) (C. Yang *et al.*, 2014). The third types of fuels used by the TCA cycle are fatty acids. Fatty acid catabolism is carried out through  $\beta$ -oxidation generating acetyl-CoA metabolites, entering the TCA cycle.

In cancer cells, glucose usage is markedly increased, as noted by Otto Warburg nearly 100 years ago. Up-regulation of high-affinity glucose transporters, such as Glucose transporter (GLUT) 1 and GLUT3, increases glucose uptake (Birnbaum, Haspel and Rosen, 1987). However, cancer cells not only increase their rate of glucose uptake but also change the fate of imported glucose. Most cancer cells preferentially use glucose to produce lactate even in normoxic conditions (J. Kim *et al.*, 2006). Compared to aerobic glycolysis, which after TCA cycle and ETC reaction generates 38 ATPs, anaerobic glycolysis only generates 2 ATP molecules. To meet their high energetic needs, cancer cells use glutamine as a fuel source to feed the TCA cycle (Anderson *et al.*, 2018). To do so, cancer cells upregulate the glutamine transporters and the enzymes in charge of the glutaminolysis process at least in part *via* the pro-oncogene MYC (Wise *et al.*, 2008).

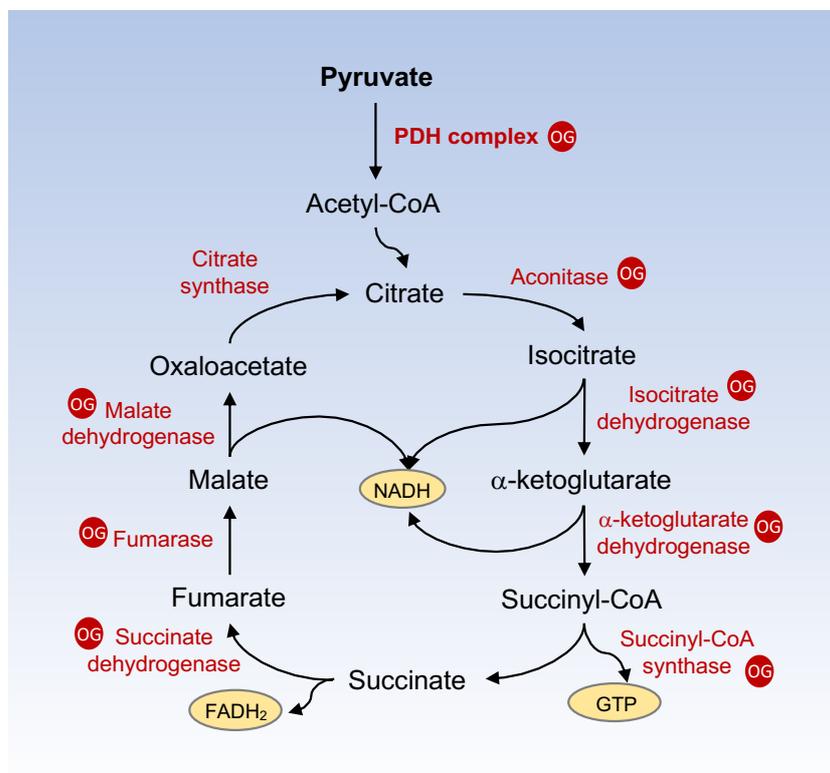


Figure 22. TCA cycle regulation by O-GlcNAcylation. Pyruvate, the final product of glycolysis, is transformed into acetyl-CoA via the pyruvate dehydrogenase (PDH) complex. Acetyl-CoA enters the TCA cycle by transformation to citrate carried out via the Citrate synthase. Aconitase converts citrate to isocitrate, which will generate

*NADH in its transformation to  $\alpha$ -ketoglutarate and succinyl-CoA. Succinyl-CoA is transformed to succinate with the generation of a molecule of GTP and, posteriorly, fumarate with the generation of FADH<sub>2</sub>. Fumarase converts fumarate to malate and finally, to oxaloacetate by the malate dehydrogenase. Oxaloacetate returns to the original point to produce citrate with the addition of a molecule of acetyl-CoA. OG indicates O-GlcNAcylated proteins.*

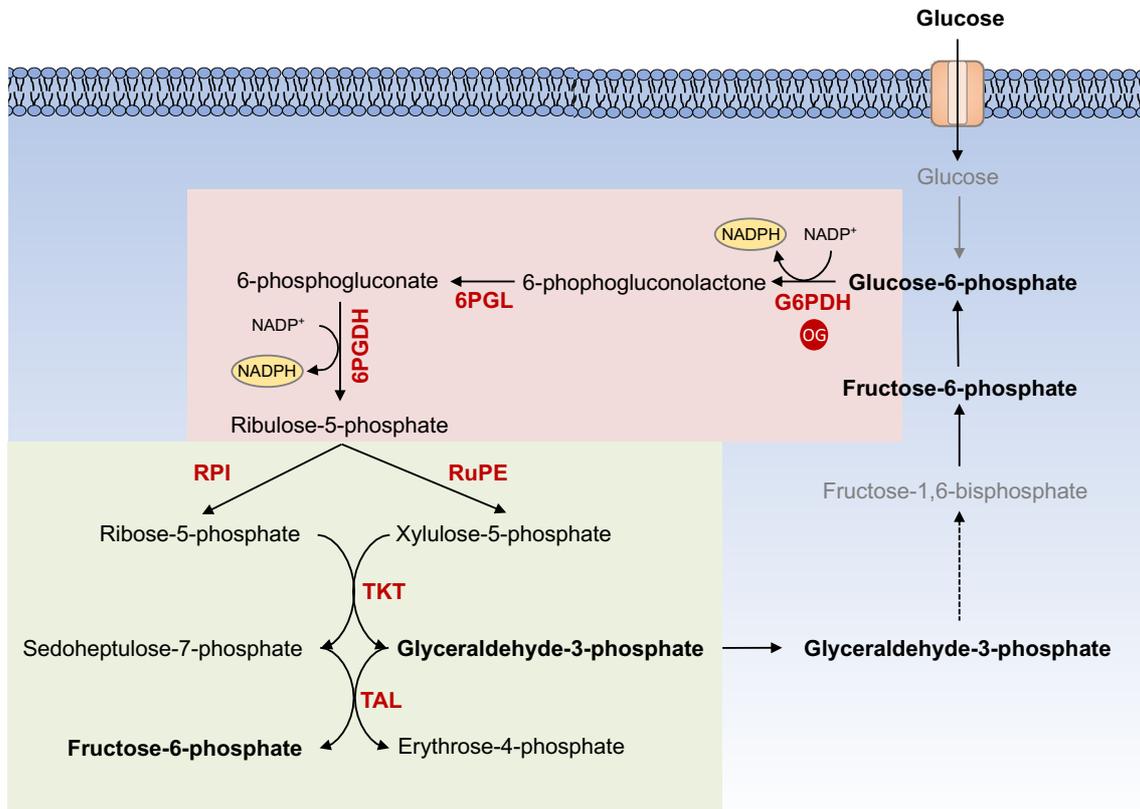
### **a) Regulation of the TCA cycle via O-GlcNAcylation**

The role of OGT in the regulation of the TCA cycle is just starting to be understood (**Figure 22**). In 2008, utilization of the UDP-GalNAc labeling approach in a cortical neuron model demonstrated the O-GlcNAcylation of alpha and beta subunits of the PDH complex (Clark *et al.*, 2008). O-GlcNAcylation of PDH was shown essential for PDH complex mitochondrial localization (Nagaraj *et al.*, 2017). Another proteomic study in isolated mitochondria from rat livers using the method  $\beta$ -elimination/Michael Addition with DTT (BEMAD), identified malate dehydrogenase O-GlcNAcylation (Cao *et al.*, 2013). Using Stable Isotope Labelling by Amino Acids (SILAC)-labelling in neuroblastoma cells with overexpression of either OGT or OGA, Tan and colleagues identified altered expression of PDH complex components, aconitase, isocitrate dehydrogenase,  $\alpha$ -ketoglutarate dehydrogenase and succinyl-CoA synthase (Tan *et al.*, 2014). In pancreatic cancer cells, fumarase was the most recent enzyme described to be O-GlcNAcylated. O-GlcNAcylation occurs at Ser75 competing for the phosphorylation site of AMPK. Fumarase phosphorylation on Ser75 promotes the formation of a complex with the protein Activating transcription factor 2 (ATF2) (Wang *et al.*, 2017). In turn, the fumarase-ATF2 complex participates in the activation of cell growth arrest genes *via* the inhibition of Lysine-specific demethylase 2A (KDM2A) demethylase activity (Y. Jiang *et al.*, 2015). Hence, O-GlcNAcylation of fumarase impairs cell cycle arrest maintaining tumor growth (Wang *et al.*, 2017). As mentioned above, the enzymatic activity and mitochondrial translocation of PGK1 is regulated by O-GlcNAcylation at Thr255. In the mitochondria, PGK1 acts as a kinase to phosphorylate the PDH complex inactivating its activity. In conclusion, O-GlcNAcylation of PGK1 promotes Warburg effect and development of colon cancer (Nie *et al.*, 2020).

### **III. The pentose phosphate pathway**

The pentose pathway (PPP) is used by cancer cells to support survival and cell growth. The PPP consists in two metabolic branches: the oxidative branch, which generates NADPH required for the generation of reduced glutathione (GSH), a major scavenger of reactive oxygen species (ROS), and fatty acid synthesis required for cell proliferation; and the non-oxidative branch, that generated pentose phosphates

necessary for generation of nucleotides (Jin and Zhou, 2019) (**Figure 23**). In cells in which maintaining redox homeostasis is more important than generating nucleotides, the oxidative branch will be accelerated and the non-oxidative branch will favor the generation of F6P to fuel the oxidative branch. However, for high proliferative cells in need of pentose phosphates, the non-oxidative PPP will be accelerated (Patra and Hay, 2014).



*Figure 23. Regulation of the pentose phosphate pathway (PPP).*

*The oxidative part of the PPP (red) converts glucose-6-phosphate into ribulose-5-phosphate by the activity of glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconolactonase (6-PGL), and 6-phosphogluconate dehydrogenase (6PGDH) generating NADPH. Ribulose-5-phosphate enters the non-oxidative path (green) where it is isomerized to ribose-5-phosphate by ribose-5-phosphate isomerase (RPI) or converted to xylulose-5-phosphate by the ribulose-5-phosphate-3-epimerase (RuPE). Finally, transketolase (TKT) and transaldolase (TAL) combine different substrates to generate different metabolites needed for cell proliferation.*

#### **a) Direct regulation of the PPP by OGT**

Glucose-6-phosphate dehydrogenase (G6PDH) is the rate-limiting enzyme of the PPP. In physiological conditions, G6PDH is highly expressed in several tissues such as liver (Rudack, Chisholm and Holten, 1971), adipose (Park *et al.*, 2005), mammary (Hilf *et al.*, 1975) and adrenal glands (Okano *et al.*, 1965). However, G6PDH is normally present as an inactive monomer and induction of its activity is being regulated by formation of an homodimer (Au *et al.*, 2000). In terms of activity regulation, one of the

main regulators of G6PDH is the NADP<sup>+</sup>/NADPH ratio. Cancer cells have elevated expression and activity of G6PDH, resulting in increased NADP<sup>+</sup> concentrations (Mele *et al.*, 2018). OGT was also demonstrated to activate G6PDH *via* O-GlcNAcylation (**Figure 23**). Interestingly, blockade of G6PDH O-GlcNAcylation decreases cell proliferation in cancer cells *in vitro* and tumor growth *in vivo* (Rao *et al.*, 2015).

#### **b) Indirect regulation of the PPP *via* O-GlcNAcylation of p53 and NRF2**

In response to DNA damage and oxidative stress, the kinase activity of the protein Ataxia telangiectasia mutated (ATM) activates not only p53, but also the interaction between G6PDH and the Heat shock protein (Hsp) 27, causing an increase in G6PDH activity (Cosentino, Grieco and Costanzo, 2011). However, the tumor suppressor p53 was also described to bind G6PDH and inhibits the formation of its active homodimer (Jiang *et al.*, 2011). These two apparently contradictory effects of p53 in the PPP could be explained by the two functions of p53: cell cycle arrest and apoptosis. In conditions of DNA damage induction, p53 blocks cell cycle to favor the repairing of DNA before re-entering the cycle, at the same time than potentiating the generation of nucleotides to maintain cell survival. Nevertheless, in cases in which DNA damage cannot be repaired, p53 induces cell death by inhibition of the PPP, blocks the generation of NADPH and increases the levels of intracellular ROS (Patra and Hay, 2014). As mentioned in **Figure 8**, p53 is activated *via* O-GlcNAcylation thereby indirectly modulating the PPP (Yang *et al.*, 2006).

The Nuclear factor erythroid 2-related factor 2 (NRF2) regulates several enzymes of the PPP, including G6PDH, through binding to its consensus sequence Antioxidant response element (ARE) on their promoters. NRF2 activates both the oxidative and non-oxidative branch for NADPH and nucleotides production (Mitsuishi *et al.*, 2012). Under stress circumstances, expression of NRF2 is induced by the oncogenic c-myc, K-Ras and B-Raf. In cancer cells, this activation is maintained through hyperactivation of the PI3K/AKT signaling pathway (DeNicola *et al.*, 2011). NRF2 is also indirectly regulated by OGT since the protein Kelch-like erythroid cell-derived protein with CNC homology (ECH) associated protein 1 (KEAP1, also known as KLHL), involved in NRF2 cytoplasmic retention, is O-GlcNAcylated (Chen, Chi and Boyce, 2017). It was demonstrated that O-GlcNAcylation of KEAP1 at Ser104 promotes its interaction with Cullin-3 (CUL3) that ubiquitinates NRF2 for its proteasomal degradation. (Chen *et al.*, 2017) (**Figure 24**).

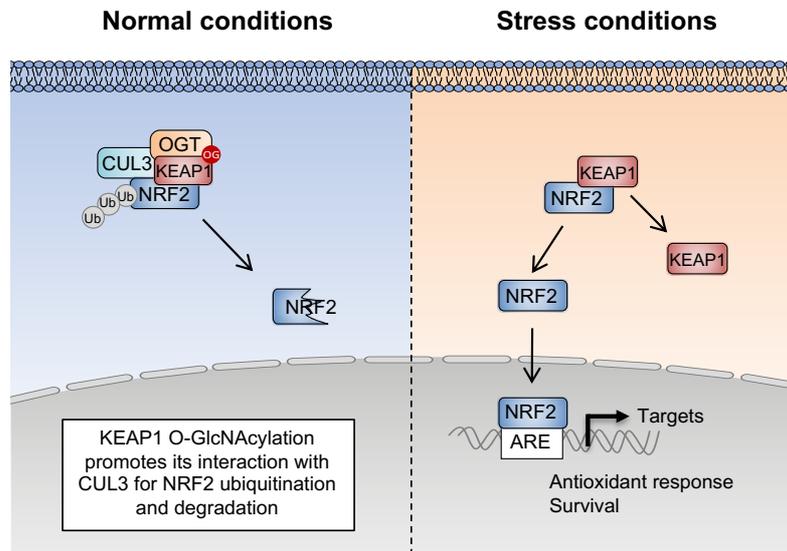


Figure 24. NRF2 regulation by KEAP1.

In normal conditions, KEAP1 is O-GlcNAcylated by OGT promoting its interaction with Cullin-3 (CUL3) that ubiquitinates NRF2 for its proteosomal degradation. Under stress conditions, the KEAP1-NRF2 interaction is lost and NRF2 translocates to the nucleus where it stimulates its targets involved in antioxidant response and survival.

#### IV. Hepatic glucose production

Under short-term fasting conditions, the liver produces glucose *via* glycogen breakdown, a process called glycogenolysis. In long-term fasting periods such as starvation, the liver generates glucose *de novo* in a process called gluconeogenesis. Gluconeogenesis is described as the reversal process of glycolysis, yet the three irreversible steps of glycolysis are catalyzed by four enzymes in the gluconeogenesis pathway: Pyruvate carboxylase (PC), Phosphoenolpyruvate carboxykinase (PEPCK), Fructose-1,6-biphosphatase (FBPase) and Glucose-6-phosphatase (G6Pase) (Zhang *et al.*, 2019) (Figure 25).

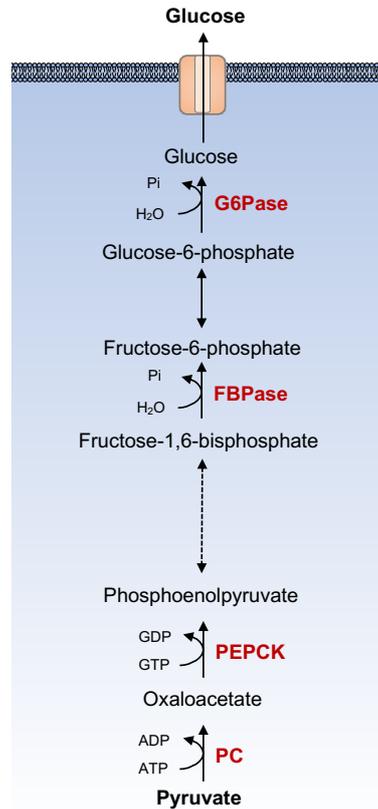


Figure 25. Gluconeogenesis regulation.

Gluconeogenesis starts with the transformation of lactate and alanine into pyruvate, which is carboxylated to oxaloacetate by the pyruvate carboxylase (PC) enzyme. Phosphoenolpyruvate carboxykinase (PEPCK) decarboxylates oxaloacetate to phosphoenolpyruvate which enters the gluconeogenic cycle. Several reversal steps from glycolysis are carried out for the generation of F1,6BP, which is dephosphorylated to F6P by the Fructose-1,6-bisphosphatase (FBPase) enzyme. F6P is converted to G6P and finally dephosphorylated by glucose-6-phosphatase (G6Pase) to glucose to be secreted.

### a) Indirect regulation of gluconeogenesis via O-GlcNAcylation

During short-term fasting, glucagon stimulates the activation of cyclic AMP-responsive element-binding protein (CREB) via PKA-mediated phosphorylation at Ser133 (Gonzalez and Montminy, 1989). CREB activation promotes interaction with different co-factors such as CBP/p300, which acetylates histones promoting activation of CREB target gene transcription or CRTC2 (Altarejos and Montminy, 2011). CREB not only upregulates the expression of gluconeogenic genes such as PC, PEPCK and G6Pase but also PGC-1 $\alpha$  which is a critical co-factor for the regulation of prolonged glucagon-mediated gluconeogenesis (Herzig *et al.*, 2001). As mentioned in **Figure 8**, CRTC2 is O-GlcNAcylated at Ser70 and Ser171 competing with phosphorylation and stimulating its nuclear translocation and binding to CREB (Dentin *et al.*, 2008) (**Figure 26**). Prolonged fasting induces the activation of PGC-1 $\alpha$  which acts as a co-factor of HNF4 and Forkhead box protein O1 (FoxO1) proteins for the activation of

gluconeogenesis (Herzig *et al.*, 2001). HCF-1-mediated PGC-1 $\alpha$  O-GlcNAcylation promotes its stability by recruitment of the deubiquitinase BAP1 and facilitates the recruitment of OGT to FoxO1 for its activation (Housley *et al.*, 2009; Ruan *et al.*, 2012). In addition, in response to high glucose or glucosamine concentrations and/or oxidative stress, FoxO1 is O-GlcNAcylated and promotes the transcription of G6Pase and PEPCK (Kuo *et al.*, 2008; Housley *et al.*, 2009). The Notch1 receptor, a known partner of FoxO1 in the regulation of gluconeogenesis, is also O-GlcNAcylated. Indeed, increased glucosamine concentration was shown to activate O-GlcNAcylation and glucose production through the cleavage and activation of FoxO1 and Notch1 in mESCs. These data suggested a role of O-GlcNAcylation in regulating self-renewal maintenance in mESCs (Jeon *et al.*, 2014). Finally, bile acids (BAs) activation of the nuclear receptor Farnesoid X receptor (FXR) induces the expression of the Small heterodimer partner (SHP). SHP protein acts as a transcriptional repressor of gluconeogenic genes such as PEPCK, FBPase and G6Pase resulting in decreased glucose production (Potthoff *et al.*, 2011). FXR is also a target of OGT: its O-GlcNAcylation at Ser62 promotes FXR stability and enhanced its activity (Berrabah *et al.*, 2014) (**Figure 26**).

### Regulation of gluconeogenesis

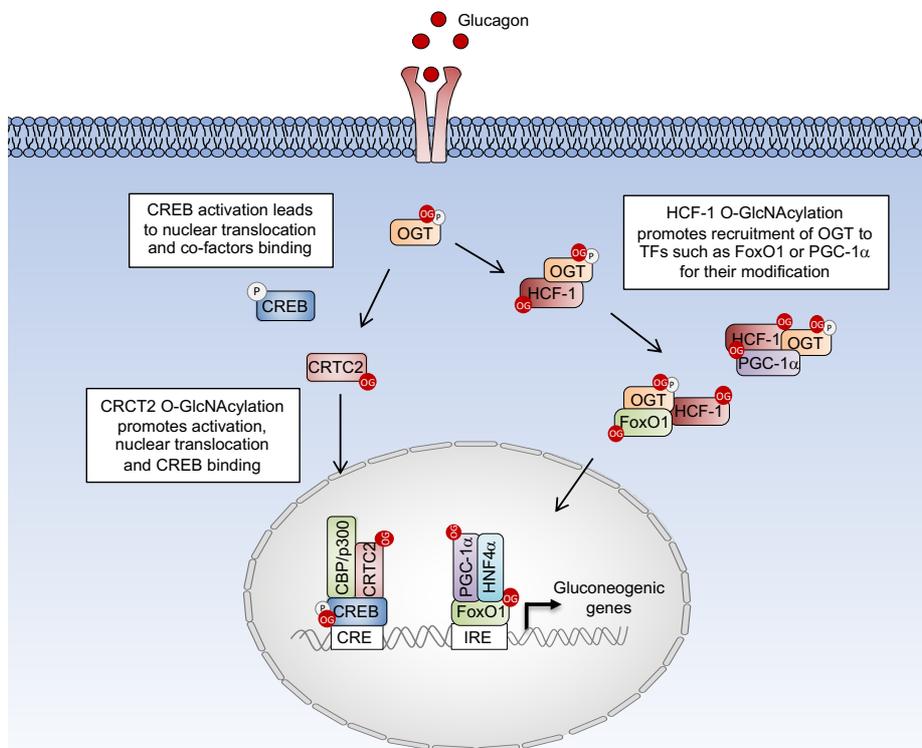


Figure 26. Regulation of gluconeogenesis by O-GlcNAcylation

*Glucagon release causes phosphorylation-mediated activation of CREB that translocates to the nucleus, binding to its co-factor and activating gluconeogenic gene expression. CRCT2, one of CREB cofactor, is O-GlcNAcylated promoting its activation and nuclear translocation. HCF-1 O-GlcNAcylation promotes OGT recruitment to PGC-1 $\alpha$  and FoxO1 for their PTM. Activation of FoxO1 and PGC-1 $\alpha$  by O-GlcNAcylation promotes nuclear translocation and activation of gluconeogenic targets. CREB, cyclic AMP-responsive element-binding protein. CRCT2, CREB regulated transcription coactivator 2. HCF-1, Host cell factor 1. PGC-1 $\alpha$ , Peroxisome proliferator-activated receptor gamma coactivator 1-alpha. FoxO1, Forkhead box protein O1. HNF4 $\alpha$ , Hepatocyte nuclear factor 4 $\alpha$ . CRE, cAMP response elements. IRE, insulin response elements. OG indicates O-GlcNAcylated proteins*

## **2. Role of OGT in lipid metabolism**

The liver is involved in the metabolism of fatty acids and triglycerides (TGs) including uptake, biosynthesis, stores, secretion and oxidation (Alves-Bezerra and Cohen, 2017). Vertebrates liver use fats from different sources: fats consumed in the diet, mobilized fat stored from specialized tissues such as adipose tissue, or, in the liver, converting excess of carbohydrates to fat (Nelson and Cox, 2017).

Under fed conditions, the liver's primary fuel is glucose rather than fat. Dietary fat present in the form of chylomicrons is mostly directed to muscle and adipose tissue. In the blood, the extracellular lipoprotein lipase enzyme will hydrolyze TGs into fatty acids and glycerol. The remnants of chylomicrons, containing TGs, cholesterol and apolipoproteins, are taken up by the liver. TGs within the liver are directed to be oxidized or to provide precursors for ketone bodies synthesis.

In fasting conditions, lipolysis in white adipose tissue increases the levels of plasma free fatty acids (FFAs). FFAs are taken up by the liver and directed toward  $\beta$ -oxidation for ketone body production, used as a fuel when glucose is scarce (see review in Alves-Bezerra and Cohen, 2017).

### **I. *De novo* lipid biosynthesis from glucose**

Excess glucose uptake by the liver can be directed to the generation of fatty acids by *de novo* lipogenesis. The generation of fatty acids in the liver has normally two possible fates: 1) in highly proliferative cells, cells require the generation of phospholipids for the synthesis of plasmatic membranes, constituted of a glycerol group, two fatty acids and a phosphate-linked head group; 2) the second will be the storage of fatty acids in form of TGs, constituted of the glycerol-3-phosphate group bound to three fatty acids. These TGs can be stored in the liver, however the majority of them are packed into VLDLs and secreted to be stored in peripheral tissues such as adipose tissue.

### **a) SREBP1c positively regulates *de novo* lipogenesis**

SREBP1 proteins are transcription factors involved in the activation of glycolysis but only SREBP1c is involved in the synthesis of fatty acids and TGs in the liver (Horton, Goldstein and Brown, 2002) (**Figure 27**). In hepatocytes, insulin activation of AKT signaling activates the synthesis and processing of SREBP1c, the predominant SREBP isoform (Owen *et al.*, 2012). In 2012, Moon and colleagues demonstrated in *Scap* liver-specific knock-out mice that SREBP1c is required in mediating hepatic steatosis and fatty liver development during carbohydrate-induced hypertriglyceridemia and insulin resistance (Moon *et al.*, 2012). In addition, AMPK-mediated phosphorylation of SREBP1c blocks its cleavage and nuclear translocation (Bullen *et al.*, 2014). Considering that OGT O-GlcNAcylates AMPK potentially potentiating its activity, it would be interesting to elucidate the possible regulation of SREBP1c *via* AMPK O-GlcNAcylation in the control of hepatic lipogenesis.

### **b) Stimulation of *de novo* lipogenesis *via* ChREBP**

In response to glucose activation, ChREBP not only activates the expression of *L-pk*, but also of lipogenic genes such as Acetyl-CoA carboxylase (*Acc*), Fatty acid synthase (*Fasn*), Stearoyl-CoA (*Scd1*) and Elongation of long-chain fatty acids family member 6 (*Elovl6*) (Ortega-Prieto and Postic, 2019) (**Figure 27**). Indeed, administration of fructose in mice induces ChREBP-dependent hepatic steatosis (Kim *et al.*, 2016). In addition, hepatic overexpression of ChREBP in mice leads to liver steatosis dissociated from insulin resistance (Benhamed *et al.*, 2012). Moreover, liver-specific knock-out mice of ChREBP show a reduction in *de novo* lipogenesis and TGs in *ob/ob* mice (Dentin *et al.*, 2006). As mentioned previously, ChREBP is a glucose responsive transcription factor regulated *via* several PTMs including O-GlcNAcylation. Regulation of *de novo* lipogenesis by OGT was shown in 2011 by our team. ChREBP is post-translationally activated *via* O-GlcNAcylation at Thr517 and Ser839 in hepatocytes increasing its DNA binding affinity and activity (Guinez *et al.*, 2011; Yang *et al.*, 2017). However, OGT was recently highlighted to regulate *de novo* lipogenesis also through a ChREBP novel partner, HCF-1 (Lane *et al.*, 2019). HCF-1 O-GlcNAcylation is stimulated in response to glucose or carbohydrate stimulus. In a HCF-1-dependent manner, OGT is recruited to ChREBP and induces its O-GlcNAcylation. This study demonstrates that the effect of OGT in *de novo* lipogenesis control not only requires ChREBP but also HCF-1 (Lane *et al.*, 2019).

### c) LXR directly and indirectly controls *de novo* lipogenesis

Liver X receptors (LXRs) are members of the superfamily of nuclear hormone receptors activated by oxysterols, molecules generated by cholesterol oxidation (Chen *et al.*, 2007). LXRs bind to LXR-responsive elements (LXRE) in the promoter of its target genes in cooperation with the nuclear receptor Retinoid X receptor (RXR). LXR regulates *Acc*, *Fasn* and *Scd1* through binding to its promoter, or indirectly by upregulating ChREBP and SREBP1c expression (Repa *et al.*, 2000; Schultz, 2000; Cha and Repa, 2007) (**Figure 27**). O-GlcNAcylation of LXR increases the expression of lipogenic genes but also increases its ability to activate the transcription of SREBP1c and ChREBP, which reinforces the up-regulation of *de novo* lipogenesis pathway (Anthonisen *et al.*, 2010; Bindsbøll *et al.*, 2015).

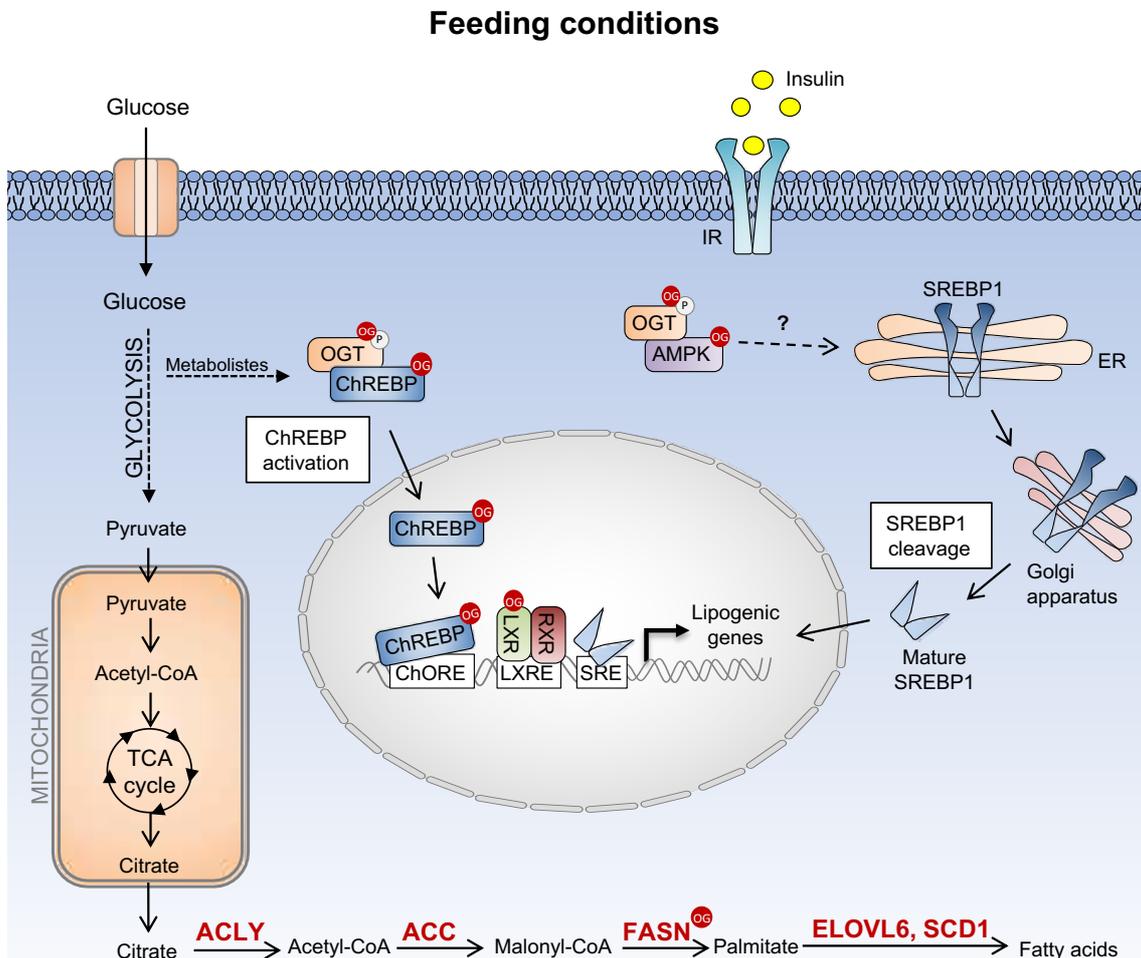


Figure 27. Regulation of *de novo* lipogenesis by OGT and O-GlcNAcylation  
 Pyruvate produced during glycolysis is transported into the mitochondria for the generation of acetyl-CoA. The acetyl-CoA molecules can enter the TCA cycle or be transported back to the cytosol for *de novo* biosynthesis of lipids. For the transport of acetyl-CoA, the Citrate synthase (CS) enzyme is required to link two acetyl-CoAs for the generation of citrate. Citrate is

transported to the cytosol by the Citrate transport protein (CTP) and, once in the cytosol, the ATP-citrate lyase (ACLY) enzyme converts citrate back to acetyl-CoA. Transformation of acetyl-CoA to malonyl-CoA is catalyzed by the Acetyl-CoA carboxylase (ACC) enzyme. Malonyl-CoA will be used as a substrate to build the carbon chain of fatty acids. Fatty acid synthase (FASN), the key rate-limiting enzyme in this pathway, catalyzes the conversion of malonyl-CoA into the fatty acid palmitate. Palmitate, a 16-carbon saturated fatty acid, is the main product of *de novo* lipogenesis pathway. However, several reactions can convert palmitate into complex fatty acids via elongation carried out by the protein elongation of long-chain fatty acids family member 6 (ELOVL6) or saturation catalyzed by the Stearoyl-CoA desaturase-1 (SCD1). OGT activates ChREBP and LXR inducing the expression of their target genes of *de novo* lipogenesis pathway. Furthermore, OGT potentiates the activity of the AMP-activated protein kinase (AMPK). Finally, OGT stabilizes FASN via O-GlcNAcylation, which increases its enzymatic activity. OG indicates O-GlcNAcylated proteins.

#### **d) Negative regulation of *de novo* lipogenesis via FoxO1 and FXR**

The transcription factor FoxO1 was implicated in the regulation of hepatic lipid metabolism. AKT regulates the activity of FoxO1 by phosphorylation at Thr24, Ser256 and Ser319 which leads to Casein kinase 1 (CK1)-mediated phosphorylation at Ser322 and Ser325 (Rena *et al.*, 1999; Rena, 2002). Phosphorylation of FoxO1 promotes its binding to the protein 14-3-3 causing nuclear export and Skp-2-mediated degradation (Huang *et al.*, 2005). Hepatic O-GlcNAcylation of FoxO1 was reported to increase in diabetic patients in response to glucose consumption (Housley *et al.*, 2008). FoxO1 is O-GlcNAcylated at Thr317, Ser550, Thr648 and Ser654. Increased FoxO1 O-GlcNAcylation was reported to activate the expression of gluconeogenic and enzymes involved in hepatic detoxification (Housley *et al.*, 2008). Its role in the regulation of lipogenesis is still unclear since *FoxO1* liver-specific transgenic mice expressing an active FoxO1 mutant, display a reduction of glycolysis and *de novo* lipogenesis resulting in decreased TG concentrations (W. Zhang *et al.*, 2006; Zhang *et al.*, 2016). However, other groups reported opposite effect with increased hepatic TGs concentrations when overexpressing FoxO1 in the liver (Altomonte *et al.*, 2003; Qu *et al.*, 2006). While the mechanisms are not clearly elucidated, one of the possible way by which FoxO1 controls *de novo* lipogenesis could be through its regulation of SREBP1c (Deng *et al.*, 2012). Elucidating the role of OGT in the regulation of FoxO1 in the context of lipid metabolism would be of high interest for the field.

Interestingly, the nuclear receptor FXR, mostly known to regulate bile acid metabolism, also participates to the regulation of FASN expression (Shen *et al.*, 2011). In response to high glucose concentrations, FXR interacts with OGT and is O-GlcNAcylated at Ser62 (AF-1 domain) leading to enhanced activity and stability (Berrabah *et al.*, 2014). Interestingly, FXR was also reported to interact with ChREBP through its AF-1 domain in immortalized human hepatocytes. FXR-ChREBP

interaction causes a decrease in ChREBP recruitment to the promoter of its target genes (Caron *et al.*, 2013). Interestingly, whether O-GlcNAcylation affects the binding of FXR to ChREBP is not known.

#### **f) OGT post-translationally regulates FASN**

FASN, the enzyme catalyzing the conversion of malonyl-CoA to palmitate, interacts with OGT and is post-translationally stabilized *via* O-GlcNAcylation (Baldini *et al.*, 2016). Immortalized human hepatocytes cultured in physiological (5mM) or high concentrations of glucose (25mM) treated with O-GlcNAc stimulators show insulin-mediated transcriptional activation of *Fasn* and increased levels of O-GlcNAcylation. Cells treated with 6-diazo-5-oxonorleucine (DON), an inhibitor of GFAT, exhibited reduced expression of *Fasn*. Interestingly, when DON treatment was complemented with glucosamine, a molecule entering the HBP bypassing GFAT, expression of *Fasn* was rescued. *In vitro*, increased in O-GlcNAcylation levels led to an increased interaction between FASN and the deubiquitinating enzyme ubiquitin-specific protease-2a (USP2A), which protected FASN from degradation (Baldini *et al.*, 2016). Furthermore, in conditions of oxidative-stress (treatment with H<sub>2</sub>O<sub>2</sub>) in U2OS human osteosarcoma cells, O-GlcNAcylation levels of FASN are induced. In this context, the authors reported that FASN overexpression promotes its interaction with OGA which blocks its activity and increases O-GlcNAcylation levels (Groves *et al.*, 2017).

## **II. Lipid oxidation**

Mitochondrial fatty acid  $\beta$ -oxidation (FAO) is the major pathway involved in the degradation of long-chain fatty acids. FAO not only provides fuel to the TCA cycle but also stimulates the hepatic synthesis of ketone bodies such as (R)-3-hydroxybutyrate (3HB) and acetoacetate (AcAc). In addition, FAO generates reduced FADH<sub>2</sub> and NADH used in the ETC.

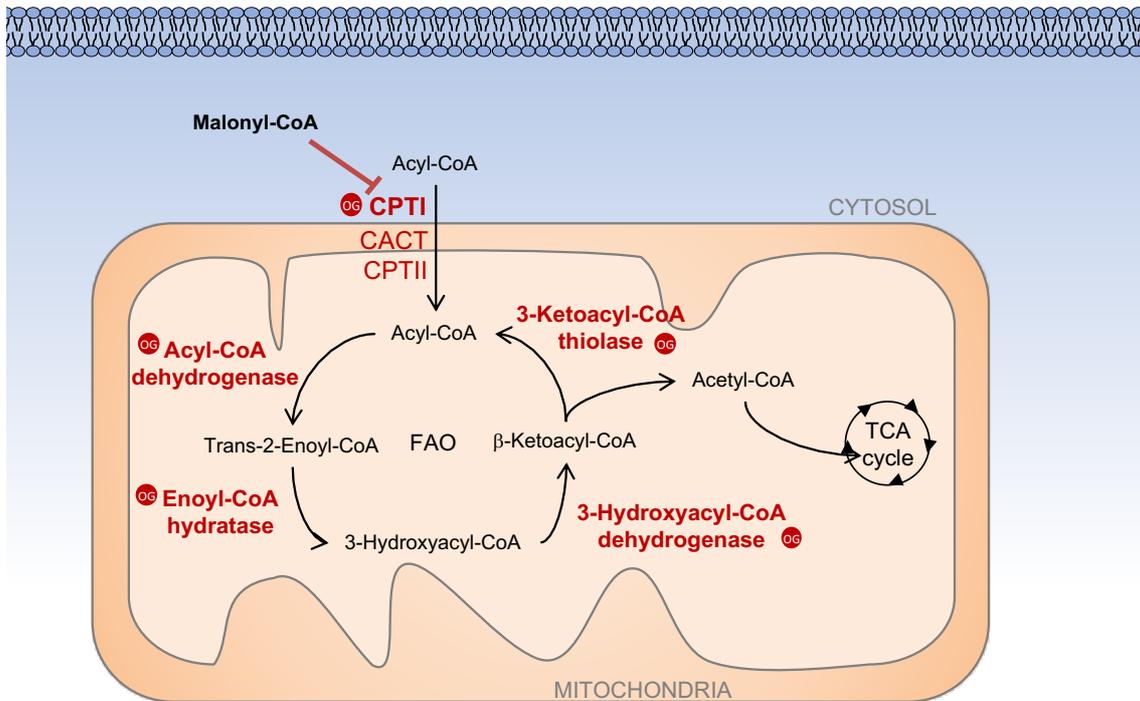


Figure 28. Regulation of the  $\beta$ -oxidation pathway

After fatty acids uptake their activation to acyl-CoAs in the cytosol and their transport to the mitochondria are required. Considering that mitochondrial membrane is impermeable to acyl-CoAs, the carnitine cycle is necessary for its import. The system requires L-carnitine and is composed by two acyl-transferases, Carnitine palmitoyltransferases I and II, CPTI and CPTII, but also the protein Carnitine acyl-carnitine translocase (CACT). CPTI catalyzes the conversion of acyl-CoA to acylcarnitine which will be transported to the inner mitochondrial membrane via CACT to finally be reconverted to acyl-CoA by the protein CPTII (Nelson and Cox, 2017). Inside the mitochondria,  $\beta$ -oxidation degrades acyl-CoAs by a cycling process consisting in four enzymatic steps, each one releasing two carbons in the form of acetyl-CoA. 1) Acyl-CoA dehydrogenase transforms acyl-CoA to trans-2-enoyl-CoA. 2) Enoyl-CoA hydratase generates 3-Hydroxyacyl-CoA by hydration of trans-2-enoyl-CoA. 3) 3-Hydroxyacyl-CoA dehydrogenase converts (S)-3-hydroxyacyl-CoA to  $\beta$ -ketoacyl-CoA. 4) 3-ketoacyl-CoA thiolase cleaves  $\beta$ -ketoacyl-CoA in a molecule of acyl-CoA and another of acetyl-CoA. OG indicates O-GlcNAcylated proteins.

The FAO pathway is regulated at different steps *via* transcriptional or post-transcriptional mechanisms. Carnitine palmitoyltransferase I (CPTI), the rate-limiting enzyme of FAO, constitutes a family of three members: CPTIA expressed in the liver, CPTIB present in muscle, and CPTIC enzymatically active in the brain. CPTI is negatively regulated by malonyl-CoA (Zammit, 2008); however, CPTIA is 30 to 100-fold more resistant to malonyl-CoA-mediated allosteric inhibition than CPTIB suggesting a role in cancer cells to maintain both lipogenesis and FAO activated at the same time (Qu *et al.*, 2016).

A proteomic study performed in heart of streptozotocin (STZ)-induced diabetic rats, revealed that CPTI is modified by O-GlcNAcylation, potentiating its activity. CPTI is not the only O-GlcNAcylated protein in this pathway, the four enzymes controlling

the FAO cycle (Acyl-CoA dehydrogenase, Enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase) are also O-GlcNAcylated (Ma *et al.*, 2016) (**Figure 28**).

The transcriptional control of FAO enzymes is regulated by Peroxisome proliferator-activated receptors (PPARs). PPARs are generally located in the nucleus (Lefebvre *et al.*, 2006). After ligand-induced activation, PPARs shuttle to the nucleus pairing with their partner RXR. The PPAR-RXR heterodimer binds to the Peroxisome proliferator response element (PPRE) consensus sequence on the promoter of their target genes (Capelli *et al.*, 2016). In the liver, the most abundant and better characterized is PPAR $\alpha$ , which is responsible for the transcriptional activation of CPT1 (Song *et al.*, 2010). While PPAR $\alpha$  has not been described to be O-GlcNAcylated, the fact that PPAR $\gamma$  is O-GlcNAcylated (Ji *et al.*, 2012) rises the possibility of OGT as a potential regulator of PPAR $\alpha$ .

PGC-1 $\alpha$ , expressed in the liver in fasted animals, is associated with increased FAO *via* the transcriptional activation of CPT1. PGC-1 $\alpha$  is also a driver of the fasting response in the liver *via* its control of gluconeogenesis (Yoon *et al.*, 2001), lipid catabolism (Estall *et al.*, 2009) and detoxification of ROS produced by the mitochondria (Besse-Patin *et al.*, 2017). As indicated previously (**Figure 9**), PGC-1 $\alpha$  is O-GlcNAcylated, resulting in an increase in its activity and stability (Ruan *et al.*, 2012; Lane *et al.*, 2019). Decreased PGC-1 $\alpha$  protein content was recently reported in adipose tissue from adipocyte-specific *Ogt* knock-out mice, associated with impairment in adipose tissue browning in these mice (Ohashi *et al.*, 2017).

#### **4. Role of OGT in bile acid metabolism**

BAs play an essential role in lipid absorption and cholesterol homeostasis. However, over the last years, BAs emerged as unexpected players of the regulation of glucose homeostasis and secretion of glucoregulatory hormones (Hofmann, 2009). Formed from cholesterol in the liver, BAs constitute around 90% of total cholesterol catabolism. There are two types of BAs (**Figure 29**). Primary BAs, synthesized in the liver, are conjugated to amino acids and transported to the gallbladder for storage. Primary BAs are secreted to the duodenum to facilitate absorption of dietary lipids, cholesterol and liposoluble vitamins. Secondary BAs, formed in the large intestine where resident bacteria catalyzes the 7 $\alpha$ -dehydroxylation of primary BAs. BAs, both primary and secondary, can return to the liver *via* portal circulation where lithocholic

acid (LCA) and chenodeoxycholic acid (CDCA) are converted into the 6 $\alpha$ -hydroxylated hyodeoxycholic (HDCA) and hyocholic acids (HCA), respectively (Monte *et al.*, 2009; Perreault *et al.*, 2013). Nevertheless, BAs species and composition vary between human and rodent (Ahmad and Haeusler, 2019). High-affinity BAs transporters are predominant in the liver and the ileum and regulate the metabolism of BAs (Ahmad and Haeusler, 2019). BAs possess detergent properties, which can lead to cytotoxicity when present in high concentrations. Liver accumulation of BAs can lead to oxidative stress, apoptosis and hepatocyte damage (Monte *et al.*, 2009). Cholestasis is the phenomena of impaired bile production and/or reduction of secretory flow. It can be due to impairment of BAs secretion by hepatocytes or due to an obstruction at the level of the bile ducts (Trauner, Meier and Boyer, 1998).

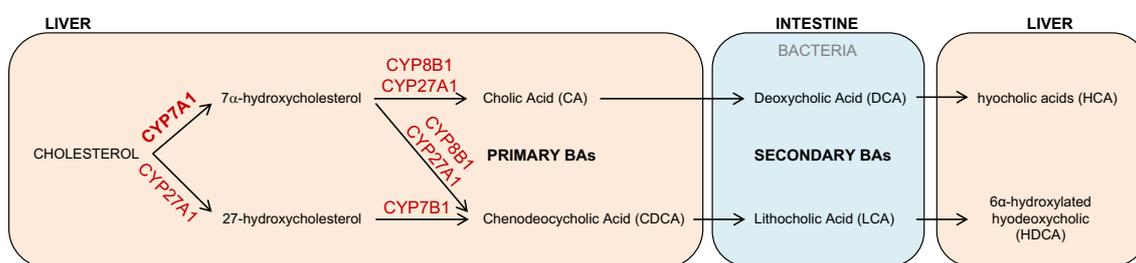


Figure 29. Metabolism and transformation of bile acids (BAs) across tissues. BAs are originated from cholesterol metabolism by the enzymes CYP7A1 and CYP27A1 to 7 $\alpha$ -hydroxycholesterol and 27-hydroxycholesterol. Primary BAs, Cholic acid (CA) and Chenodeoxycholic acid (CDCA), are generated in the liver, transferred to the gallbladder and, finally, secreted to the intestine. In the intestine, host bacteria transform the primary BAs to secondary BAs, Deoxycholic acid (DCA) and Lithocholic acid (LCA). Primary and secondary BAs can return to the liver where they are converted to Hyocholic acids (HCA) and 6 $\alpha$ -hydroxylated hyodeoxycholic (HDCA).

## I. Importance of FXR in bile acid metabolism

FXR, the first discovered BA-responsive nuclear receptor, acts as the main regulator in this pathway (Makishima *et al.*, 1999). FXR is highly expressed in the liver, kidney and intestine. However, there is some controversy about its potential beneficial (Y. Zhang *et al.*, 2006; Fang *et al.*, 2015) or deleterious (F. Li *et al.*, 2013; C. Jiang *et al.*, 2015) effects in BAs metabolism. **Table 2** and **Table 3** summarize some of these “contradictory” effects.

<b>FXR beneficial effects</b>
Upregulation of SHP, repression of CYP7A1 and decrease in BAs content
Increase secretion of FGF15 (FGF19 in humans) which represses CYP7A1
Reduce BAs uptake <i>via</i> repression of BAs receptors in liver and intestine
Promote liver detoxification by stimulation of pathways such as glucuronidation
Increase BAs excretion by transcriptional stimulation of transporters in liver and kidney
Induction of nuclear receptors expression involved in detoxification and BAs synthesis

Table 2. Beneficial effects of FXR on glucose and BA metabolism.

<b>Beneficial effects provoked by lack of FXR</b>
Decrease hepatic gluconeogenesis through inhibition of pyruvate carboxylase
Increase thermogenesis by increasing body weight gain
Increase secretion of GLP1
Delayed intestinal absorption of glucose
Activation of glycolytic genes by preventing FXR/ChREBP interaction (an interaction which represses ChREBP activity)

Table 3. Beneficial effects provoked by lack of FXR.

FXR interacts with OGT and is O-GlcNAcylated in response to high glucose concentrations. FXR O-GlcNAcylation (Ser62) increases its stability and activity. Point mutation of this residue decreases O-GlcNAcylation of FXR and correlates with inhibition of its transcriptional activity (Berrabah *et al.*, 2014). The authors demonstrated *in vivo* that FXR is O-GlcNAcylated also in fed conditions increasing the expression of its target genes (SHP and cytochrome P450 7A1 (CYP7A1)) and decreased bile acid content.

## **5. Role of OGT on signaling pathways key for cell metabolism**

Upon secretion of different stimuli such as insulin or growth factors, the liver activates several signaling cascades that regulate glucose and lipid metabolism pathways but also a wide variety of cellular processes such as proliferation, differentiation, apoptosis and stress responses. Here is presented a brief recapitulation of several of these signaling pathways and the impact of OGT on their regulation.

## I. The PI3K-AKT-mTORC signaling pathway

The PI3K-AKT-mTOR signaling cascade is an insulin signaling pathway that regulates diverse cellular processes (Matter *et al.*, 2014). Upon its release, insulin binds to its receptor in the membrane surface recruiting PI3K to the phosphorylated insulin receptor substrate (IRS). PI3K promotes the phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) promoting the recruitment of PDK1 and AKT (Walker *et al.*, 1999). AKT is phosphorylated on Thr308, by PDK1, and at Ser473 *via* the mammalian target of rapamycin (mTORC) 2 (Titchenell, Lazar and Birnbaum, 2017) (**Figure 30**). These phosphorylations activate AKT generating a cascade of reactions that modulate multiple downstream pathways such as glycolysis or lipid synthesis.

Upon insulin stimulation, OGT translocates from the nucleus to the cytoplasm thanks to its PIP-binding domain (Whelan, Lane and Hart, 2008; Yang *et al.*, 2008). This translocation facilitates OGT phosphorylation by the insulin receptor (IR) increasing its activity (Whelan *et al.*, 2010). Furthermore, in cells expressing IR and insulin growth factor-1 (IGF1) receptor such as hepatocytes, pancreatic beta cells or endothelial cells, OGT modifies several proteins in the PI3K/AKT/mTOR pathway. However, the physiological relevance of some of these modifications is still not well understood. Nevertheless, IRS-1 and AKT are O-GlcNAcylated, a modification which inhibits their activity either by impairing the interaction with PI3K in 3T3-L1 adipocytes (Whelan *et al.*, 2010) and with PDK1 in MCF-7 breast cancer cells (Wang *et al.*, 2012) or by competing with phosphorylation-mediated activation in rat primary adipocytes (Shi *et al.*, 2015) or the pancreatic beta cell line INS-1 (Kang *et al.*, 2008).

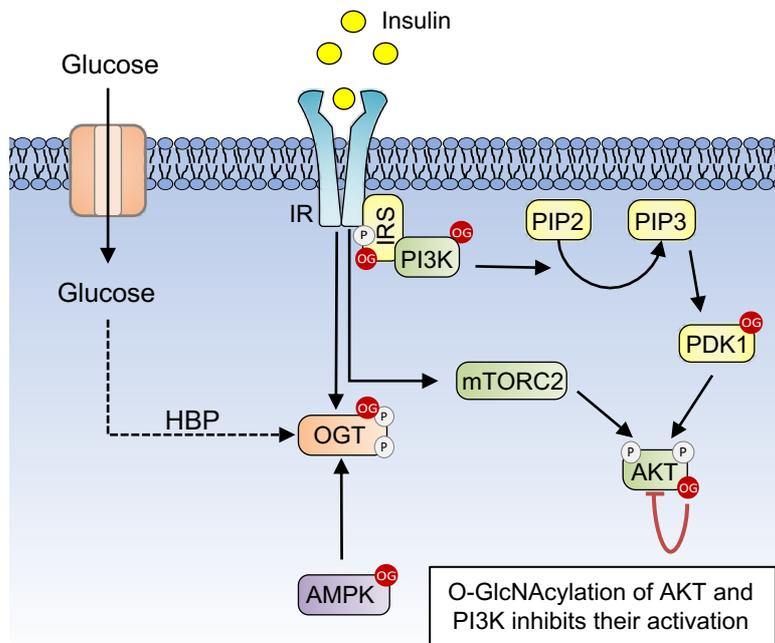


Figure 30. Regulation of PI3K/AKT/mTOR signaling pathway by OGT. Insulin binding to its receptor at the cellular membrane induces the recruitment of PI3K to the phosphorylated insulin receptor substrate (IRS). PI3K phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) inducing the recruitment of PDK1 and AKT. AKT is phosphorylated by PDK1 and mTORC2 thereby activating different cellular processes. Upon insulin release, OGT is recruited to the membrane where it is phosphorylated by the insulin receptor (IR) and AMPK potentiating its activity. OG indicates O-GlcNAcylated proteins.

## II. The MAPK-ERK signaling pathway

The MAPK-ERK signaling pathway is the major signaling cascade regulating cell proliferation and survival. Under physiological conditions, growth factor secretion activates the MAPK-ERK signaling cascade. Growth factors bind to their receptor in the plasma membrane recruiting the growth factor receptor-bound protein 2 (Grb2) which is associated to the Son of sevenless (SOS) protein. SOS exchanges GDP, associated to the membrane-binding RAS protein, with GTP activating the protein RAS. RAS recruits the B-RAF protein, which phosphorylates the protein MAP kinase kinase (MEK). Finally, MEK-mediated phosphorylation of ERK modulates a wide range of cellular proteins and transcription factors localized both in the cytoplasm and the nucleus *via* phosphorylation (Smalley and Smalley, 2018). Excessive activation of upstream proteins in the ERK pathway induces several diseases such as cancer and inflammation (Maik-Rachline, Hacoheh-Lev-Ran and Seger, 2019; Guo *et al.*, 2020).

The MAPK/ERK signaling pathway activates the transcriptional regulation and activity of OGT (Zhang *et al.*, 2015). The authors show that overexpression of a constitutive form of MEK1 in H1299 cells (lung cancer human cell line) induces the expression and

activity of OGT. Inversely, treatment of several cancer cell lines (DU145, BPH-1 and H1299) with U0126, an inhibitor of MEK proteins, reduces the expression and activity of OGT, suggesting that hyperactive MAPK/ERK signaling could lead to hyper-O-GlcNAcylation in different cell types.

### **III. The p38 MAPK signaling pathway**

The p38 MAPK pathway is strongly activated *in vivo* by inflammatory cytokines, environmental stresses and to a lesser extent by serum growth factors. The activation of this pathway translates in activation of cell differentiation, apoptosis or autophagy. Canonical activation of p38 MAPK proteins occurs *via* the phosphorylation of Tyr and Thr residues in the conserved TGY motif. The enzymes catalyzing this phosphorylation are the MKK (also called MAPK kinase or MAP2Ks) 3, 4 and 6, which previously need to be phosphorylated by the so-called MAP3Ks (or MAPK kinase kinase). The exact kinases involved depend on the stimuli activating the cascade (Rodríguez-Carballo, Gámez and Ventura, 2016). The importance of the p38 MAPK signaling pathway in the regulation of immune responses has been largely highlighted in the context of carcinogenesis. For a detailed review see Martínez-Limón *et al.*, 2020.

In 2008, OGT was demonstrated to interact with p38 under glucose deprivation conditions in the Neuro-2a neuroblastoma cell line. P38 recruits OGT to specific targets for O-GlcNAc modification, suggesting a role for OGT in responding to cellular stress such as defective brain glucose metabolism (Cheung and Hart, 2008). Recently, increased O-GlcNAcylation in tumor microenvironment was shown to promote tumor proliferation through the inhibition of p38 MAPK (Moriwaki and Asahi, 2017). The authors transplanted B16 melanoma cells subcutaneously into *Ogt*-transgenic mice overexpressing OGT and observed significant tumor growth compared to wild-type mice. The tumors presented significant downregulation of p38 MAPK and upregulation of ERK1/2 signaling (Moriwaki and Asahi, 2017).

### **IV. JNK signaling pathway**

The c-Jun N-terminal kinases (JNKs), members of the mitogen-activated protein kinase (MAPK) family, regulate many important physiological processes such as immune response and embryonic development *via* their impact in cytoskeletal protein dynamics and cell death/survival pathways. The JNK pathway activation occurs in response to inflammation, oxidative stress and/or DNA damage. Activation of this pathway requires phosphorylation of two MAP2Ks, MKK4 and MKK7. MKK4 and

MKK7 synergistically phosphorylate the JNK protein for its activation. Activated JNK can phosphorylate cytoplasmic proteins or be translocated into the nucleus for indirect alteration of gene expression (Zeke *et al.*, 2016). The activation of JNK has also been implicated in the malignant transformation of cells and tumorigenesis (Seki, Brenner and Karin, 2012). However, its function has been related not only to apoptosis but also to cell survival depending on the cell type and lineage studied.

Stimulation of O-GlcNAcylation by Thiamet-G treatment in wild-type mice treated with acetaminophen exhibited increased hepatotoxicity due, at least in part, to an increase in the JNK signaling pathway (McGreal *et al.*, 2018). These data suggest that the JNK signaling pathway is regulated by OGT although the molecular mechanisms involved are still unclear.

## **6. Role of OGT in the regulation of circadian rhythms**

Circadian rhythms are a series of endogenous autonomous oscillators of physiological activities that create cycle of 24 hours day/night (Reppert and Weaver, 2002). Circadian rhythms disruption is a common issue in modern society and has been associated to severe consequences on human health (Xie *et al.*, 2019). The core regulator of the circadian clock is the suprachiasmatic nucleus of the hypothalamus controlled mostly by the light/dark cycle. The suprachiasmatic nucleus controls the peripheral clocks *via* secretion of regulatory factors, synchronized mostly by internal signals, food and temperature (Dibner, Schibler and Albrecht, 2010; Xie *et al.*, 2019). The molecular clock of the circadian rhythms system consists in a Transcription-translation feedback loop (TTFL) complex composed of a core: the Circadian Locomotor Output Cycles Kaput (CLOCK), Brain and Muscle ARNT-Like 1 (BMAL1), and the Repressor proteins Period-1 (PER1), PER2, PER3, Cryptochrome-1 (CRY1) and CRY2 (Gekakis, 1998). Two sets of proteins collaborate with the TTFL core to maintain expression oscillations. One of them is formed by the receptor tyrosine kinase-like orphan receptor (ROR) and the nuclear receptors REV-ERB (Solt, Kojetin and Burris, 2011). The second one comprises D-box-related genes including D site-binding protein (DBP), Thyrotroph embryonic factor (TEF), and the Hepatic leukemia factor (HLF) (Preitner *et al.*, 2002; Sato *et al.*, 2004).

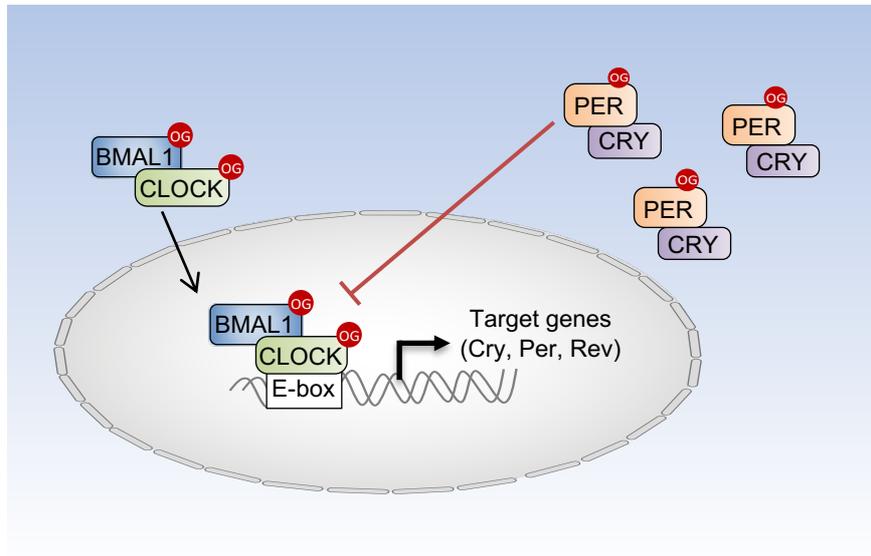


Figure 31. Simplified view of circadian rhythm regulation by O-GlcNAcylation  
 At the beginning of the cycle, Circadian Locomotor Output Cycles Kaput (CLOCK) and Brain and Muscle ARNT-Like 1 (BMAL1) act as a heterodimer and activate transcription of Period (PER) and Cryptochrome (CRY) genes (Gekakis, 1998). After several hours, the proteins PER and CRY accumulate, dimerize, and form a complex which by translocation to the nucleus inhibit the CLOCK/BMAL1 heterodimer (Xu et al., 2015). OG indicates O-GlcNAcylated proteins.

### I. O-GlcNAcylation stabilizes CLOCK and BMAL1

OGT modifies several important proteins involved in circadian rhythms (Li et al., 2013). OGT overexpression in dexamethasone-synchronized U2OS cells (U2OS-B6) is associated with increased amplitude of the clock oscillations and increased expression of *Per2* and *Cry1*. Cre-induced recombination of *Ogt* in mouse primary hepatocytes of OGT<sup>lox/Y</sup> mice decreases BMAL1 and CLOCK target gene expression. The authors also reported that O-GlcNAcylation of BMAL1 and CLOCK promotes protein stability through inhibition of their ubiquitination, and thereby increasing the expression of genes such as *Per* and *Cry*. Finally, the authors suggested a role for O-GlcNAc diurnal rhythms on the circadian regulation of physiological process in peripheral tissues ( Li et al., 2013) (Figure 31).

### II. OGT modifies PER2 via competition with phosphorylation

PER2 is O-GlcNAcylated in a region known to regulate human sleep phase (Ser662). O-GlcNAcylation on this residue competes with CK1-dependent phosphorylation of PER2 (Kaasik et al., 2013). The authors suggested that the O-GlcNAcylation/phosphorylation switch of PER2 could provide a precise mechanism for controlling the molecular clock. Although the exact mechanism is still unknown, the

authors proposed a role for OGT in controlling epigenetic modifiers such as the Polycomb repressor complexes as one of the possible explanations (Kaasik *et al.*, 2013).

### III. REV-ERB $\alpha$ controls OGT protein stability

REV-ERB ( $\alpha$  and  $\beta$ ) are cyclically expressed nuclear receptors with important roles in the regulation of circadian clock and in metabolic control. REV-ERB $\alpha$  is particularly important in the regulation of hepatic metabolism since it acts as a transcriptional repressor recruiting the HDAC3/NCOR repressor complex (Bugge *et al.*, 2012; Sun *et al.*, 2013). In 2018, REV-ERB $\alpha$  was described to interact with OGT in HepG2 cells. *Rev-erb $\alpha$*  knock-down was associated with decreased levels of O-GlcNAcylation and OGT, suggesting a role for REV-ERB $\alpha$  in regulating OGT stability and activity. *In vivo* validation further demonstrated a decreased in O-GlcNAc and OGT protein in liver of *Rev-erb $\alpha$*  knock-out mice. Interestingly, the authors also demonstrated that the O-GlcNAcylation of AKT is REV-ERB $\alpha$  dependent. Indeed, overexpression of REV-ERB $\alpha$  increased AKT O-GlcNAcylation in HepG2 cells and *Rev-erb $\alpha$*  knock-out mice exhibit decreased AKT phosphorylation. In addition, REV-ERB $\alpha$  also regulated the nuclear activity of OGT impacting TET activity and increasing DNA 5hmC levels (Berthier *et al.*, 2018).

#### CHAPTER 2: RELEVANT POINTS

1. OGT tightly controls metabolism of glucose, lipids and bile acids *via* modulation of a wide variety of pathways and signaling cascades
2. OGT is regulated by circadian rhythms but also impacts its regulation through the control of BMAL1, CLOCK and PER proteins

# Chapter 3: *Ogt* knock-out models and effect of dosage compensation

## 1. The effect of dosage compensation in OGT expression

The *Ogt* gene is located in the region Xq13.1 of the X chromosome. In order to modulate the transcription of genes located in the X chromosome between males (XY) and females (XX) the X chromosome requires a mechanism called ‘dosage compensation’ (Figure 32). In mammals, this process is called X-inactivation and consists in the inactivation or silencing of one of the two X chromosomes in females (Lyon, 1961). X chromosome inactivation occurs during early embryogenesis but differently for embryonic or extraembryonic tissues. In the extraembryonic tissue, the paternal X chromosome (Xp) is inactivated; however, during the early blastocyst stage there is a reactivation of the inactive X chromosome (Xi) so that the inner cell mass

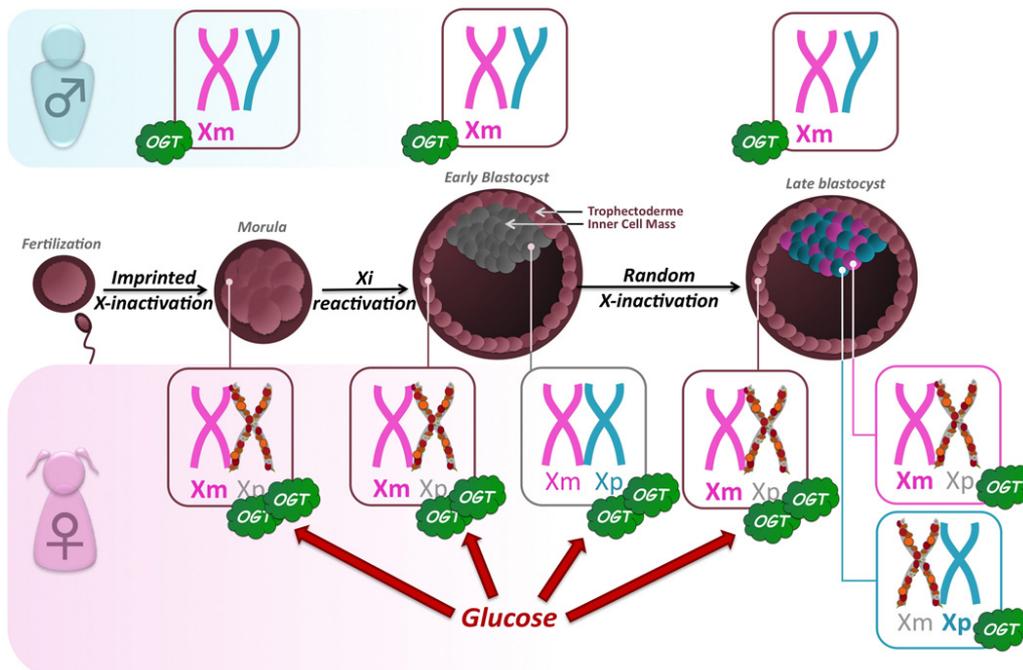


Figure 32. Effect of “Dosage compensation” in the regulation of OGT. In males, the X chromosome is always activated. However, dosage compensation is the mechanism of X-inactivation carried out in female embryos to compensate for the extra X chromosome. Initially, the inactivation occurs on the paternal X chromosome (Xp). Between morula and early blastocyst step, the Xp is reactivated in the inner cell mass and every cell will experience a random X chromosome inactivation. This process contributes to the regulation of OGT expression between males and females avoiding abnormally high OGT expression in females. From (Olivier-Van Stichelen, Abramowitz and Hanover, 2014)

undergoes a random X-inactivation where half of the cell silences the Xp and the other half the maternal X chromosome (Xm) (Takagi and Sasaki, 1975).

The process of chromosome inactivation requires several steps (Figure 33). The first step is the counting of X chromosomes and selection of the chromosome to be silenced. The second step consists in the inactivation of the selected chromosome in *cis*. The regulatory element X-inactivation center (XIC) of the future Xi generates non-coding RNAs, such as *XIST*, increasing when the inactivation process starts. These RNAs coat the entire Xi and recruit the PcG complexes to deposit histone repressive marks, such as H3K27me3 and H2AK119Ub by the PRC2 and PRC1 respectively (Plath *et al.*, 2003; de Napoles *et al.*, 2004). Finally, the last step consists in the maintenance of the X-inactivated stage *via* hypoacetylation of H3

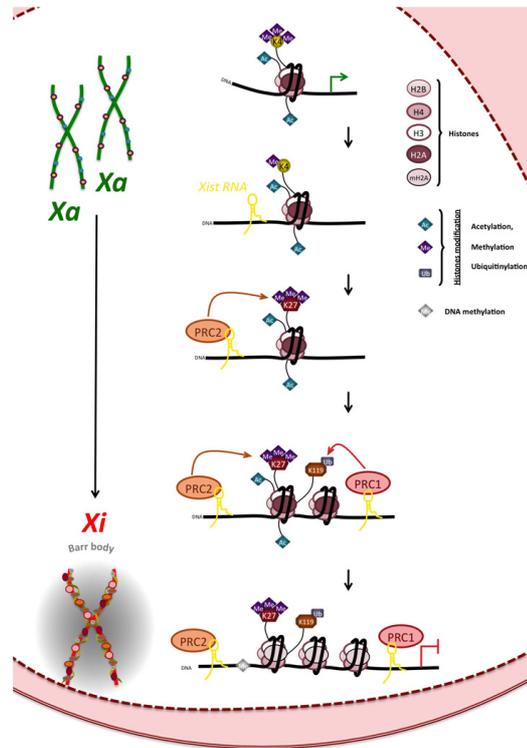


Figure 33. X-inactivation process. *Xist* RNA produced by the future inactivated X chromosome binds to the DNA and recruits the PRC1 and PRC2 complexes for the trimethylation of H3K27 and H2A ubiquitination. Methylation of DNA and substitution of H2A by macroH2A ensure the X chromosome long-term inactivation. From Olivier-Van Stichelen, Abramowitz and Hanover, 2014

and H4 but also replacement of H2A by macroH2A, known to inhibit both the acetylation of histones and the recruitment of transcription factors (Mermoud *et al.*, 1999; Gilbert, Pehrson and Sharp, 2000). OGT is essential for embryonic development since *Ogt* global knock-out causes lethality 4.5 days post coitus (blastocyst) (Shafi *et al.*, 2000). Interestingly, heterozygous *Ogt* knock-out are viable when inheriting the mutant allele in the Xp (O'Donnell *et al.*, 2004). *Ogt* was suggested to escape the extraembryonic Xi but it is silenced during the random Xi. Furthermore, tamoxifen-inducible *Ogt* global knock out also causes lethality four weeks after tamoxifen injection indicating that OGT is also essential for survival of adult mice (Ida *et al.*, 2017). Reactivation of Xi in females is associated with several diseases such as autoimmune diseases and cancer (Kaneko and Li, 2018). *Xist* has been suggested to act as a tumor suppressor down-regulating oncogenes in the female X chromosome. Indeed, reactivation of Xi results in increased aggressiveness and lethality in blood cancer (Yildirim *et al.*, 2013). Overall, the possession in females of two *Ogt* alleles could

increase the risk of developing diseases such as cancer or metabolic syndrome especially when considering the possibility of Xi reactivation.

## 1. Generation of tissue/cell-specific knock out of OGT

The fact that global knock-out of *Ogt* causes early lethality prompted several groups to generate inducible or tissue/cell specific knock out mice. Several groups shed light into the role of OGT by characterizing some of these models. I will summarize here the key findings obtained.

### I. Knock-out of *Ogt* in adipocytes: role in hyperphagia, adipose - brain cross-talk and brown adipose tissue browning

In 2018, Li and colleagues published an adipocyte-specific knock-out of *Ogt* (OGT<sup>Adipoq</sup>) obtained using the Adiponectin-Cre transgenic mice. The authors compared mice fed in chow and high-fat diet (HFD). In HFD conditions, the adipocyte-specific ablation of *Ogt* caused a decrease in HFD-induced obesity, reflected by a decrease in body weight and fat mass accumulation. OGT<sup>Adipoq</sup> mice also exhibited improved insulin sensitivity and glucose tolerance accompanied by a reduction in HFD-induced hyperphagia. The authors then proposed that adipocyte OGT trans-activates lipid desaturation via expression of the *Scd* genes. SCD proteins facilitate the accumulation of N-arachidonyl ethanolamine (AEA), which by indirect cross-talk with the brain induced hyperphagia in condition of food overload (Li *et al.*, 2018). The same team published a follow up study describing the inducible deletion of *Ogt* knock-out in adipocytes. This model was characterized by a reduction of visceral fat mass due to increase in lipid droplet-associated perilipin 1 (PLIN1)-mediated lipolysis. Indeed, increased O-GlcNAcylation was shown to inhibit PLIN1-mediated lipolysis in visceral adipose tissue, thereby contributing to diet-induced obesity. The authors concluded that O-GlcNAcylation is essential for maintaining visceral fat mass during fasting but also increases subcutaneous and

#### ADIPOSE TISSUE

**Adipocytes** *Li et al. (2018)*  
Adiponectin-Cre  
Fed with high-fat diet (HFD)

- Abolished HFD-induced obesity
- Decrease weight and fat mass accumulation
- Reduced serum leptin
- Reduced HFD-induced hyperphagia
- Ameliorated insulin resistance and glucose tolerance

**Adipocytes** *Yang et al. (2020)*  
Adipoq-Cre ER

- No significant metabolic changes
- Reduced visceral fat (especially during fasting) by promoting lipolysis
- Significant decrease in epididymal WAT adipocytes sizes

**Adipocytes** *Ohashi et al. (2017)*  
Adipoq-Cre  
UCP1-Cre

- Reduction in BAT mass
- Impaired BAT browning
- Intolerance to cold-induced thermogenesis

visceral fat when mice are fed HFD diet (Yang *et al.*, 2020). Another study examined brown adipose tissue (BAT) function in OGT<sup>Adipoq</sup> mice (Ohashi *et al.*, 2017). In parallel to a significant reduction in O-GlcNAcylation in BAT, histological analysis of OGT<sup>Adipoq</sup> mouse BAT revealed a WAT-like appearance that correlated with decreased PGC-1 $\alpha$  and *Ucp1* expression. BAT-specific *Ogt* knock-out mice were later generated using *Ucp1*-Cre transgenic mice. These mice showed marked intolerance to cold exposure that was partially explained by the reduction in PGC-1 $\alpha$  stability and protein level. Altogether, it was concluded that O-GlcNAcylation plays an essential role in cold-induced thermogenesis in BAT (Ohashi *et al.*, 2017).

## II. Muscle *Ogt* knock-out: essential role in cardiomyocyte development and skeletal muscle insulin resistance

Muscle *Ogt* deficient mice have a lean phenotype associated with an increase in whole body energy expenditure and insulin sensitivity compared to wild-type mice (H. Shi *et al.*, 2018). This correlates with increased glucose uptake and enhanced glycolytic gene expression in skeletal muscles. Interestingly, muscle *Ogt* deficient mice also exhibit elevated expression and circulating levels of Interleukine (IL)-15. This elevation in IL-15 was due to impaired transcriptional repression *via* EZH2 (H. Shi *et al.*, 2018), known to be stabilized and activated by OGT (Chu *et al.*, 2014).

*Ogt* depletion in cardiomyocytes *in vivo* was associated with impaired heart development, leading to neonatal lethality shortly after birth. RNA sequencing analysis allows the identification of 753 genes dysregulated in cardiomyocytes from E11.5 *Ogt* knock-out compared to wild-type mice. Most upregulated genes were related to morphogenesis, homeostasis and muscle contraction consistent with the cardiac morphological phenotype. In particular, the expression of *Angiopoietin-1* (*Angpt1*), essential for correct heart development, was markedly down-regulated in cardiomyocytes from *Ogt* knock-out mice (Mu *et al.*, 2020).

MUSCLE	
<b>Skeletal muscle</b> HSA-Cre	Shi <i>et al.</i> (2018)
<ul style="list-style-type: none"> <li>- Lean phenotype</li> <li>- Increase energy expenditure</li> <li>- Increase insulin sensitivity</li> <li>- Enhanced glucose uptake</li> <li>- Increase glycolytic enzymes expression</li> </ul>	
<b>Cardiomyocytes</b> TroponinT-Cre	Mu <i>et al.</i> (2020)
<ul style="list-style-type: none"> <li>Heart developmental defects</li> <li>- Hypertrabeculation</li> <li>- Biventricular dilation</li> <li>- Atrial septal defects</li> <li>- Ventricular septal defects</li> <li>- Coronary vessel development</li> </ul>	

### III. Pancreatic knock-out of *Ogt*: role in ER stress and AKT signaling

In 2015, Alejandro and colleagues generated a  $\beta$ -cell specific *Ogt* knock-out mice through the breeding of OGT<sup>lox/lox</sup> with RIP-Cre mice. These mice presented a severe age-dependent hyperglycemia leading to severe glucose intolerance at 20-weeks of age.  $\beta$ -cell *Ogt* knock-out mice exhibited increased cell apoptosis at 12-weeks compared to controls which correlated with decreased insulin secretion due, at least partially, to a reduction in Ca<sup>2+</sup> signaling. Increased ER stress was also observed in islet from  $\beta$ -cell *Ogt*

knock-out mice, with a significant increase in C/EBP-homologous protein (CHOP) expression together with a downregulation of the pro-survival AKT1/2 and Pancreatic and duodenal homeobox 1 (Pdx1) signaling pathway. Rescued AKT expression in islets from  $\beta$ -cell *Ogt* knock-out mice restored  $\beta$ -cell proliferation, but no difference in O-GlcNAcylated levels or apoptosis was observed. Interestingly, deletion of at least one allele of *Chop* in  $\beta$ -cell *Ogt* knock-out mice was sufficient to improve hyperglycemia and  $\beta$ -cell mass. These results provided a direct link between O-GlcNAcylation and survival of  $\beta$ -cell *via* the regulation of ER stress and AKT signaling pathway (Alejandro *et al.*, 2015). Lastly, the genetic deletion of *Ogt* in pancreatic embryonic epithelium was reported to cause pancreatic hypoplasia due to reduced levels of Pdx1 protein and increased apoptosis (Baumann *et al.*, 2020).

PANCREAS	
<b>Pancreatic epithelium</b>	
RIP-Cre	Alejandro <i>et al.</i> (2015)
-	Hyperglycaemia
-	Impaired insulin secretion
-	Increased ER-stress induced apoptosis
-	Decreased Akt signalling pathway leading to decreased proliferation
<b>Pancreatic epithelium</b>	
RIP-Cre	Baumann <i>et al.</i> (2020)
Pdx1-Cre <sup>Late</sup>	
-	Development of pancreatic hypoplasia
-	Decrease in pancreatic mass
-	Reduction in Pdx1 <sup>+</sup> cells
-	Increase cell apoptosis
-	Differential cell population

### IV. Intestine *Ogt* knock-out: regulation of epithelial barrier and differentiation process

Ablation of *Ogt* in intestinal epithelial cells (IECs) using Villin-Cre (OGT<sup>Vil</sup> mice) was associated to disrupted epithelial barrier caused by intestinal damage, inflammation and microbial dysbiosis, a homeostatic disequilibrium in Paneth cells. The authors proposed that the O-GlcNAcylation of Signal transducer and activator of transcription 1 (STAT1) may contribute to the intestinal defect of OGT<sup>Vil</sup> mice (Zhao *et al.*, 2018). Interestingly, OGT deficiency in IECs also promoted L cell differentiation *via* impaired FoxO1-induced repression of Neurogenin3 expression, a key gene involved in

differentiation of enteroendocrine cells. At the mechanistic level, it was suggested that short-chain fatty acids generated by the intestinal microbiota, could stimulate O-GlcNAcylation of FoxO1. Activation of FoxO1 through O-GlcNAcylation did in turn inhibit the differentiation of enteroendocrine cells to L cells (Zhao *et al.*, 2020).

INTESTINE	
<b>Intestinal epithelial cells</b>	
Vil-Cre	Zhao <i>et al.</i> (2018)
<ul style="list-style-type: none"> <li>- Disrupt epithelial barrier</li> <li>- Microbial dysbiosis</li> <li>- Paneth cell dysfunction</li> <li>- Intestinal inflammation</li> </ul>	
<b>Intestinal epithelial cells</b>	
Vil-Cre	Zhao <i>et al.</i> (2020)
<ul style="list-style-type: none"> <li>- Promotion of L cell differentiation</li> <li>- Expression of Neurogenin3 via FOXO1 inactivation</li> </ul>	

## V. Knock-out of *Ogt* in macrophages: enhanced TLR-induced immune response and necroptosis

*In vitro* and *in vivo* studies of macrophage-specific deletion of *Ogt*, generated by crossing of OGT<sup>lox/lox</sup> with Lysosome M-Cre mice, revealed impaired antiviral immune response. In this context, it was proposed that O-GlcNAcylation of Mitochondrial antiviral-signaling protein (MAVS), by promoting its ubiquitination, could lead to downstream antiviral innate immune signaling activation (Li *et al.*, 2018). Surprisingly, Li *et al.* (2019) demonstrated that induction of sepsis in myeloid-specific *Ogt* knock-mice led to exacerbated macrophage inflammation and necroptosis activation compared to wild-type mice. Depletion of Receptor interacting serine/threonine kinase 3 (RIPK3) reduced this effect suggesting that RIPK3 plays an important role in this immunometabolic cross-talk (Li *et al.*, 2019). Our team recently demonstrated that Lipopolysaccharide (LPS) treatment by inducing GFAT2 expression, one of the key limiting enzymes of the HBP, potentiated O-GlcNAcylation in macrophages (Al-Mukh *et al.*, 2020). *Ogt* deficiency impaired LPS induced O-GlcNAcylation and resulted in increased Nitric oxide synthase 2 (NOS2) expression and cytokine production, reinforcing the concept that inflammatory processes are regulated by OGT.

MACROPHAGES	
<b>Myeloid cells</b>	
LysM-Cre	Li <i>et al.</i> (2018)
<ul style="list-style-type: none"> <li>- Impaired antiviral immune system</li> </ul>	
<b>Myeloid cells</b>	
LysM-Cre	Li <i>et al.</i> (2019)
<ul style="list-style-type: none"> <li>- Enhance TLR-induced immune response</li> <li>- Enhanced necroptosis</li> </ul>	
<b>Myeloid cells</b>	
LysM-Cre	Al-Mukh <i>et al.</i> (2020)
<ul style="list-style-type: none"> <li>- Enhanced inflammatory cytokines production in a FoxO1-dependent manner</li> </ul>	

## VII. Brain *Ogt* knock-out: role in neurodegeneration and feeding behavior

Several groups published neuron-specific *Ogt* knock-out models (Ruan *et al.*, 2014; Lagerlöf *et al.*, 2016; Wang *et al.*, 2016; Su and Schwarz, 2017; Wheatley *et al.*, 2019). Constitutive deletion of *Ogt* in neurons led to progressive neurodegeneration, with excessive accumulation of Tau protein in the forebrain, a pathogenic feature typically associated to Alzheimer's disease, neuronal death and memory loss (Wang *et al.*, 2016). Inducible deletion of *Ogt* in the brain induced obesity by impairing satiety (Lagerlöf *et al.*, 2016) and recapitulated features of aging brain but with no signs of neurodegeneration (Wheatley *et al.*, 2019). *Ogt* deficiency in AgRP<sup>+</sup> neurons promoted WAT browning, leading in turn to improved glucose metabolism and energy homeostasis (Ruan *et al.*, 2014). Finally, deletion of *Ogt* in sensory neurons highlighted the importance of OGT in sensory neuron survival and maintenance (Su and Schwarz, 2017).

BRAIN	
<b>AgRP neurons</b>	
AgRP-Cre	Ruan <i>et al.</i> (2014)
<ul style="list-style-type: none"> <li>- Inhibition of neuronal excitability</li> <li>- Promotes WAT browning</li> <li>- Protects against diet-induced obesity and insulin resistance</li> </ul>	
<b>Excitatory neurons (Forebrain)</b>	
CamK2a-Cre	Wang <i>et al.</i> (2016)
Progressive neurodegeneration <ul style="list-style-type: none"> <li>- Pathogenic processing of Tau and amyloid precursor protein</li> <li>- Neuronal death</li> <li>- Gliosis</li> <li>- Memory loss</li> </ul>	
<b>Neurons (Hypothalamus)</b>	
CamK2a-CreER <sup>T2</sup>	Lagerlöf <i>et al.</i> (2016)
Regulates feeding behaviour <ul style="list-style-type: none"> <li>- Obesity from overeating</li> <li>- Impairs satiety</li> </ul>	
<b>Sensory neurons</b>	
Brn3a-CreER <sup>T</sup>	Su <i>et al.</i> (2017)
Nav1.8-Cre	
<ul style="list-style-type: none"> <li>- Essential for sensory neurons survival and maintenance</li> <li>- Hyposensitivity to thermal and mechanical stimuli</li> <li>- Decreases epidermal innervation</li> <li>- Cell body lost</li> </ul>	
<b>Neurons (Forebrain)</b>	
CamK2a-CreER <sup>T2</sup>	Wheatley <i>et al.</i> (2019)
Recapitulates features of brain aging <ul style="list-style-type: none"> <li>- Neuron dendritic spines reduction</li> <li>- Altered synaptic function</li> <li>- Impairs hippocampal-dependent learning and memory</li> <li>- No neurodegeneration</li> </ul>	

## VI. Knock-out of *Ogt* in hepatocytes: consequences on liver injury, fibrosis and regeneration

In 2018, McGreal and colleagues published the first liver-inducible knock-out of *Ogt* generated through the adenoviral expression of Cre in *Ogt* conditional hepatocytes. The authors studied this model in the context of liver injury induced by acetaminophen (McGreal *et al.*, 2018). Inducible *Ogt* liver knock-out mice showed decreased liver injury in response to acetaminophen with faster replenishment of hepatic GSH. Limitation in hepatotoxicity in these mice was due in part, to decreased activation of JNK signaling (McGreal *et al.*, 2018). Recently, a constitutive version generated by

crossing OGT<sup>lox/lox</sup> with Alb-Cre mice was published (B. Zhang *et al.*, 2019). Constitutive deletion of Ogt in the liver led to hepatomegaly, associated with hepatocyte ballooning, liver injury, inflammation and early stage fibrosis compared to wild-type mice at four weeks of age. Microarray analysis highlighted global changes in 5 week-old *Ogt* liver knock-out mice compared to wild-types, especially increased markers of fibrosis and necroptosis (up-regulation of RIPK3 and Mixed lineage kinase domain like pseudokinase (MLKL)) (B. Zhang *et al.*, 2019).

### **CHAPTER 3: RELEVANT POINTS**

1. OGT expression is regulated by X chromosome dosage compensation
2. As OGT is essential for embryo development, tissue/cell-specific knock were generated and provided important insights in OGT and O-GlcNAcylation functions in specific cell/tissues.

# Chapter 4: Role of OGT in pathophysiological conditions

## 1. Liver characteristics and cellular types

The liver is the largest internal organ in humans constituting between 2-5% of total body weight. Liver's primary function is the maintenance of normal blood glucose concentration and constitutes the main deposit of glucose and energy storage. However, the liver plays other important functions such as blood toxins removal and bile acids production. Structurally, the liver is composed by polygonal lobules separated by connective tissue called the Glisson's capsule. In the center of the lobule is located the central vein and, in the periphery, a portal triad is found constituted by the bile ducts, nerves and branches of the hepatic artery and portal vein (**Figure 34**).

The parenchymal cells of the liver, called hepatocytes, are arranged radially and constitute 70% of the total cell number (Ding *et al.*, 2016). However, other cellular types are important for the complex liver function in metabolism, coagulation, detoxification and immune response such as the Hepatic stellate cells (HSCs), Kupffer cells (KCs), Liver sinusoidal endothelial cells (LSECs) and cholangiocytes.

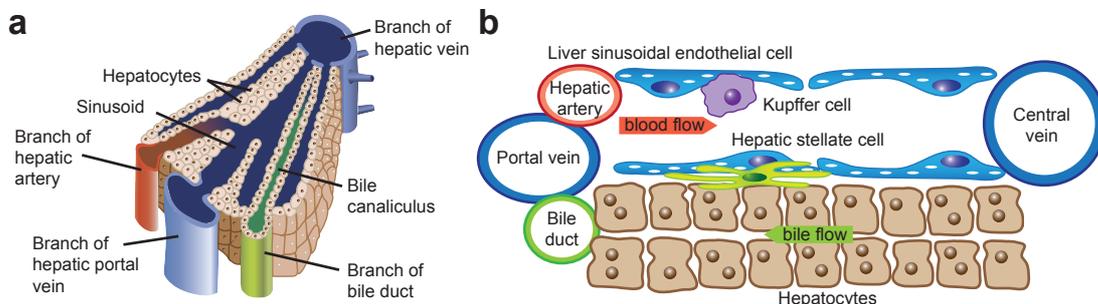


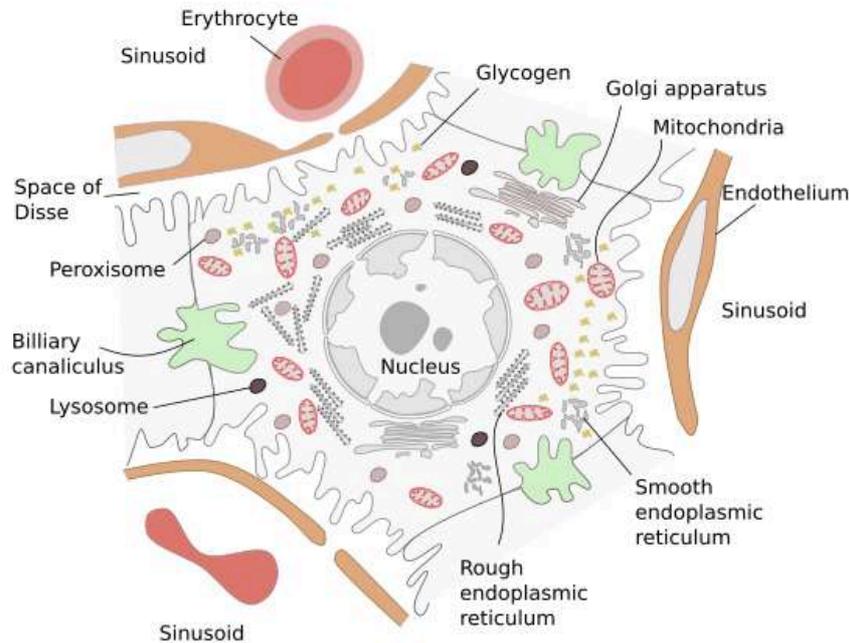
Figure 34. Hepatic lobule structure and cell types.

a. The liver is composed by several lobules constituting its functional units. Each lobule is composed of a central vein and layers of hepatocytes that radiate towards the portal triad. The portal triad is formed by a branch of hepatic artery, hepatic portal vein and bile duct. b. Sinusoids, located between different lobules, are discontinuous vessels built of fenestrated liver sinusoidal endothelial cells. Kupffer cells are located in the sinusoids. HSCs are located in the Disse space.

### I. Hepatocytes: the major population of hepatic parenchymal cells

Hepatocytes are the hepatic parenchymal cells constituting about 70% of the total cell number within the liver (Ding *et al.*, 2016). Hepatocytes are responsible for many functions which ensure liver homeostasis, such as carbohydrate, protein and lipid

metabolism, detoxification and immune cell activation (Macswen *et al.*, 2003). Importantly, hepatocytes interact through the fenestrated LSECs with all the different intrahepatic cell populations (**Figure 35**).



*Figure 35. Hepatocyte morphology.*

*Hepatocytes present distinct apical and basolateral membranes. The apical membrane contains a canicular domain, forming the biliary canaliculus where the hepatocytes excrete the BAs produced in the cells; and the lateral domain, connecting the biliary canaliculus and the basolateral membrane. The basolateral or sinusoidal membrane located next to the Disse space contain microvilli which infiltrate into the sinusoid through the LSECs fenestrations.*

Hepatocytes are classified in 3 zones based on their function and blood perfusion:

- **Zone 1:** located next to the portal triad are the best perfused and the first to regenerate after liver injury due to the proximity to nutrients and oxygenated blood. The hepatocytes are involved in oxidative metabolism such as  $\beta$ -oxidation, amino acid catabolism, gluconeogenesis, cholesterol and bile formation.
- **Zone 2:** pericentral region of the hepatocytes, located between the zones 1 and 3.
- **Zone 3:** located next to the central veins and consequently with lowest perfusion. Hepatocytes localized in this zone are mostly involved in detoxification, ketogenesis, glycolysis, lipogenesis, glycogen synthesis and glutamine formation.

The Yes-associated protein (YAP) and Wnt/ $\beta$ -catenin pathway are master regulators of liver zonation. Periportal hepatocytes are characterized by higher activation of YAP signaling, involved in proliferation, survival and differentiation (Patel, Camargo and Yimlamai, 2017). Hepatocyte-specific deletion of Yap in mice not only causes defects in hepatocyte survival but profound defect in bile duct development

(Zhang *et al.*, 2010). However, pericentral hepatocytes show higher activation of Wnt/ $\beta$ -catenin signaling, involved in cell fate determination, polarity and proliferation, with expression of its traditional target genes such as Axin2, glutamine synthetase (GS) and cytochrome P450 enzymes. Hepatocyte-specific deletion of  $\beta$ -catenin shows a periportal phenotype throughout the whole liver (Russell and Monga, 2018).

## **II. Cholangiocytes, the epithelial cells of bile ducts**

Cholangiocytes are the lining epithelial cells in bile ducts comprising between the 3-5% of total hepatic cell mass (Alpini, 2002). Even though the proportion of cholangiocytes in the liver is small, cholangiocytes are essential for correct bile formation and liver homeostasis. Indeed, cholangiocytes are damaged in a wide variety of human diseases called cholangiopathies, which potentially lead to liver failure.

Cholangiocytes are polarized and specialized cells divided in different subtypes (**Figure 36**). Within the Canal of Heiring reside immature cholangiocytes, poorly differentiated and considered progenitor cells, which participate in epithelium renewal and regeneration, called HPCs for hepatic progenitor cells. However, cholangiocytes increase their degree of differentiation (cell polarity, response to hormones and receptor expression and transporters) along the biliary tree, passing from being called small cholangiocytes to large cholangiocytes (Alpini *et al.*, 1996; Han *et al.*, 2013; Banales *et al.*, 2019).

Under physiological conditions, cholangiocytes contribute to the composition and volume of bile secretion. In response to injury, cholangiocytes become activated and start proliferating. In addition, cholangiocytes transdifferentiate to a neuroendocrine-like phenotype to sustain biliary proliferation itself but it also play a role in immune responses, hepatic inflammation and liver fibrosis development (Franchitto *et al.*, 2013). Resulting from cholangiocytes trans-differentiation, there is a *de novo* synthesis of neuroendocrine factors which modulate biliary damage, such as secretin (Glaser *et al.*, 2010; Meng *et al.*, 2014), vascular endothelial growth factor (VEGF) (Gaudio *et al.*, 2006), follicle-stimulating hormone (FSH) (Mancinelli *et al.*, 2009) and estrogens (Svegliati-Baroni *et al.*, 2006).

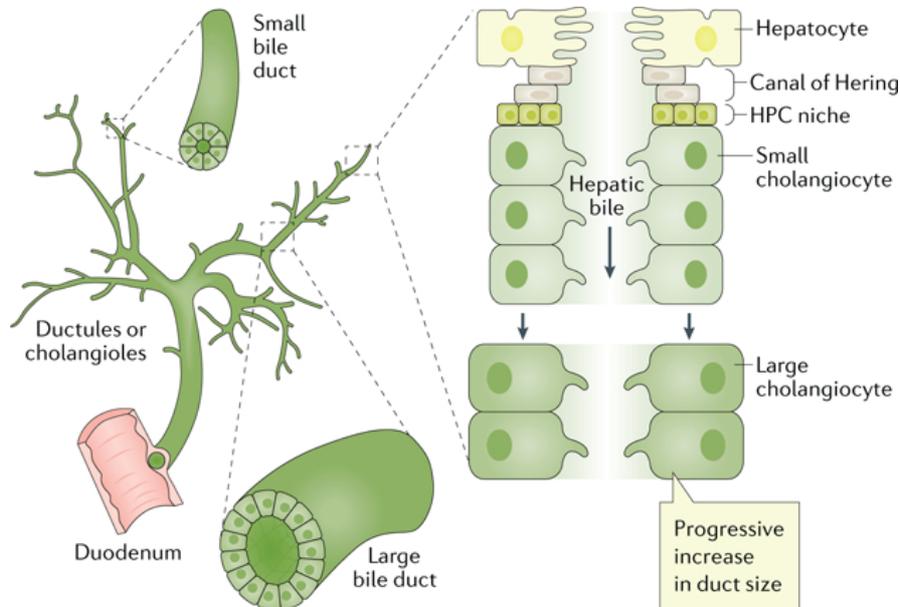


Figure 36. Bile duct structure.

Hepatic bile is produced by hepatocytes, secreted into hepatocyte canaliculus to posteriorly transfer through the small and large bile duct where it is modified to ductal bile. The Canal of Hering connects the hepatic canaliculus and the ductules or cholangiocytes. Hepatocyte progenitor cell (HPCs) niche is also located in the interface between the Canal of Hering and the cholangiocytes. Cholangiocytes divide in small and large depending upon the level of specialization.

### III. Hepatic stellate cells (HSCs), source of extracellular matrix

HSCs are mesenchymal cells belonging to the myofibroblast family which contribute to around 15% of cells in the liver (Friedman, 2008b). HSCs are classically recognized as the main extracellular matrix (ECM) source during liver injury. In a homeostatic liver, HSCs are quiescent and located in the Disse space (Friedman, 2008a). However in the context of an injury or microenvironmental stress, HSCs become activated, differentiating into proliferative myofibroblast phenotype and generating abnormal amounts of ECM that induce fibrotic scar tissue in liver fibrosis (Kisseleva *et al.*, 2012). HSCs activation is a complex process in liver fibrosis which can be induced by a large number of cell-surface, cytoplasmic and/or nuclear molecular signals and pathways (see review on Hou and Syn, 2018).

### IV. Liver macrophages (Kupffer cells) and bone marrow-derived macrophages

KCs are the resident, self-renewing and non-migratory phagocytes in the liver. They account for 20-35% of all non-parenchymal cells in the liver and represent the largest population of resident macrophages in the body, between 80 to 90% (Zeng *et al.*, 2013; Li *et al.*, 2014). KCs play a critical role in the innate immune response and their sinusoids location allows efficient pathogen phagocytosis from portal or arterial

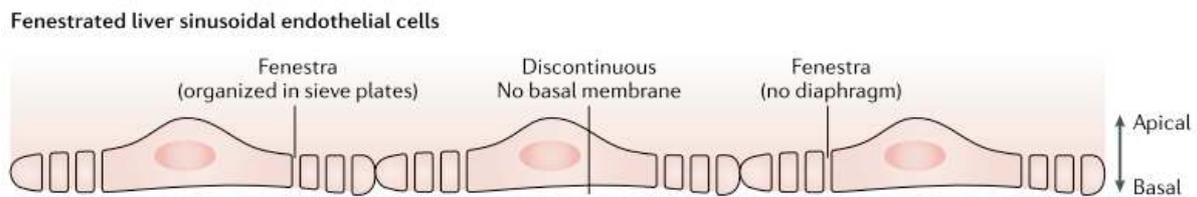
circulation. KCs are also important for clearance of particles, erythrocytes (dead or dying), hepatic parenchymal cells and endotoxins derived from the gastrointestinal tract, ensuring liver homeostasis (Dixon *et al.*, 2013). Moreover, they are considered a type of antigen-presenting cell (APC) providing a link between the innate and the adaptive immune system. Generally, KCs exert a protective role in situations such as drug-induced liver injury and toxin-induced fibrosis. However, dysregulation in the control of inflammatory responses can contribute to pathological conditions such as non-alcoholic fatty liver (NAFL) / non-alcoholic steatohepatitis (NASH) (Tacke, 2017).

KCs origin has been suggested to involve two mechanisms: replenishment by local self-proliferation and recruitment from monocytes derived from bone marrow (Davies *et al.*, 2013). In 2012, murine KCs were suggested to originate from the embryonic yolk sac (Schulz *et al.*, 2012). However, bone marrow derived macrophages (BMDM) can also differentiate into KCs. Macrophages carry out a “specialization” process called polarization. *In vitro*, macrophages change their polarization state based on the type of stimuli such as cytokines, microbial products, pathogens or other modulators (Murray *et al.*, 2014). However, *in vivo*, the polarization process is more complex, as macrophages are sufficiently plastic to integrate multiple stimuli. Hence, the traditional M1/M2 nomenclature for macrophage polarization (Mills *et al.*, 2000) referring to M1 for macrophages activated by IL1 $\beta$ , IL-6, tumor necrosis factor alpha (TNF $\alpha$ ) or LPS with the consequence of activating an inflammatory response and M2 for those activated by IL-4, IL-10, IL-13 or Transforming Growth Factor (TGF)- $\beta$  leading to anti-inflammatory response have been questioned over the past years (Nahrendorf and Swirski, 2016).

## V. Liver sinusoidal endothelial cells (LSECs)

LSECs are the most abundant hepatic non-parenchymal cell population. LSECs constitute a vascular population receiving blood from the hepatic artery and portal vein, hence having the capacity of removing and recycling nutrients from the hepatocytes (Shetty, Lalor and Adams, 2018). Morphologically and functionally, LSECs present similarities to lymphatic endothelial cells (Lalor *et al.*, 2006) since both have minimal basement membrane (**Figure 37**), relatively organized cell junctions (Géraud *et al.*, 2012) and share scavenger receptors such as lymphatic vessel endothelial hyaluronic acid receptor (LYVE1) (Choi *et al.*, 2013) or Prospero homeobox protein 1 (PROX1) (Tanaka and Iwakiri, 2016). A characteristic that distinguishes LSECs from other hepatic endothelial populations is their fenestrated organization. The fenestration or

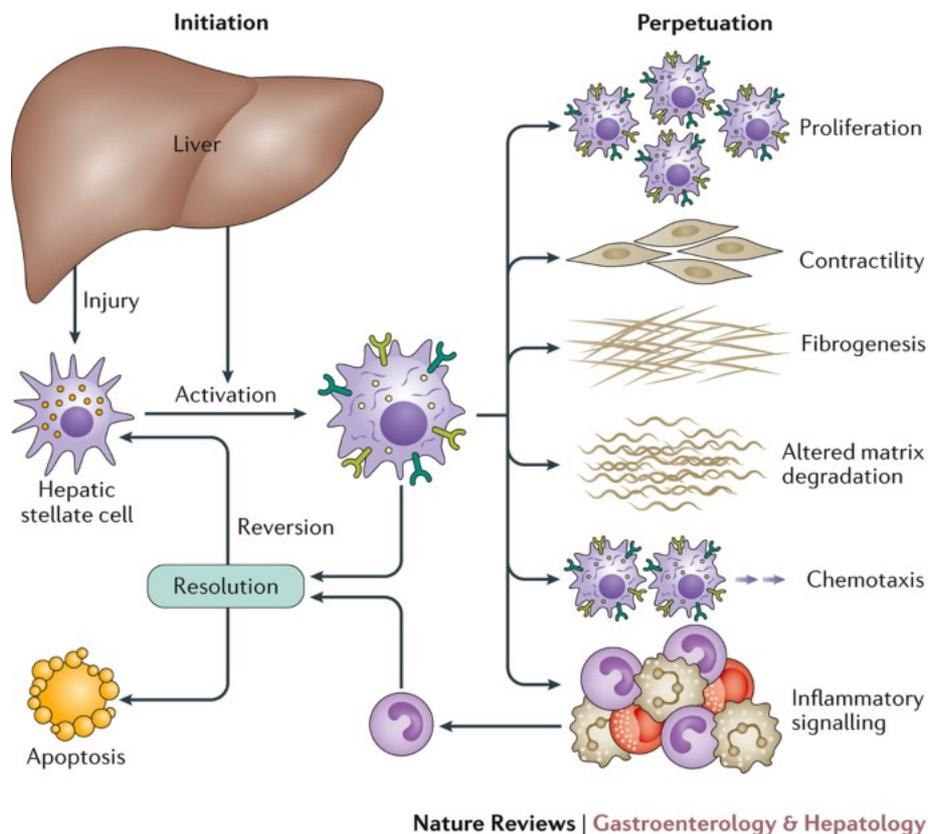
membrane pores in LSECs are organized into sieve plates and lack a diaphragm or basal lamina conferring the characteristic of high permeability to LSECs (Poisson *et al.*, 2017). All of these characteristics confer to the LSECs the filtration and scavenger functions in the liver. LSECs also play a role in innate and adaptive immunological function including antigen presentation (Lalor *et al.*, 2002) or leukocytes recruitment (Xu *et al.*, 2003). Moreover, LSECs play a crucial role in the cellular crosstalk that regulates chronic liver diseases, such as fibrosis and carcinogenesis (see more in review from Shetty, Lalor and Adams, 2018).



*Figure 37. Organization of Liver sinusoidal endothelial cells (LSECs). LSECs are polarized cells presenting fenestrations organized in sieve plates. Due to the fenestrations, LSECs present a characteristic of high permeability. LSECs do not have basal membrane or diaphragm. Shetty, Lalor and Adams, 2018*

## 2. Liver regeneration

In almost all acute and chronic liver diseases, the regeneration process starts with a ductular reaction beginning at the periportal area (Roskams *et al.*, 2004). This reaction presents a ductular morphology although it does not necessarily mean ductular origin. The origin could be proliferating cholangiocytes, differentiating hepatic stem cells or de-differentiated hepatocytes. However, the specific origin of the ductular reaction is difficult to elucidate without the help of lineage tracing (Van Haele, Snoeck and Roskams, 2019). The liver enormous regenerative capacity allows however to rapidly compensate for these damages in an exceptionally well-regulated process.



*Figure 38. Resolution of liver injury via regeneration. Liver injury can occur through a large number of stimuli. Initiation of liver injury induces activation of hepatic stellate cells and the consequent induction of several mechanisms such as proliferation, liver fibrosis and inflammation signaling. However, when the liver state improves, it leads to resolution of fibrosis that implicates cell apoptosis and inactivation of hepatic stellate cells. From Tsuchida and Friedman, 2017.*

## I. 2/3 Partial hepatectomy: the most used model of liver regeneration

The most used model for the study of liver regeneration is the rodent two-third partial hepatectomy (PHx). In this model, two-third of the liver is resected leaving a remnant third undamaged. Traditional view of liver regeneration implicates the activation of mature quiescent adult hepatocytes of the remnant liver to enter the cell cycle. This traditional view divides the process of liver regeneration in three phases. The first one, called the priming phase, consists in the transcriptional activation of more than 100 genes for the preparation of hepatocytes to face growth factor signaling. The second phase, corresponds to the activation of growth factor receptors such as epidermal growth factor receptor (EGFR) or c-Met, known to be essential for liver regeneration (Paranjpe *et al.*, 2016). The third and last step consists in the cessation of proliferation activating pathways such as TGF- $\beta$  or integrin signaling pathways, which mediates the communication between ECM and epithelial cells (**Figure 38**).

## II. Hepatic stem cells activation during liver regeneration

In case of hepatocyte proliferation impairment, hepatic stem cells from the biliary compartment can generate hepatocytes for hepatic repair. The Notch pathway is one of the identified mechanisms involved in the differentiation of hepatic stem cells during regeneration. The Notch pathway targets the SRY-related HMG box transcription factor 9 (Sox9), which promotes cell proliferation and regulates stem cell homeostasis and differentiation. However, the molecular mechanism involved is still unclear. Among potential factors, HDAC1 was recently shown to favor differentiation of hepatic stem cells into hepatocytes in mice with liver injury following choline-deficient methionine-supplemented (MCD) diet. HDAC1 expression is also induced in reactive ducts and hepatocytes of patients with cirrhosis. These pathways open up the possibility of inducing hepatic stem cells differentiation for the treatment of advanced liver disease patients (see review in Ko *et al.*, 2019).

Recent studies associated changes in histone and DNA methylation with HSCs activation (Sheen-Chen *et al.*, 2014). DNA methylation correlates with conversion of quiescent HSCs into hepatic myofibroblasts, while its inhibition controls hepatic wound healing and limits liver fibrogenesis (Bian *et al.*, 2012; Perugorria *et al.*, 2012; Sheen-Chen *et al.*, 2014). In the case of histone methylation, activation of HSCs is accompanied by induction of the methyltransferases EZH2 and Absent, small, or homeotic discs 1 (ASH1), transferring methyl groups to H3K27 and H3K4, respectively (El Taghdouini and van Grunsven, 2016). The role of EZH2 and ASH1 is essential for maintaining the profibrogenic phenotype of activated HSCs specially *via* regulation of PPAR $\gamma$ , a negative regulator of HSCs activation during liver fibrosis (Zhang, Lu and Zheng, 2012).

## 3. Endoplasmic reticulum (ER) stress-induced liver injury

The ER is the most important cellular compartment when talking about protein folding and maturation. Within the ER, polypeptide chains bind to the binding-immunoglobulin protein GRP-78 (also called BiP), which initially prevents polypeptide aggregation and posteriorly, facilitates their folding to acquire native conformation. Subsequently, the peptides will be post-translationally modified, a critical process for the correct protein folding. However, aberration in protein folding, for example in the case of highly proliferative or malignant cells which can overwhelm the protein folding

machinery, resulting in the accumulation of misfolded protein commonly known as ER stress (Adams *et al.*, 2019).

## I. Unfolded protein response to ER stress

As a response to ER stress, cells activate a mechanism called ER-associated degradation, where proteins are translocated into the cytosol for ubiquitination and proteasomal degradation. When this process is insufficient to cope with the amount of stress, cells activate the unfolded protein response (UPR) (**Figure 39**), a cell signaling pathway readjusting the ER capacity to fold proteins and to restore protein homeostasis. In metazoans, there are three UPR signal activator proteins: inositol requiring enzyme 1 $\alpha/\beta$  (IRE1) (Cox, Shamu and Walter, 1993; Mori *et al.*, 1993), PKR-like ER kinase (PERK) (Harding, Zhang and Ron, 1999) and activating transcription factor 6 $\alpha/\beta$  (ATF6) (Haze *et al.*, 1999). These proteins have an ER luminal domain (LD) which recognizes directly or indirectly the misfolded proteins (Walter and Ron, 2011). The IRE1 and PERK proteins possess in their cytosolic portion a kinase domain that has the capacity of autophosphorylate in response to misfolded protein recognition (Shamu and Walter, 1996; Tirasophon, Welihinda and Kaufman, 1998; Harding, Zhang and Ron, 1999). IRE1 leads to translation and splicing of the potent transcriptional factor X-box-binding protein 1 (*XBPI*), improving protein folding capacity but also activates the protein degradation and transport pathways (Calton *et al.*, 2002). PERK activation leads to phosphorylation of the eukaryotic translation initiation factor-2 $\alpha$  (eIF2 $\alpha$ ), a component of the eIF2 complex, which results in attenuation of global translation but also, transcriptional activation of specific genes such as the activating transcription factor 4 (ATF4) which contributes to increase ER folding capacity and antioxidant response *via* activation of NRF2 (Harding, Zhang and Ron, 1999; Vattam and Wek, 2004). However, ATF6 differs from both of the previous mechanism. ATF6 located in the ER, translocates into the Golgi apparatus upon activation where it is cleaved. The bZIP cytosolic domain of ATF6 then migrates to the nucleus where it activates UPR target genes (Haze *et al.*, 1999; Shen *et al.*, 2002).

In chronic ER stress where cells are unable to restore ER protein homeostasis, ER stress leads to a switch favoring apoptosis and cell death. PERK sustained activation leads to the upregulation of CHOP, a transcription factor involved in apoptosis regulation, which promotes the transcriptional activation of the DNA damage-inducible protein 34 (GADD34). GADD34 reverses the phosphorylation of eIF2 $\alpha$  leading to the activation of translation, including proteins involved in the ER stress-induced apoptosis

(Novoa *et al.*, 2001). IRE1 persistent activation can lead to apoptosis through its interaction with the Tumor necrosis factor receptor-associated factor 2 (TRAF2). TRAF2 activates apoptosis *via* activation of JNK and p38 MAPK signaling pathways.

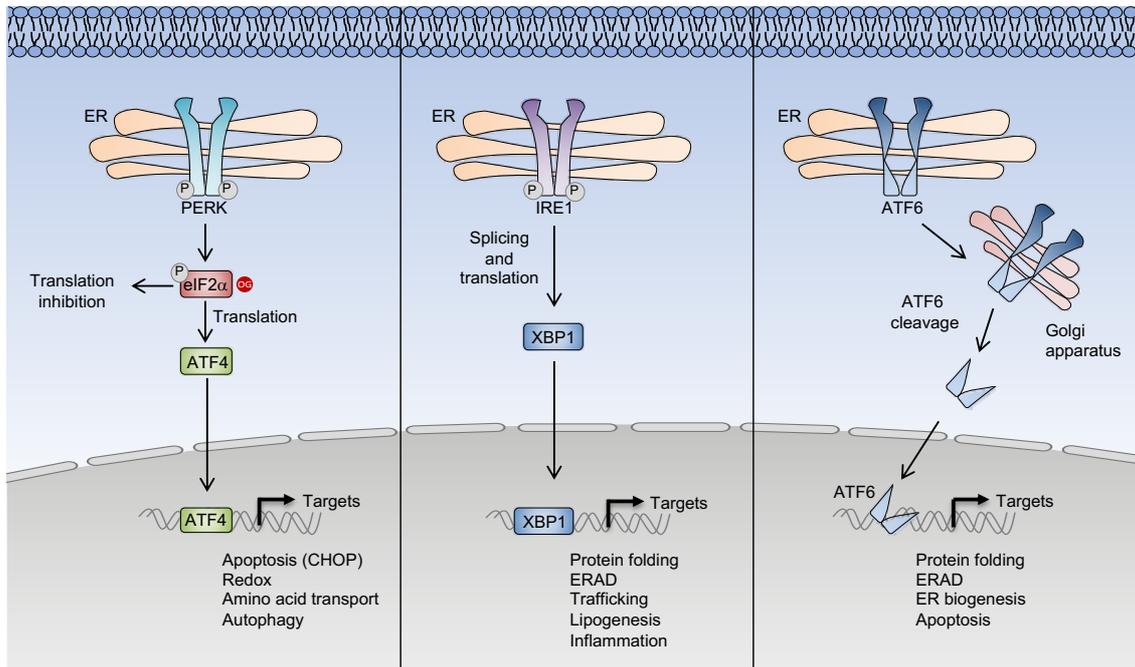


Figure 39. Mechanisms of Unfolded protein response (UPR) to ER stress. In chronic ER stress, PERK and IRE1 are autophosphorylated promoting the translation of ATF4 and XBP1, respectively. In contrast, ATF6 carries out a process of maturation that requires its cleavage at the level of the Golgi apparatus. These three proteins translocate to the nucleus and activate UPR target genes. eIF2 $\alpha$  is described to be O-GlcNAcylated.

## II. O-GlcNAc reduces ER stress and limits apoptosis

eIF2 $\alpha$  was described to be O-GlcNAcylated at Ser219, Thr239 and Thr241 in HepG2 cells. Point mutation on these residues to prevent O-GlcNAcylation increases the phosphorylation of eIF2 $\alpha$  at Ser51 and consequent translation of CHOP. These results suggested a role for OGT in the regulation of CHOP-induced apoptosis (Jang *et al.*, 2015). Moreover, in cardiomyocytes treated with ER stress activators, adenoviral overexpression of OGT or pharmacological inhibition of OGA, attenuates stress-induced CHOP activation and cell death suggesting that enhanced O-GlcNAcylation could represent a partially adaptative response to reduce ER-stress induced cell death (Ngoh *et al.*, 2009). Similar results were shown in a rabbit model of renal ischemia/reperfusion injury, in which glucose administration suppressed the levels of CHOP (Suh *et al.*, 2014). In addition, O-GlcNAcylation of HIF-1 $\alpha$  also regulates its stability by inhibiting its proteasomal degradation. Overexpression of degradation resistant mutants of HIF-1 $\alpha$  in cells depleted in OGT prevented the ER stress-mediated

CHOP response (Ferrer *et al.*, 2014). In cardiomyocytes, XBP1 was reported to stimulate the HBP pathway leading to increase in O-GlcNAcylation (Wang *et al.*, 2014). Since O-GlcNAcylation was reported to reduce ER stress-induced apoptosis (Ngoh *et al.*, 2009), XBP1 could be part of a positive feed-back loop with O-GlcNAcylation to ameliorate ER stress. Lastly, O-GlcNAc was demonstrated to limit ER stress *via* formation of stress granules (SG) and processing bodies (PB), which functions are the regulation of mRNA translation and degradation (Ohn *et al.*, 2008). Taken all together, these data suggest a critical role for OGT and O-GlcNAc in limiting ER stress and preventing apoptosis.

## **5. Effect of OGT and/or O-GlcNAcylation in NAFLD development**

Non-alcoholic fatty liver disease (NAFLD) consists in a heterogenous disease which histologically can be divided in NAFL and NASH (**Figure 40**). Hepatic insulin resistance is strongly associated to the development of NAFLD impacting whole-body energy metabolism. NAFL is characterized by the presence of at least 5% of hepatic steatosis without the presence of liver injury in the form of hepatocyte ballooning. However, NASH corresponds to the presence of at least 5% of hepatic steatosis accompanied by inflammation and liver injury with or without fibrosis (Stefan, Häring and Cusi, 2019). NAFLD has also been recognized as an emerging risk factor to develop HCC.

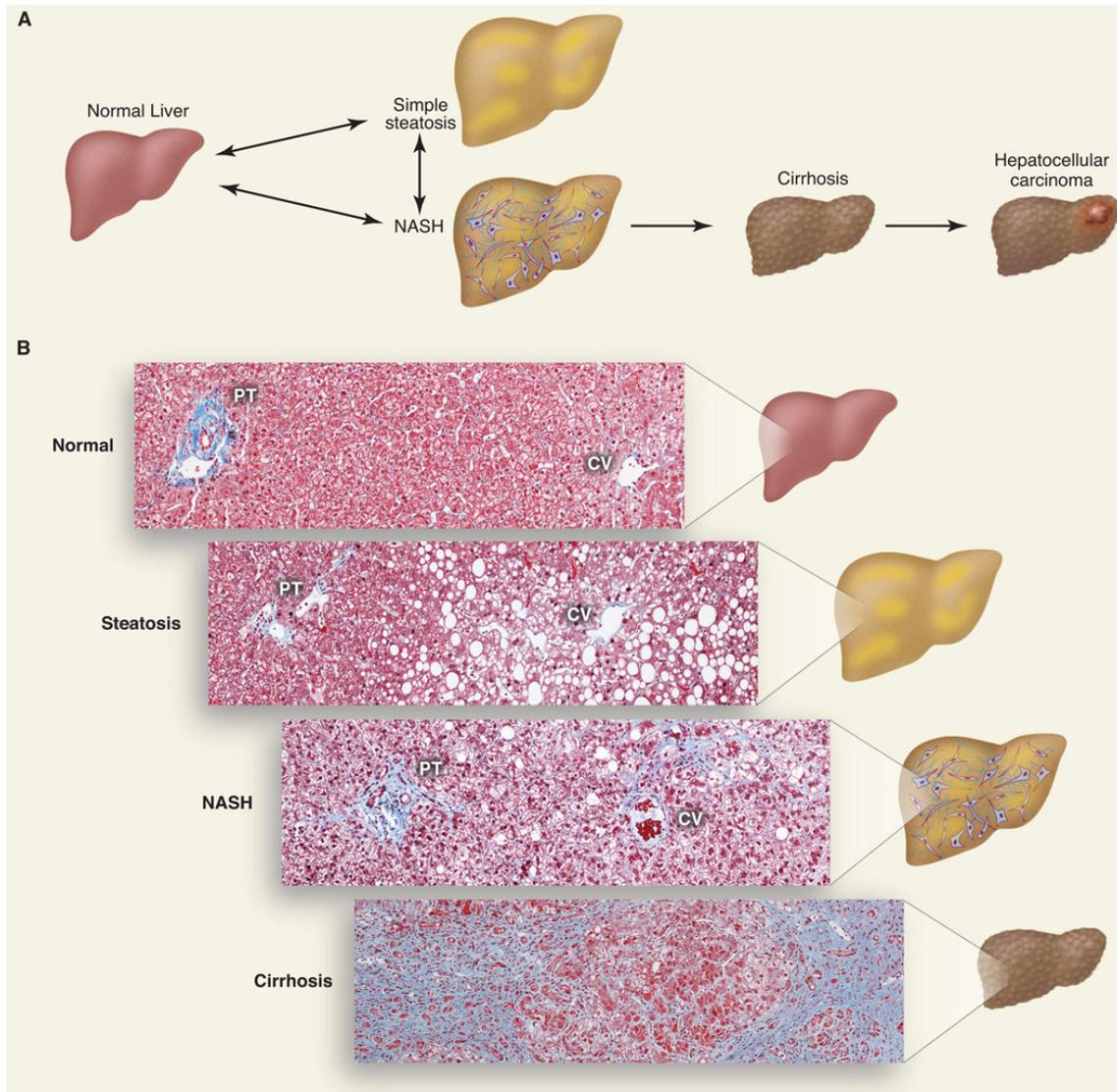


Figure 40. Non-alcoholic fatty liver disease (NAFLD) spectrum.

A) Schematic representation of NAFLD progression. Normal liver accumulates fat in form of TGs in hepatocytes promoting the development of steatosis. Steatosis can also be associated with inflammation, fibrosis and cell death which is referred as non-alcoholic steatohepatitis (NASH). NASH progression can lead to cirrhosis known to be a risk factor for the development of HCC. B) Histological sections representative of the different stages in NAFLD progression. Collagen is shown in blue by Masson's trichrome staining. PT: Portal triad. CV: Central vein. From Cohen, Horton and Hobbs, 2011.

## I. Involvement of OGT in hepatic insulin resistance

Hepatic insulin resistance causes deregulation in key metabolic pathways including gluconeogenesis and glycogen synthesis. As mentioned in **Chapter 2**, OGT regulates several enzymes and transcriptional regulators of these pathways. In addition, *in vivo* hepatic overexpression of OGT impairs insulin signaling causing insulin resistance and dyslipidemia in mice (X. Yang *et al.*, 2008).

In diabetic conditions, gluconeogenesis deregulation is one of the main liver features contributing to hyperglycemia. As previously mentioned in **Chapter 2**, OGT regulates CRT2, FoxO1, PGC-1 $\alpha$  and HCF-1 transcriptional regulators of hepatic glucose production (Dentin *et al.*, 2008; Housley *et al.*, 2008; Ruan *et al.*, 2014). High fat diet-fed mice and *db/db* mice show elevated levels of O-GlcNAcylation in the liver (Dentin *et al.*, 2008). Decreased O-GlcNAcylation *via* hepatic overexpression of OGA caused a decrease in gluconeogenesis and hyperglycemia (Dentin *et al.*, 2008). High fat diet-fed mice and *db/db* mice also show elevated levels of HCF-1 correlating with an increased in gluconeogenesis and hyperglycemia. HCF-1 and OGT knock-down restore glucose homeostasis in mice (Ruan *et al.*, 2012).

## II. OGT regulation of lipogenesis in NAFL and NASH

NAFLD is characterized by the accumulation of fatty acids in the form of TGs (Cohen, Horton and Hobbs, 2011). O-GlcNAcylation could be a possible mechanism controlling the paradoxical maintenance of lipogenesis under conditions of insulin resistance (**Figure 27**). OGT suppresses insulin signaling at the same time that it potentiates lipogenesis *via* ChREBP O-GlcNAcylation. Indeed, ChREBP is hyper O-GlcNAcylated in liver of diabetic mice and decreasing O-GlcNAcylation *via* overexpression of OGA decreases ChREBP activity and steatosis (Guinez *et al.*, 2011). LXR, another transcription factor involved in lipogenesis and known to be O-GlcNAcylated, positively correlates with intrahepatic fat, inflammation and fibrosis in NAFL and NASH patients (Ahn *et al.*, 2014). LXR activation has the potential to modulate cholesterol homeostasis, improve insulin sensitivity and induce anti-inflammatory responses. However, LXR also stimulates hepatic lipogenesis and hypertriglyceridemia, this effect is considered as a side effect in the treatment of steatosis with LXR agonists (Ni *et al.*, 2019). However, OGT, which is associated with the development of metabolic diseases such as NAFLD and cancer, also modifies and activates two transcription factors which negatively regulate hepatic lipogenesis, FXR and FoxO1 (Housley *et al.*, 2008; Berrabah *et al.*, 2014). FXR is a liver nuclear receptor whose activation causes beneficial effects such as improved glucose metabolism, insulin sensitivity and decreased hepatic lipogenesis. However, generation of knock-out mice for FXR or/and its target SHP demonstrated that reduction of hepatic steatosis is, at least in part, mediated through SHP (Akinrotimi *et al.*, 2017). In NASH patients, deficiency in FXR causes progression and exacerbated development of NASH. In contrast, FXR activation is associated with protection against liver inflammation

(Armstrong and Guo, 2017). FoxO1 suppresses the expression SREBP1c leading to decrease in TGs accumulation, upregulates FAO and promotes the expression of enzymes controlling BAs metabolism and transport. FoxO1 could potentially contribute to protection against NAFLD. However, tight regulation is necessary and both over- or under-activation of FoxO1 leads to undesirable consequences (Dong, 2017).

### **III. Role of OGT in fibrosis**

Liver fibrosis is a dynamic process consisting in accumulation of ECM, which occurs as a consequence of liver injury produced by viral hepatitis, NAFL or NASH. A critical event in fibrogenesis is the activation of HSCs, main source of ECM proteins. Under physiological conditions, quiescent HSCs control turnover of ECM by secreting limited amounts of ECM molecules, metalloproteinases and their inhibitors. However, liver injury activates HSCs to transform into fibrogenic myofibroblasts, which produce alpha-smooth muscle actin ( $\alpha$ -SMA), ECM components and ECM remodeling enzymes. Fibrosis initiation is characterized by fast induction of growth factor receptors, signaling and development of fibrogenic and contractile phenotype. The process of amplification of HSCs activation is called perpetuation. Although fibrosis can be reversed after the cession of liver injury cause, chronic injury can lead to cirrhosis, which consists in hepatic architecture disorganization associated to abnormal blood flow and eventually portal hypertension (Tsuchida and Friedman, 2017). Considering the large amount of molecules and pathways involved in the activation of HSCs, I will highlight bellow the recent knowledge regarding OGT implications in these processes.

#### **a) Transforming growth factor- $\beta$ (TGF $\beta$ ) pathway regulation**

Transforming growth factor- $\beta$  (TGF $\beta$ ) is considered as the most potent fibrogenic cytokine and it is released by several cell populations in an inactive form (Hellerbrand *et al.*, 1999). TGF $\beta$  binds to the TGF $\beta$  type I receptor (TGF $\beta$ R1), which is phosphorylated activating the SMAD cascade of proteins, especially SMAD3, activating the transcription of type I and III collagen (**Figure 41**). SMAD7, a target of this cascade, acts as a negative regulator of the SMAD pathway. TGF $\beta$  also stimulates non-canonical SMAD-independent pathways implicating the activation of MAPK pathways, including Extracellular signal-regulated kinase (ERK), p38 and JNK (Tsuchida and Friedman, 2017; Dewidar *et al.*, 2019).

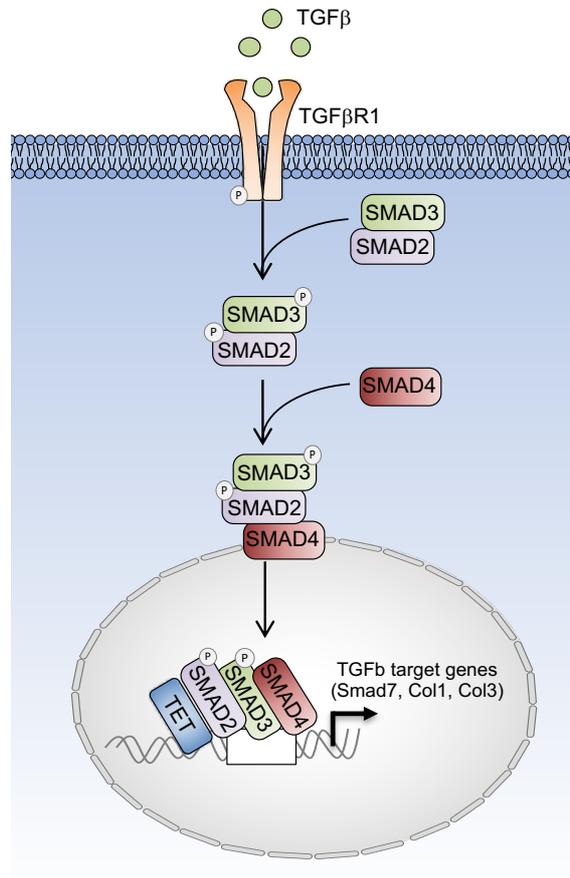


Figure 41. Transforming growth factor- $\beta$  (TGF $\beta$ ) canonical signaling pathway. Upon binding of TGF $\beta$  to its receptor TGF $\beta$ R1 on the cellular membrane, TGF $\beta$ R1 is auto-phosphorylated activating the SMAD-dependent cascade. SMAD2 and SMAD3 are phosphorylated and bind to the protein SMAD4 translocating to the nucleus and activating the transcription of its target genes in a TET demethylation manner. One of the targets of this cascade is *Smad7* which produces a negative feedback on the cascade.

Recently, high levels of the DNA demethylase TET3, previously mentioned to be O-GlcNAcylated, were reported in fibrotic liver of mice and human. The authors showed that TET3, upon binding to promoters of TGF $\beta$  target genes, demethylates and promotes TGF $\beta$  expression in HSCs. The same study demonstrated an improvement of liver fibrosis in TET3 knock-out mice (Xu *et al.*, 2020).

### b) Platelet-derived growth factor (PDGF)

Platelet-derived growth factor (PDGF) is a hepatic critical mitogen controlling HSCs proliferation and migration. Increased expression of PDGF receptor (PDGFR)  $\beta$  has been related to the development of liver injury in human and rodents (Davis, 1978). Depletion of PDGFR $\beta$  in HSCs decreases liver injury and fibrosis in carbon tetrachloride (CCl<sub>4</sub>) and bile duct ligation mouse models (Kocabayoglu *et al.*, 2015). A recent RNA-seq study performed in pancreatic adenoma of *Ogt* deficient cells,

demonstrated a down-regulation of pathways involved in development and differentiation including the PDGF-PDGFR downstream pathway suggesting the involvement of OGT in this pathway (Sharma *et al.*, 2019).

#### **c) Connective tissue growth factor (CTGF)**

Connective tissue growth factor (CTGF) is a fibrogenic cytokine expressed at low levels in normal livers but highly induced in the context of liver fibrosis (Tsuchida and Friedman, 2017). CTGF contributes to ECM formation, proliferation, migration and adhesion but also cell survival. CTGF is transcriptionally regulated by the YAP1/Tafazzin (TAZ) signaling pathway, highly associated with cancer progression. The protein Large tumor suppressor kinase 2 (LATS2), the main negative regulator of the YAP1/TAZ pathway, has recently been shown to be O-GlcNAcylated. O-GlcNAcylation of LATS2 inhibits its activity by blocking the binding with its partner the Monopolar spindle-one-binder 1 (MOB1), hence activating the YAP/TAZ pathway to promote proliferation (Kim *et al.*, 2020).

#### **d) Vascular endothelial growth factor (VEGF)**

The Vascular endothelial growth factor (VEGF) is released from LSECs and HSCs during liver injury inducing proliferation of HSCs and angiogenesis. VEGF promotes fibrogenesis but seems to be also required for tissue repair and resolution of fibrosis (L. Yang *et al.*, 2014; Kantari-Mimoun *et al.*, 2015). Specificity protein 1 (Sp1) is one of the transcriptional regulator of VEGF and it is activated through AKT phosphorylation (Pore *et al.*, 2004). In addition, Sp1 is also O-GlcNAcylated (Majumdar *et al.*, 2006). Elucidate the effect of OGT in the Sp1-mediated regulation of VEGF would be of high interest.

## **6. Role of OGT and O-GlcNAcylation in the development of cancer**

Over the past years, up-regulation of O-GlcNAcylation levels and/or OGT expression has been related to the development of nearly every cancer type such as liver (Xu *et al.*, 2017), colon (Jiang *et al.*, 2019) or breast cancer (Barkovskaya *et al.*, 2019). Indeed, transcriptomic analysis of 18 NAFLD-associated HCC biopsies shows a correlation with OGT up-regulation, an up-regulation also confirmed in additional NAFLD-HCC tumor tissues and cell lines (Xu *et al.*, 2017). Cancer cells exhibit increased global O-GlcNAcylated level either caused by increased OGT or decreased

OGA protein. Increased O-GlcNAcylation in cancer cells favors glucose uptake, glucose flux through the PPP and lipid synthesis necessary for supporting cell proliferation (Xu *et al.*, 2017). Since we are interested in the role of OGT in the liver, we will focus on its role in HCC and cholangiocarcinoma. Here, I will recapitulate some of the more important features related to OGT and HCC over the recent years (**Figure 42**).

## **I. OGT in cellular metabolic reprogramming**

In 2014, Ferrer and colleagues showed that O-GlcNAcylation regulates glucose uptake and glycolysis in cancer cells *via* HIF1 $\alpha$  and its transcriptional target GLUT1. Reducing O-GlcNAcylation decreased HIF1 $\alpha$  protein levels through degradation. In cancer cells, decrease in O-GlcNAcylation activates ER stress and apoptosis *via* CHOP. However, overexpression of HIF1 $\alpha$  and GLUT1 rescued metabolic defects (Ferrer *et al.*, 2014). The authors also demonstrated that HIF1 $\alpha$  inhibits the activity of PFK1, a key enzyme of the glycolytic pathway thereby leading to redirecting glucose to the PPP. Interestingly, OGT redirects glucose to the PPP not only through its inactivation of PFK1 but also through the activation and stabilization of G6PDH (Rao *et al.*, 2015). As mentioned in **Chapter 2**, PKM2 is highly expressed in HCC associated with poor prognosis. Inhibition of PKM2 in HCC cells inhibited cell proliferation and induced apoptosis *in vivo* and *in vitro* (Lv *et al.*, 2018). *In vivo* and *in vitro* O-GlcNAcylation of PKM2, increased in cancer cells, inhibits its pyruvate kinase activity *via* dissociation of the active tetramer favoring its nonmetabolic function in proliferation. In addition, PKM2 was recently shown to be upregulated in activated HSCs in mouse and human fibrotic liver. *In vitro* knock-down of PKM2 markedly inhibited activation and proliferation of HSCs *via* acetylation of H3K9 on the promoter of MYC and CycD1 promoters. Treatment of CCl<sub>4</sub>-injected mice with TEPP-46, an inhibitor of PFK2 dimerization, reduced their liver fibrosis *in vivo* (Zheng *et al.*, 2020).

These results suggest a role for OGT-OGA in cellular metabolic reprogramming in cancer cells.

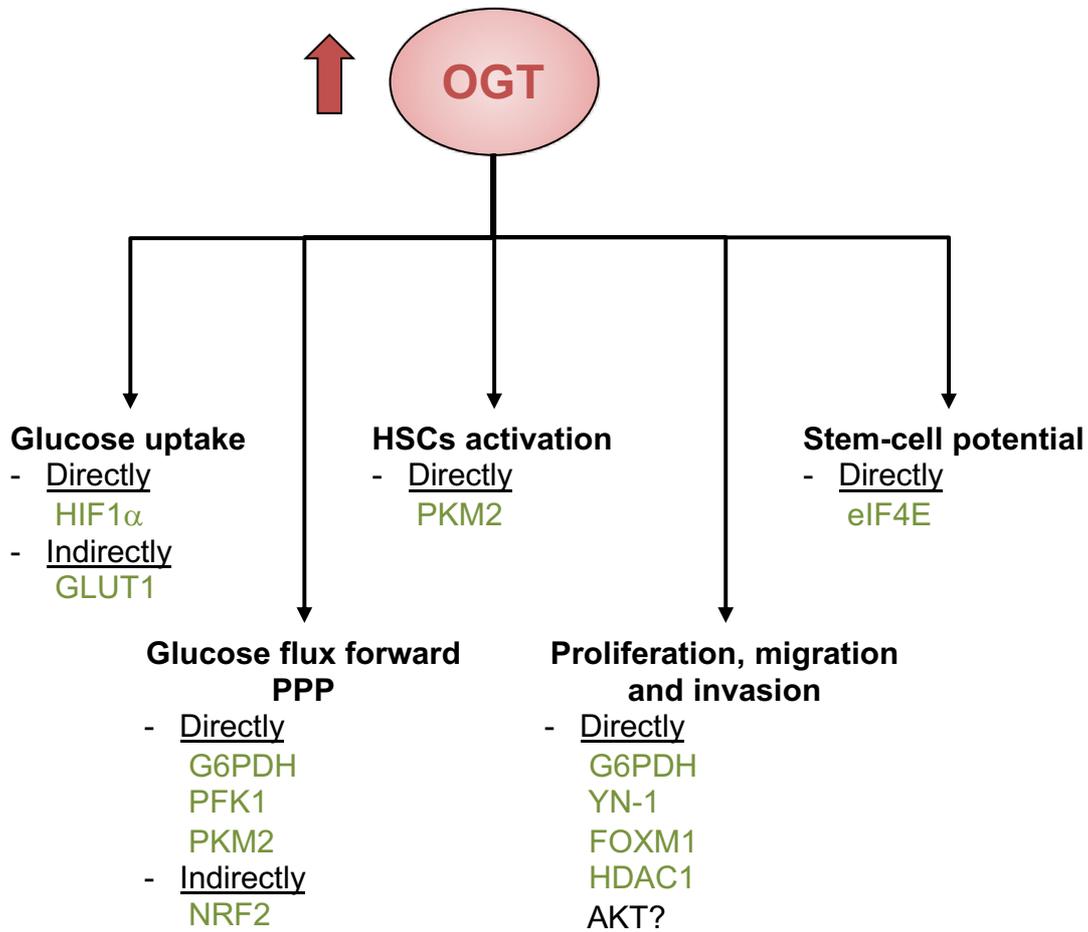


Figure 42. Direct and indirect effects of OGT signaling and metabolic pathways involved in HCC.

## II. Role of OGT in proliferation, cell migration and invasion

Functional assays *in vitro* and in nude mice with gain or loss of OGT function were used to determine the function of OGT in the context of cancer. The authors demonstrated that, *in vivo*, OGT significantly increases cell growth, clonogenicity, migration and invasion (Xu *et al.*, 2017). In xenografts, OGT promoted tumor growth and lung metastasis in nude mice (Xu *et al.*, 2017). The authors also demonstrated that OGT induces the production of palmitic acid which in turn enhanced the expression of GRP78, a marker of ER stress, and IRE1 $\alpha$  (Xu *et al.*, 2017). OGT significantly activated the JNK cascade, the NF- $\kappa$ B pathway and the DNA binding capacity of NF- $\kappa$ B (Xu *et al.*, 2017). Inhibition of OGT in xenograft mouse model also suppressed cell proliferation (Xu *et al.*, 2017), a phenotype confirmed in several other studies (L. Wang *et al.*, 2018; Wang *et al.*, 2019).

### **a) Role of OGT in the regulation of the PI3K/AKT signaling pathway in the context of cancer**

Regulation by O-GlcNAcylation of the PI3K/AKT signaling also modulates proliferation, growth and invasion in cancer cells. Most studies agreed in a positive regulation of AKT phosphorylation and activation *via* upregulation of OGT, O-GlcNAcylation or OGA overexpression in HepG2 hepatic cells (Perez-Cervera *et al.*, 2013) or cholangiocarcinoma cell lines (Phoomak *et al.*, 2018). Briefly, increase in O-GlcNAcylation favors AKT phosphorylation which inhibits proteasomal degradation of Cyclin D1 *via* phosphorylation and down-regulation of GSK3 $\beta$ . Accumulation of Cyclin D1 protein favors cells proliferation and migration (Diehl *et al.*, 1998). However, other contradictory studies suggested the opposite effect, where decreased O-GlcNAcylation by OGA overexpression induced AKT phosphorylation and activation in HepG2 cells and mouse liver (Soesanto *et al.*, 2008). An inhibitor of PI3K, GDC-0941, is being used in clinical trial of patients with cancer (Kwei, Baker and Pelham, 2012). Tumor cells resistant to this compound exhibit an increase in PI3K/AKT/mTOR signaling pathway together with increase OGT expression. Surprisingly, inhibition of *OGT* expression on these cells rescues the sensitivity to GDC-0941. These data open up a new path in which using inhibitors of OGT could potentially increase the effectivity of anti-tumoral therapies.

### **b) OGT controls cell proliferation *via* activation of HDAC1**

O-GlcNAcylation of the histone deacetylase HDAC1 is increased in HCC, suggesting that it could play an important role in the pathogenesis of HCC. Several years ago, two major sites of O-GlcNAcylation were identified in HDAC1 histone deacetylase domain. These O-GlcNAc modifications also activate phosphorylation of HDAC1 increasing its enzymatic activity. Mutation of O-GlcNAcylated site on HDAC1 (Thr114 and Ser263) induces p21 upregulation, a protein involved in cell cycle arrest. Upregulation of p21 in HDAC1 O-GlcNAc mutants altered HCC cell proliferation and occurrence *via* induction of cell cycle arrest but also caused an upregulation of E-cadherin, involved in cell differentiation and cell structure maintenance (Zhu *et al.*, 2016). Through analysis of HCC databases, mSin3A was identified as upregulated in HCC (J. Wang *et al.*, 2018). Considering the relationship between mSin3A, OGT and different epigenetic complexes mentioned in **Chapter 1** such as HDAC1 or TET proteins, it would be interesting to better understand the contribution and/or regulation of mSin3A in the context of HCC.

### **c) O-GlcNAcylation of Y-box binding protein 1 (YB-1) modulates cell proliferation**

Expression of Y-box binding protein 1 or YB-1, a protein involved in the regulation of transcription and translation *via* mRNA silencing in the cytoplasm, was found elevated in HCC tissues. Interestingly, the upregulation in YB-1 correlated with elevated OGT and O-GlcNAc levels, and indeed several residues modified by O-GlcNAcylation were identified on YB-1. However, only O-GlcNAcylation on YB-1 Thr126 was shown to positively influence YB-1 phosphorylation and function, translating into enhanced cell proliferation and HCC progression (Liu *et al.*, 2016).

### **d) Forkhead box M1 (FOXM1) is involved in angiogenesis in the context of HCC**

Forkhead box M1 or FOXM1 is a transcription factor traditionally associated with proliferation *via* regulation of the transition G1/S and G2/M of the cell cycle. Recently, FOXM1 was confirmed by western blotting and immunohistochemistry to be upregulated in HCC, correlating with HCC recurrence and shorter survival (Hu *et al.*, 2019). Interestingly, FOXM1 was previously identified to be highly O-GlcNAcylated in breast cancer cells (Caldwell *et al.*, 2010). Indeed, the authors showed that reducing O-GlcNAc levels in breast cancer cells decreased FOXM1 protein content and caused a decrease of its target genes including Skp2, which regulates cell cycle progression, and matrix metalloproteinase-2 (MMP-2), involved in angiogenesis and tumor progression (Caldwell *et al.*, 2010). These data highlight the potential control of FOXM1 by OGT also in the context of HCC.

### **e) YAP activation *via* O-GlcNAcylation promotes cell proliferation and tumorigenesis**

The Hippo-YAP pathway is a conserved growth suppressor that participates in the control of organ size during development and tumor growth during adult life. The YAP protein is strongly upregulated in HCC and its expression correlates with the induction of cell migration (C. Shi *et al.*, 2018). As mentioned before, hepatic-specific *Yap* knock-out mice exhibit increased liver size resulting from macrovesicular steatosis, but also cirrhosis and abnormal biliary system formation (Zhang *et al.*, 2010). YAP is modulated by O-GlcNAcylation at Ser109 promoting its activity in cell proliferation and tumorigenesis (Peng *et al.*, 2017).

#### **f) The oncogenic c-MYC protein is stabilized by OGT**

c-MYC is a transcription factor known to control cell proliferation, cell cycle, growth, differentiation, apoptosis and metabolism (Chen, Liu and Qing, 2018). c-MYC promotes hepatic tumorigenesis *in vivo*, *in vitro* and in human cancer. c-MYC is O-GlcNAcylated at Thr58 which reduces its phosphorylation on Ser62 and thereby its degradation (Makwana *et al.*, 2019). Increased O-GlcNAcylation level in HCC could lead to stabilization of c-MYC and induction of the tumorigenic effect.

#### **g) OGT stabilizes the EZH2 repressor**

We previously mentioned in **Figure 13** that EZH2 is O-GlcNAcylated resulting in its stabilization and histone trimethylation. EZH2 expression, which is upregulated in HCC cell lines and patients (Chen *et al.*, 2018; Xiao *et al.*, 2019), can promote tumorigenesis by several mechanisms: 1) through its canonical mechanism of silencing tumor suppressor genes by trimethylation of H3K27; 2) by methylation of proteins leading to their activation such as STAT3; or 3) by acting as a coactivator of transcription factors and activating gene expression (Zhang *et al.*, 2020). EZH2 was recently shown to regulate genes involved in the control of hepatocyte maturation and fibrogenesis (Grindheim *et al.*, 2019). The authors showed that *Ezh1/2* hepatic-specific knock-out mice exhibit liver damage at 2 months of life, evidenced by increased ductular reaction and fibrosis. Interestingly, the authors also reported a state of liver regeneration in 2 month-old *Ezh1/2* hepatic-specific knock-out mice with visible nodules in which cells re-expressed EZH2, suggesting potential escape from *Alb-Cre* recombination (Grindheim *et al.*, 2019).

### **III. Stem-cell potential**

Cancer stem cells (CSCs) are a population of cells with stem cell-like characteristics present in tumor tissues. CSCs were identified in HCC, providing the liver with high proliferative, invasive and recurrent advantage compared to the rest of hepatic cells (see more in review from Wu *et al.*, 2020). CSCs markers in HCC have been studied over the past years, highlighting CK19, EpCAM, CD133, CD90, CD24 and CD44; however, additional studies will be needed to better understand the potential of targeted CSC therapies for the treatment of HCC. Several groups suggested possible origins for the hepatic CSCs such as LPCs (Li, *et al.*, 2017), de-differentiation of mature hepatocytes and biliary cells (Nio, Yamashita and Kaneko, 2017), or derived from bone marrow stem cells (Kim *et al.*, 2010), yet the question remains unclear. However,

understanding the origin and the regulation of these cells could have a great impact on the effectiveness of HCC treatment.

#### **a) O-GlcNAcylation stabilizes the eukaryotic initiation factor 4E (eIF4E)**

Upregulation of the eukaryotic initiation factor 4E (eIF4E) expression was reported through immunostaining of HCC liver samples (Cao *et al.*, 2019). eIF4E is a key translation factor that binds to the 5'-untranslated region of the stem-related gene Sox2. O-GlcNAcylation of eIF4E was recently identified on Thr168 and Thr177 residues leading to its stability *via* protection from proteasomal degradation. The authors then suggested that high glucose promotes stem-like cell potential in HCC *via* the OGT-eIF4E axis (Cao *et al.*, 2019).

#### **b) O-GlcNAcylation regulates stability of $\beta$ -catenin**

The Wnt signaling pathway maintains CSCs' capacity of self-renewal by inhibiting their differentiation (Wu *et al.*, 2020). One of the target of this pathway is  $\beta$ -catenin, commonly known to be mutated and constitutively active in HCC (Wu *et al.*, 2020).  $\beta$ -catenin was shown to be O-GlcNAcylated on, at least, 4 residues at the N-terminus identified by MS/MS and leading to its stability and activity (Olivier-Van Stichelen *et al.*, 2014). Better understanding the role of  $\beta$ -catenin/OGT axis in the regulation of CSCs self-renewal could help better understand the role of OGT in the context of HCC.

### **IV. Cholestasis and cholangiocarcinoma (CCA)**

Primary biliary cirrhosis (PBC) or primary sclerosing cholangitis are generated through bile duct injury, generally caused by mechanical injury or by autoimmune conditions. These injuries produce a chronic loss and/or narrowing of bile duct which causes an increase of bile in the liver, called cholestasis, damaging bile duct cholangiocytes. A consequent failure of bile acid secretion leads to the production of ROS, cytokines and chemokines (Christofk *et al.*, 2008). Chronic biliary injury manifests as an abnormal proliferation of bile ducts, called ductular reaction, but also an abnormal cell response by the hepatic progenitors (Colletti *et al.*, 2000). CCA is a rare tumor of slow growing but highly metastatic that leads to late diagnosis and poor prognosis (Zografos *et al.*, 2011). High glucose concentrations were previously correlated with increased cell proliferation, adhesion, migration and invasion in CCA cell lines (Saengboonmee *et al.*, 2016). One of the mechanism proposed was an increase in STAT3 phosphorylation and nuclear translocation, accompanied by cyclin D1, vimentin and MMP-2 enhanced expression (Saengboonmee *et al.*, 2016). High O-GlcNAcylation also correlates with

shorter survival and increased aggressiveness in CCA patients (Phoomak *et al.*, 2012, 2017). Although the implication of OGT and O-GlcNAc in the development and progression of CCA is still not completely understood, Phoomak *et al.* (2017), reported increased global O-GlcNAcylation in highly metastatic CCA cell lines exposed to high glucose concentrations that correlated with increased O-GlcNAcylation of vimentin and vimentin stability, a marker of EMT associated to tumor growth and metastasis (Phoomak *et al.*, 2017). In addition, the authors also investigated the effect of O-GlcNAcylation on membranous N-glycans associated to CCA progression and identified, *via* mass spectrometry, high mannose and biantennary complex N-linked glycans to positively correlate with metastasis. Suppression of OGT using siRNAs led to a decrease in these two types of N-glycans, suggesting that OGT regulates their expression level in CCA. Down-regulation of  $\alpha$ 1,2-mannosidase IA (MAN1A1) and FoxO1 seems necessary for the formation of high-mannose N-glycans *via* the activation of ERK and AKT phosphorylation (Phoomak *et al.*, 2018). These findings suggest that OGT and O-GlcNAcylation could be important for the progression and metastasis of CCA.

#### **CHAPTER 4: RELEVANT POINTS**

1. OGT and O-GlcNAcylation play important roles in the regulation of liver regeneration and homeostasis
2. Increased O-GlcNAcylation has major consequences in the development and worsening of liver injury, NAFLD and different types of liver cancer

# Results

## Objectives of study

As mentioned in the introduction, OGT is an enzyme controlling O-GlcNAcylation, an important post-translational modification (PTM). O-GlcNAcylation consists in the addition of GlcNAc moieties to Ser/Thr residues of target proteins located in different cellular compartments such as the cytoplasm, the nucleus and/or the mitochondria. OGA has the opposite role, hydrolyzing GlcNAc moieties from OGT-targeted proteins. O-GlcNAcylation is a tightly regulated reaction influenced by fluxes through the hexosamine biosynthesis pathway (HBP) in conditions of high carbohydrate uptake or cellular stress. Deregulation in the homeostasis of this cycling reaction has been reported associated with a variety of pathological conditions such as metabolic syndrome, diabetes or cancer (Nie and Yi, 2019).

Our team previously reported that the carbohydrate responsive transcription factor ChREBP is regulated by O-GlcNAcylation through OGT (Guinez *et al.*, 2011). ChREBP contains a low glucose inhibitory domain (LID) and a glucose responsive activation conserved element (GRACE), which is activated in response to glucose *via* PTMs including O-GlcNAcylation. Upon activation, ChREBP translocates to the nucleus where it binds to the carbohydrate responsive element (ChoRE) on the promoter of its target genes involved in the regulation of glycolysis and *de novo* lipogenesis in the liver. Recently, several ChREBP O-GlcNAcylation sites were identified by mass spectrometry and O-GlcNAcylation of ChREBP on serine 839 (Ser839) was reported essential under high glucose concentrations for heterodimerization of ChREBP with its obligatory transcriptional partner Max like Protein X (Mlx) and for enhanced DNA-binding activity.

Considering the implications of ChREBP in the context of gluco-lipotoxicity we aimed to better understand the role of OGT in the regulation of ChREBP, glucose metabolism and lipogenesis in the liver *in vivo*. Because the study of OGT function in *Ogt* global knock-out mice has been limited by its embryonic or early postnatal lethal phenotype, generation of tissue/cell-specific *Ogt* knock-out mice appeared necessary to better understand the role of OGT.

With this purpose, my PhD work included two research objectives:

The research objective 1 implicates the study of the OGT-ChREBP axis with the purpose of gaining further insight into ChREBP regulation by OGT in the liver and to provide further functional evidence of newly identified ChREBP O-GlcNAcylation sites.

The research objective 2 consisted in the metabolic characterization of hepatocyte-specific *Ogt* knock-out mice (OGT<sup>Δ</sup>) to better understand the role of O-GlcNAcylation and OGT in the regulation of liver homeostasis.

**Research article 1: Importance of the OGT-  
ChREBP axis for ChREBP transcriptional  
activity in the liver**

**Importance of the OGT-ChREBP axis for ChREBP transcriptional activity in the liver**

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**Manuscript in preparation**

**Abbreviations used are:** ACC, acetyl coA carboxylase; ChoRE, carbohydrate responsive element; ChREBP, carbohydrate responsive element binding protein; FAS, fatty acid synthase; FoxO1, forkhead box protein O1; GFP, green fluorescent protein; GRACE, glucose responsive activation conserved element; HCF-1, host cell factor 1; HBP, hexosamine biosynthesis pathway; LID, low glucose inhibitory domain; LPK, L-pyruvate kinase; LXR, Liver X receptors; Mlx, Max like Protein X; OGA, O-GlcNAcase; OGT, O-GlcNAc transferase; qPCR, quantitative real-time PCR; PTMs, post-translational modifications; SCD1, Steroyl CoA desaturase.

## **Abstract**

Over the past years, ChREBP has emerged as a major player in the development of metabolic syndrome, but the molecular mechanisms regulating its glucose-dependent activation remain largely unknown. The regulation of ChREBP activity occurs at the level of subcellular localization, DNA binding and transcriptional activity. We and others previously reported that high glucose concentrations induce OGT-mediated ChREBP O-GlcNAcylation, which enhances ChREBP DNA binding and protein stability. In the current study, we wished to gain further insight into ChREBP regulation by OGT in liver and to provide further functional evidence of the newly identified ChREBP O-GlcNAcylation sites. Our study confirms that the OGT-ChREBP axis is important for ChREBP transcriptional activity in liver cells *in vitro* and *in vivo*. Our study also reports that ChREBP and OGT directly interact, and that catalytic activity of OGT is required for ChREBP O-GlcNAcylation and transcriptional activity. However, despite the previous identification of Ser839 as a potential major O-GlcNAcylation site for ChREBP function we observed that its mutation did not impair ChREBP O-GlcNAcylation and/or transcriptional activity in liver cells. Our results suggest that residues important for the control of ChREBP activity by O-GlcNAcylation still remain to be identified.

**Key words :** ChREBP, OGT, O-GlcNAcylation, lipogenesis

## Introduction

In the liver, the transcriptional response to dietary carbohydrates is mediated through the transcription factor Carbohydrate Responsive Element Binding Protein (ChREBP). Highly enriched in the liver, ChREBP contains a low glucose inhibitory domain (LID) and a glucose responsive activation conserved element (GRACE) located in its N-terminus (Li *et al.*, 2006). Activation of the GRACE domain by glucose promotes ChREBP transcriptional activity and binding to the carbohydrate responsive element (ChoRE) of its target genes including L-pyruvate kinase (L-PK), a rate-limiting enzyme in glycolysis, Fatty acid synthase (FAS), Acetyl-CoA carboxylase (ACC) or Stearoyl-CoA desaturase (SCD1), key enzymes of *de novo* lipogenesis (Kawaguchi *et al.*, 2001). More recently, another isoform of ChREBP, ChREBP $\beta$ , originating from an alternative first exon promoter, was identified in adipose tissue and liver (M. A. Herman *et al.*, 2012). This alternative splicing which results in a constitutively active and potent ChREBP isoform, is lacking the LID domain associated with inhibition of ChREBP activity (M. A. Herman *et al.*, 2012). In this context, the full length ChREBP isoform was renamed ChREBP ( $\alpha$ ). The regulation of ChREBP $\beta$  by ChREBP $\alpha$  suggests the existence of a feed forward loop, potentially exacerbating the response to glucose under hyperglycemic conditions. Although ChREBP $\beta$  was suggested as an interesting potential therapeutic target to prevent and/or treat type 2 diabetes and obesity-related metabolic diseases (Abdul-Wahed *et al.*, 2017), the mechanism of regulation and action of this isoform needs further elucidation.

More information concerning ChREBP ( $\alpha$ ) activation is currently available. In response to glucose, ChREBP ( $\alpha$ ) undergoes several post-translational modifications (PTMs) (dephosphorylation, acetylation and/or O-GlcNAcylation) (Ortega-Prieto and Postic, 2019) that will either modulate its cellular location and/or its transcriptional activity (Bricambert *et al.*, 2010, Guinez *et al.*, 2011). Among described PTMs, O-GlcNAcylation is a reversible posttranslational modification that depends on glucose concentrations and on its flux through the hexosamine biosynthesis pathway (HBP). O-GlcNAcylation, a highly dynamic process, is controlled by the action of two enzymes: the O-GlcNAc transferase (OGT), which transfers the monosaccharide N-acetylglucosamine (GlcNAc) to serine and/or threonine residues on target proteins, and the O-GlcNAcase (OGA), which hydrolyses the sugar. It was reported that ChREBP O-GlcNAcylation stabilizes the ChREBP protein and increases its transcriptional activity toward its target glycolytic and lipogenic genes in the liver (Guinez *et al.*, 2012).

Reducing ChREBP O-GlcNAcylation levels *via* OGA overexpression prevented hepatic steatosis of obese *db/db* mice, suggesting that O-GlcNAcylation of ChREBP may represent an important regulatory mechanism of hepatic metabolism under physiological and pathological situations.

Direct modification of ChREBP by O-GlcNAcylation was recently validated in cell-free coupled transcription/translation system and in cells by chemo-enzymatic and metabolic labeling (Yang et al., 2017). Importantly, the authors identified several ChREBP O-GlcNAcylation sites by mass spectrometry. In particular, O-GlcNAcylation of ChREBP on serine 839 (Ser839) was reported essential under high glucose concentrations for heterodimerization of ChREBP with its obligatory transcriptional partner Max like Protein X (Mlx) and for enhanced DNA-binding activity.

In the present work, we wished to gain further insight into ChREBP regulation by OGT in liver and to provide further functional evidence of the newly identified ChREBP O-GlcNAcylation sites. Our study confirms that the OGT-ChREBP axis is important for ChREBP transcriptional activity in liver cells *in vitro* and *in vivo*. However, despite the identification of Ser839 as a potential major O-GlcNAcylation site for ChREBP function (Yang et al., 2017), we observed that its mutation did not impair ChREBP O-GlcNAcylation and/or transcriptional activity in liver cells. Our results suggest that residues important for the control of ChREBP activity by O-GlcNAcylation still remain to be identified.

## **Experimental procedures**

### **Animals**

Ten to twelve week-old adult male *Chrebp*<sup>+/+</sup> and *Chrebp*<sup>-/-</sup> (Iroz et al., 2017) were used for *in vivo* experiments. Procedures were carried out according to the French guidelines for the care and use of experimental animals (Animal authorization agreement n° CEEA34.AFB/CP.082.12, Paris Descartes Ethical Committee). Mice were maintained in a 12 hours light/dark cycle with water and standard diet (61.3% carbohydrate, 13.5% fat, 25.2% protein; SAFE A03) unless specified. For Glucose challenge experiments, mice were allowed *ad libitum* access to standard diet in addition to a 20% glucose solution (Sigma, G7021).

### **Injection of adenovirus *in vivo***

Adenovirus coding GFP or OGT (Dentin et al., 2008) produced by the Laboratoire de thérapie génique (Nantes) were delivered through penis vein injection ( $3 \cdot 10^9$  pfu/mouse) to adult mice. Four days later, nutritional protocols were applied as specified.

### **Primary cultures of hepatocytes**

Hepatocytes were isolated from liver of adult C57BL6/J mice and cultured as previously described (Dentin et al., 2004). Hepatocytes were incubated under low glucose concentrations (5 mM) for 4 h and then infected with specific adenovirus (shControl or shOGT (1 pfu/cell, Genecust) overnight as described (Guinez et al., 2011). Cells were then cultured in the presence of low (5 mM) or high (25 mM) glucose for 24 h.

### **Culture and transfection experiments in HEK293T**

Human embryonic kidney cells (HEK293T) were grown in 6-wells plates in 5 mM D-glucose DMEM supplemented with 10% fetal calf serum (Sigma). Transfections of HEK293 cells with OGT<sup>WT</sup> (Guinez et al., 2011), OGT<sup>H498A</sup> (Lazarus et al., 2011) ChREBP<sup>WT</sup>-Flag (Ma et al., 2005) and ChREBP<sup>S839A</sup>-Flag plasmids (1µg of plasmid/well) were performed using Lipofectamine 2000 and OptiMEM.

### **Site-specific mutagenesis and luciferase reporter assays**

ChREBP<sup>S839A</sup> mutant (Serine 839 into Alanine) was obtained through site-directed mutagenesis with oligonucleotides previously described (Yang et al., 2017). HEK293T were transfected using 250 ng of a *L-pk* luciferase reporter construct containing three

ChoRE sequences (Lou et al., 2009) as previously described (Guinez et al., 2011) or an empty pcDNA plasmid. Co-transfections were performed using 250 ng of OGT<sup>WT</sup>, OGT<sup>H498A</sup>, ChREBP<sup>WT</sup>-FLAG or ChREBP<sup>S839A</sup>-FLAG (as indicated on the Figures) and 250 ng of  $\beta$ -galactosidase plasmid for normalization. The luciferase assay was conducted using the dual luciferase substrate system (E1501; Promega, Madison, WI).

### **Western blotting analysis**

Whole liver or cells were lysed in lysis buffer (20mM Tris HCl [pH 7.5], 150mM NaCl, 5mM EDTA, 50mM NaF, 30mM NaP, 1% Triton X-100, EDTA-free protease inhibitor cocktail [Roche, 4693132001], orthovanadate 1mM, Thiamet-G 10 $\mu$ M [Sigma, SML0244]). Western blot was performed using 30 $\mu$ g of lysate from cells and liver tissue, subjected to 10% SDS-PAGE gel electrophoresis and transferred to nitrocellulose membranes. Antibodies used are: anti-OGT (Sigma, SAB4200311), anti-ChREBP (Novus), anti-RL2 (Abcam, ab2739) and anti-FLAG (Sigma, A8592). GAPDH (Santa Cruz, sc-25778) and  $\beta$ -actin (CST #4970) were used to normalize loading as indicated on Figure legend.

### **Immunoprecipitation and wheat germ agglutinin purification**

For ChREBP immunoprecipitation, cells were lysed on IPH buffer (20 mmol/L Tris/HCl, 150 mmol/L NaCl, 0.5% NP-40 [v/v], protease inhibitors and the OGA inhibitor Thiamet-G (Sigma)). Proteins (1 mg of proteins) were incubated with 2  $\mu$ g of anti-FLAG antibody (Sigma, A8592) and placed at 4°C overnight. Bound proteins were recovered after addition of 25  $\mu$ l of Sepharose-labeled protein G (Sigma) for 1 h at 4°C. Beads were gently centrifuged for 1 min and washed four times for 1 min each. Bound proteins were analyzed by Western blot with a polyclonal anti-OGT antibody (Sigma, SAB4200311). For wheat germ agglutinin ([WGA], a GlcNAc-binding lectin) precipitation, 1 mg of proteins was incubated with 25  $\mu$ l of WGA agarose beads (Sigma, L1882). Then, proteins were eluted from the beads in a 2x Laemmli buffer and separated by SDS-PAGE. Revelation of ChREBP O-GlcNAcylation was performed by incubation with FLAG antibody.

### **Quantitative Real-Time PCR**

Total RNA was extracted using the SV Total RNA isolation system (Promega, Z3101). RNA samples (2 $\mu$ g) were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA was measured by qPCR (LightCycler®

480 SYBR Green I Master, Roche) using the primers described in the Table X. Gene expression was normalized over expression of the TATA-box binding protein (TBP) mRNA levels. Primers used for *Ogt*, *ChREBP*, *ChREBP $\beta$* , *Pklr*, *Acc*, *Fas*, *Scd1*, *Elovl6* were described previously (Guinez et al., 2011; Iroz et al., 2017).

### **Statistical Analysis**

Data represent at least three independent experiments reported as means  $\pm$  S.E.M, and analyzed with analysis of variance using Prism 5.0, GraphPad software. A student's T-test was used when comparing two groups (followed by Mann-Whitney post hoc test) or two-way ANOVA when comparing three or more groups followed by a Bonferroni post hoc test. Statistical significance was defined as  $p < 0.05$ .

## Results

**OGT potentiates the effect of ChREBP on lipogenic gene expression and triglyceride accumulation.** To address the importance of OGT in the ChREBP-mediated glucose response, OGT overexpression studies were conducted *in vivo*. Ten to twelve week-old *Chrebp*<sup>+/+</sup> and *Chrebp*<sup>-/-</sup> mice (Iroz et al., 2017) were injected with an adenovirus vehicle containing the GFP or the OGT protein. Four days later, nutritional protocols were applied: *Chrebp*<sup>+/+</sup> and *Chrebp*<sup>-/-</sup> were given 24-hour access to a bottle of glucose-free water (Fed) or a bottle containing 20% glucose solution (Glucose Challenge) (**Figure 1**). Blood glucose concentrations of GFP-*Chrebp*<sup>-/-</sup> mice were higher than GFP-*Chrebp*<sup>+/+</sup> mice under glucose challenged conditions (**Figure 1A**). Interestingly, OGT overexpression in liver of glucose challenged *Chrebp*<sup>+/+</sup> and *Chrebp*<sup>-/-</sup> mice reduced blood glucose concentrations (a decrease that does not reach statistical difference) (**Figure 1A**). A comparable 4-fold increase in *Ogt* expression was observed in liver of glucose challenged *Chrebp*<sup>+/+</sup> and *Chrebp*<sup>-/-</sup> mice in OGT infected mice (**Figure 1B**). Western Blot analysis confirmed the absence of both ChREBP isoforms ( $\alpha$  (94kDa) and  $\beta$  (72kDa)) in liver of *Chrebp*<sup>-/-</sup> mice (**Figure 1C**). Glucose challenge increased ChREBP protein content ( $\alpha$  and  $\beta$ ) in liver of *Chrebp*<sup>+/+</sup> mice (**Figure 1C**). While no further increase in ChREBP protein content could be detected in *Chrebp*<sup>+/+</sup> mice overexpressing OGT (**Figure 1C**), *Chrebp* $\beta$  mRNA levels were found significant increased compared to GFP conditions when OGT was overexpressed in the liver of glucose challenged *Chrebp*<sup>+/+</sup> mice (**Figure 1D**). Interestingly, a similar profile was observed for lipogenic ChREBP target genes, namely *Acc*, *Fas*, *Elovl6* (**Figure 1D**). This effect on gene expression paralleled with a significant increase in triglyceride concentrations when OGT was overexpressed in liver of glucose challenged *Chrebp*<sup>+/+</sup> mice (**Figure 1E**). This stimulatory effect of OGT on lipogenic gene expression and triglyceride concentrations was dependent on ChREBP since the effect was blunted in liver of *Chrebp*<sup>-/-</sup> mice (**Figure 1D** and **1E**). Altogether, our findings indicate that the stimulatory effect of OGT on lipogenic gene expression and triglyceride concentrations is highly dependent on hepatic ChREBP activity.

### **OGT silencing blunts the stimulatory effect of glucose on ChREBP target genes.**

To confirm the importance of modulating OGT for ChREBP activity, OGT was silenced through an shRNA strategy in mouse hepatocytes (**Figure 2A**). Western Blot (**Figure 2B**) and qPCR analysis (**Figure 2C**) confirmed the efficiency of the shOGT

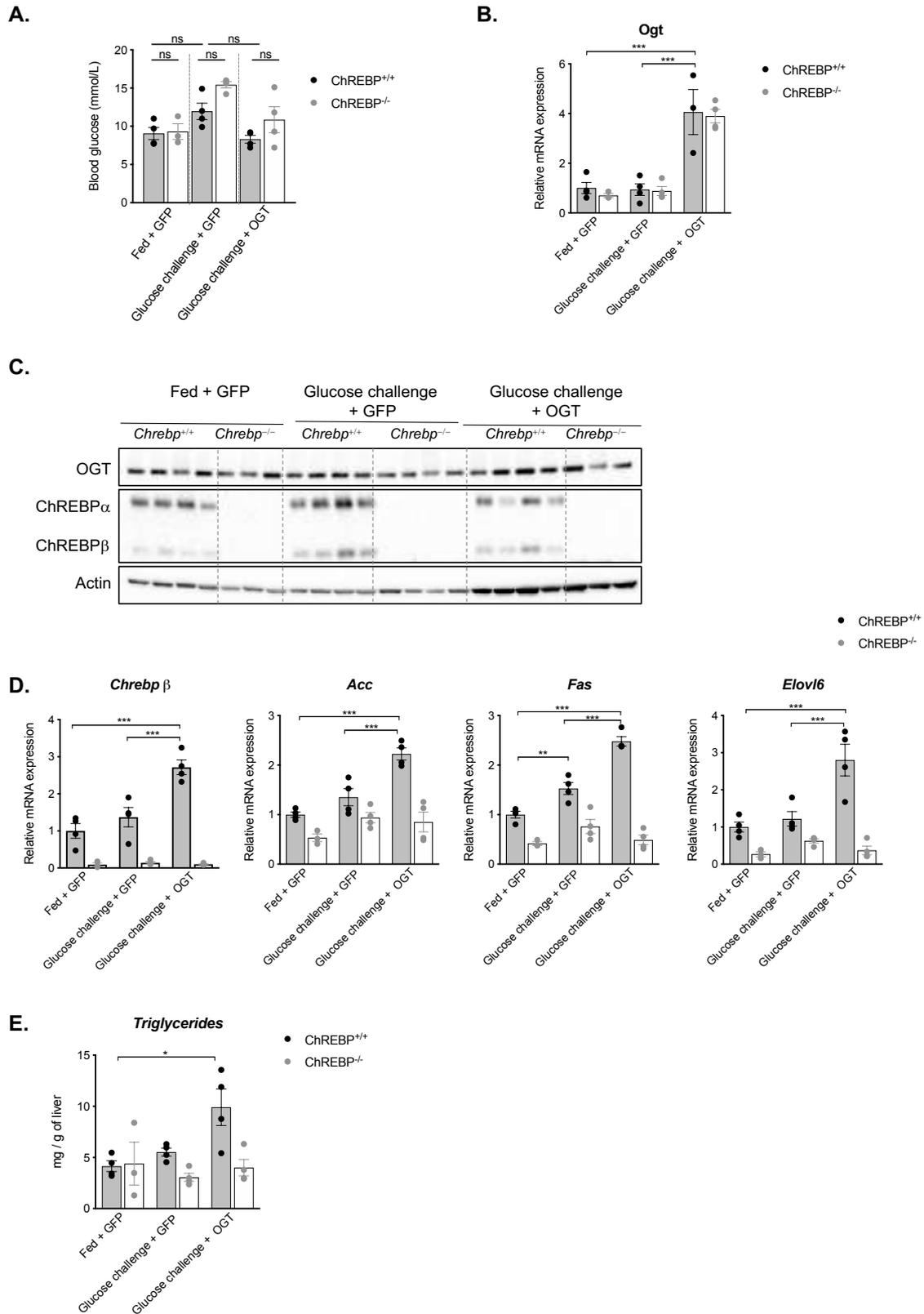
approach. Upon OGT silencing, we observed a decrease in ChREBP mRNA levels under both low (5 mM) and high glucose (25 mM) concentrations (**Figure 2D**). Importantly, the stimulatory effect of high glucose concentrations (25 mM) on ChREBP target genes (*Chrebpβ*, *Pklr*, *Scd1*, and *Elovl6*) was significantly reduced in shOGT conditions (**Figure 2E**). Of note, a significant decrease in *Chrebpβ* and *Pklr* expression was also observed under low glucose (5 mM) concentrations (**Figure 2E**). Our results underline the significant contribution of OGT to the glucose-mediated effect of ChREBP on its target genes.

**O-GlcNAcylation of ChREBP is dependent on the O-GlcNAc transferase activity of OGT.** We next examined whether ChREBP directly interacted with OGT and/or could be modified by O-GlcNAcylation in HEK293T cells (**Figure 3A**). We tested the interaction between ChREBP and wild type OGT (OGT<sup>WT</sup>) but also with a mutant isoform, carrying a mutation on Histidine 498 (His498), located between the reactive serine hydroxyl and the GlcNAc binding pocket, thereby impairing OGT catalytic activity (Lazarus et al., 2011). A ChREBP-Flag plasmid (Ma et al. 2005) was transfected in the absence or in the presence of either OGT<sup>WT</sup> or OGT<sup>H498A</sup> (**Figure 3B**). Immunoprecipitation of cell lysates with a Flag antibody and immunoblotting with anti-OGT antibody revealed that ChREBP co-immunoprecipitated with OGT (**Figure 3C**). Interestingly, the interaction between ChREBP and OGT was stronger with OGT<sup>H498A</sup> than with OGT<sup>WT</sup> (**Figure 3C**). However, despite stronger interaction, recovery of ChREBP on WGA beads suggested that ChREBP was less O-GlcNAcylated in presence of the catalytic mutant of OGT and the levels were similar to cell expressing only the endogenous OGT (**Figure 3C**). To determine whether O-GlcNAcylation of ChREBP directly impacts on its activity, HEK293T cells co-expressing ChREBP and either OGT<sup>WT</sup> or OGT<sup>H498A</sup> were transfected with a *L-pk* luciferase reporter construct containing three ChoRE sequences (Lou et al., 2009) (**Figure 3D**). We observed that the presence of OGT<sup>WT</sup> potentiated the effect of ChREBP on the *L-pk* luciferase reporter construct (**Figure 3D**). This effect was not observed when OGT<sup>H498A</sup>, which does not increase O-GlcNAcylation of ChREBP, was expressed (**Figure 3C**). Altogether, our results show that O-GlcNAcylation of ChREBP increases its transcriptional activity and is dependent on OGT catalytic activity.

**Mutation of Ser839 does not impair ChREBP O-GlcNAcylation or its transcriptional activity.** Since we report here that ChREBP O-GlcNAcylation results

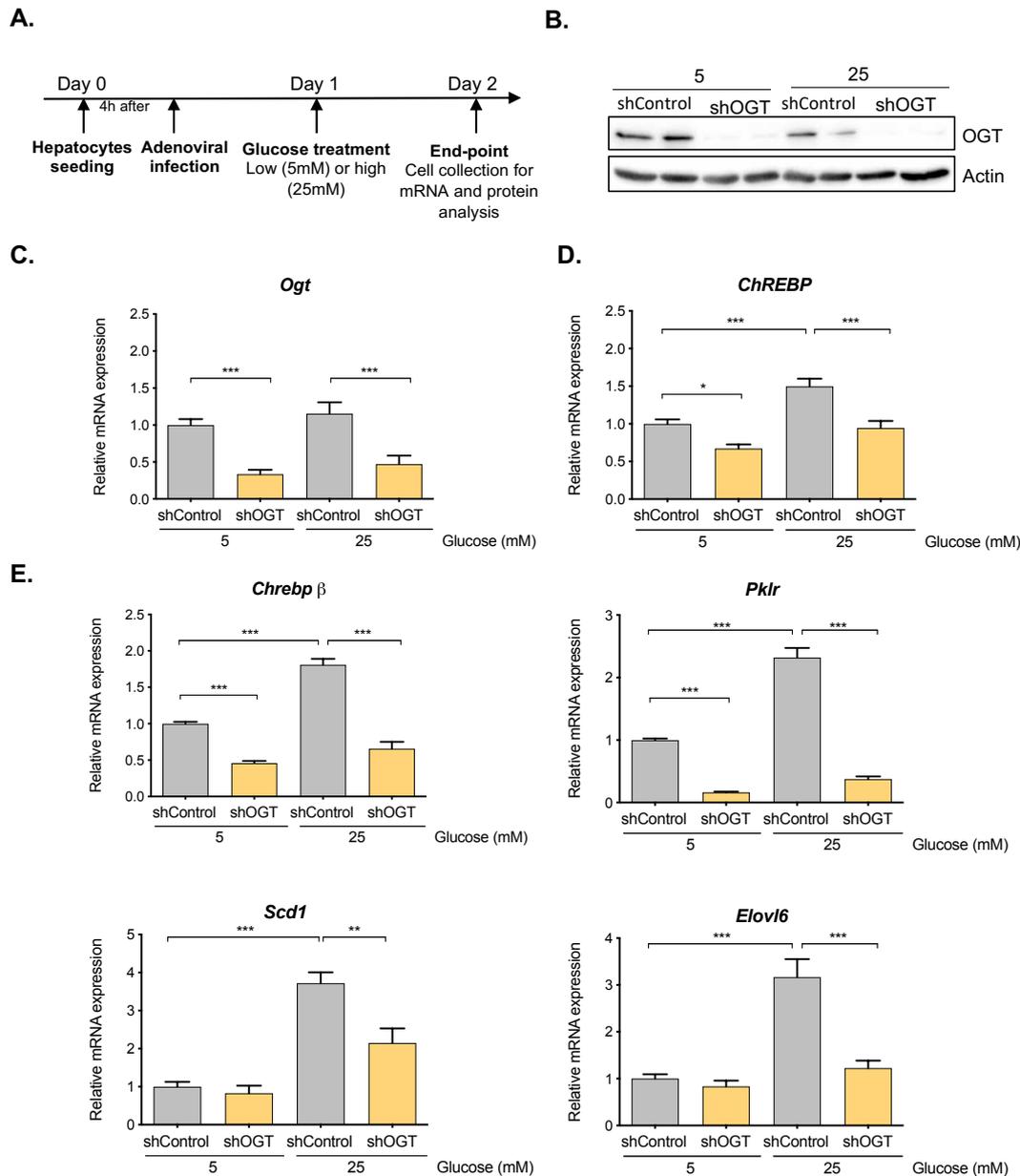
in an increase in its transcriptional activity, we aimed at providing further functional evidence of the newly identified ChREBP O-GlcNAcylation sites (Yang et al., 2017). We focused on Ser839, since it was reported that O-GlcNAcylation on this particular residue is important for ChREBP activity in liver cells (Yang et al., 2017). ChREBP<sup>WT</sup>-Flag and ChREBP<sup>S839A</sup>-Flag plasmids were transfected in HEK293T cells under low and high glucose concentrations (**Figure 4B**). To determine if the mutation of ChREBP led to a modification of its transcriptional activity, luciferase assay was performed. HEK293T cells were transfected with *L-pk* luciferase reporter, and with ChREBP<sup>WT</sup>-Flag or ChREBP<sup>S839A</sup>-Flag plasmids (Figure 4B). We observed that under low or high glucose concentration, there was no difference in ChREBP transcriptional activity despite the mutation at Ser839. As a control, Western blot analysis of ChREBP<sup>WT</sup>-Flag or ChREBP<sup>S839A</sup>-Flag transfection was done (**Figure 4C**). In agreement, global O-GlcNAcylation level of this ChREBP mutant was not decreased as revealed by its retention in WGA and it was comparable to the one measured in ChREBP<sup>WT</sup> (**Figure 4D**).

# Figures

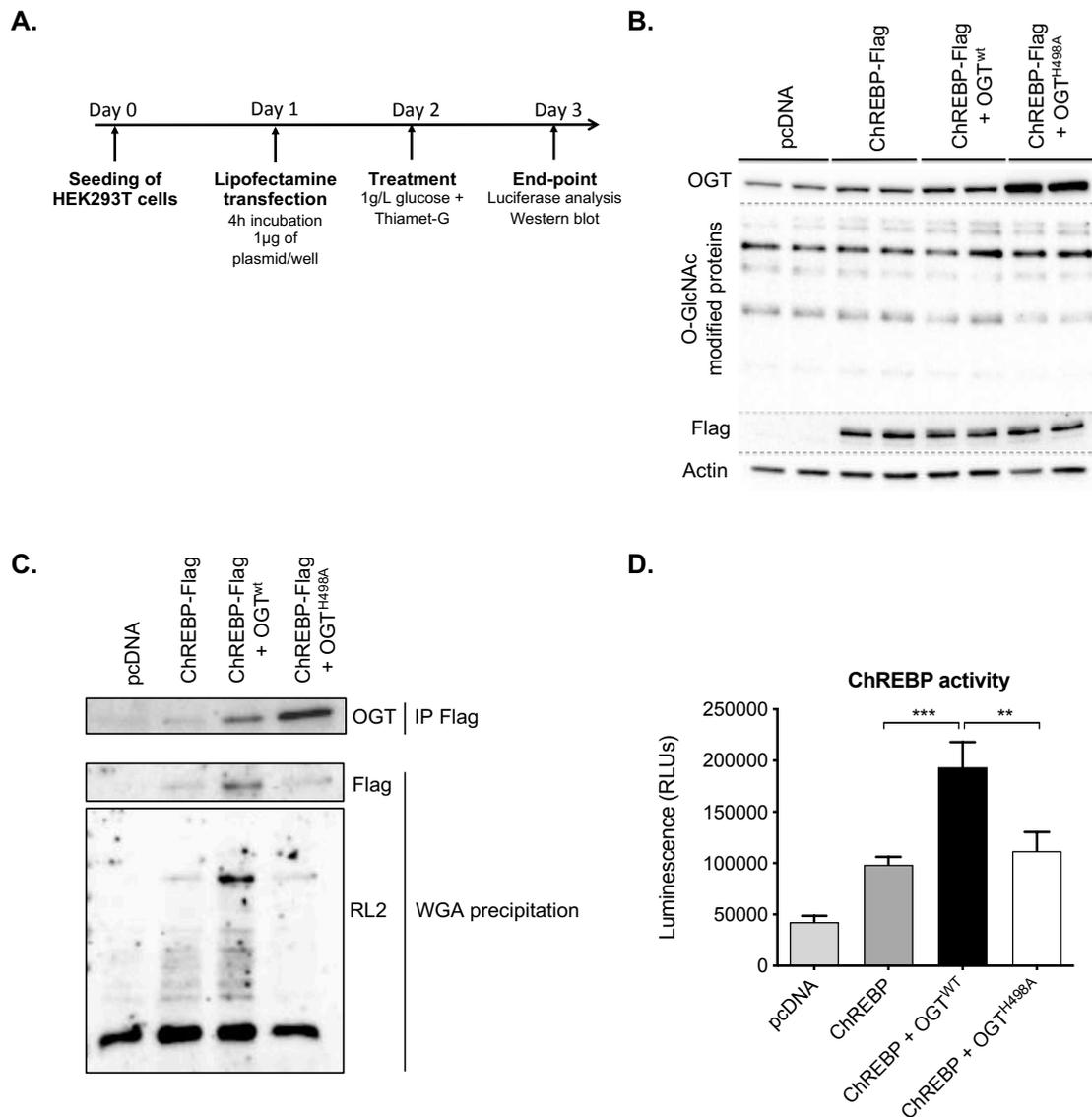


**Figure 1. The stimulatory effect of OGT on lipogenic gene expression is dependent on ChREBP.** Adult male ChREBP<sup>+/+</sup> and ChREBP<sup>-/-</sup> mice were injected intravenously with a single dose of  $3 \times 10^9$  pfu GFP or OGT adenovirus on Day 1. Seven days later,

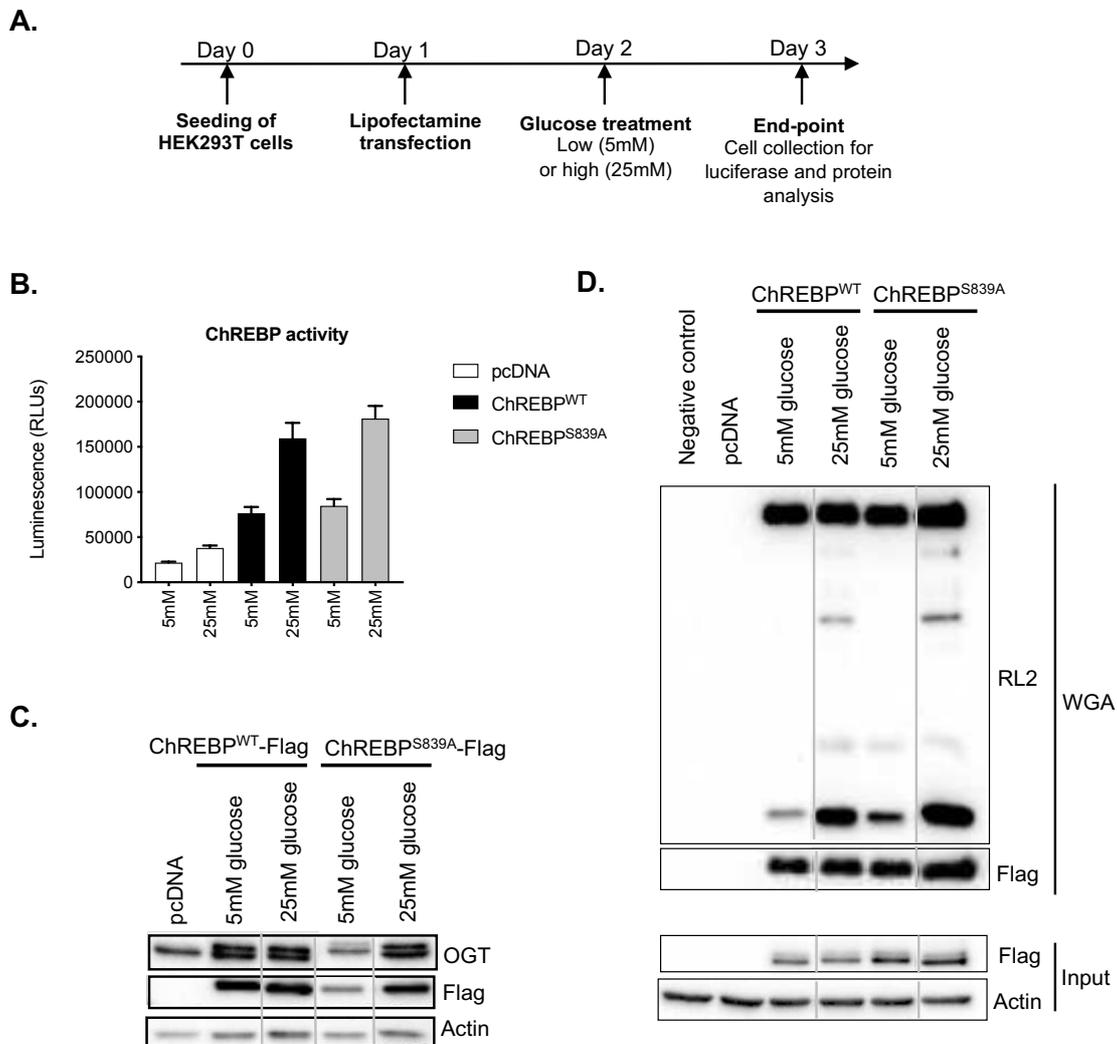
liver samples were harvested for analyses. **A.** Blood glucose in mg/dl was recovered at time of harvest from tail snip. **B.** Western Blot analysis of protein extracted from whole liver lysate.  $\beta$ -actin was used as loading control. Three to four representative samples are presented. **C.** RT-qPCR analysis of *Ogt* expression. **D.** RT-qPCR analysis of *Chrebp $\beta$* , *Acc*, *Fas*, *Elovl6* expression. **E.** Triglyceride concentrations expressed as mg/g of liver. Figures are presented as means  $\pm$  SEM from 3 to 4 individual mice per group. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001 by unpaired Student t-test (Mann-Whitney).



**Figure 2. OGT knockdown impairs the stimulatory effect of ChREBP on its target genes.** **A.** Mouse hepatocytes were incubated under low glucose concentrations (5 mM) and adeno-infected with 5 pfu/cell of control (shCTRL) or shOGT for overnight. Cells were then incubated for 24 h under low glucose (5 mM) or high glucose concentrations (25 mM). **B.** Representative Western blot of OGT is shown. Two representative samples are presented. **C.** RT-qPCR analysis of *Ogt* expression **D.** RT-qPCR analysis of *Chrebp* expression **E.** RT-qPCR analysis of *Chrebp* $\beta$ , *Pklr*, *Scd1* and *Elovl6* expression. Data are means  $\pm$  SEM.  $n = 3$  independent cultures with conditions done in triplicates. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  by one-way ANOVA. Bonferroni post-test.



**Figure 3. O-GlcNAcylation of ChREBP depends on OGT catalytic activity.** **A.** HEK293T cells were transfected with ChREBP-Flag plasmid (Ma et al. 2005) in the absence or in the presence of OGT<sup>WT</sup> or OGT<sup>H498A</sup> to a final concentration of 1 $\mu$ g of plasmid/well. On the second day, cells were treated with 1g/L of glucose and 10 $\mu$ M Thiamet-G. **B.** Representative Western blot analysis of OGT, O-GlcNAcylated and Flagged proteins (ChREBP) are shown. Two representative samples are presented. **(C).** Immunoprecipitation of cell lysates with a Flag antibody and immunoblotting with OGT antibody. Immunoprecipitation of cell lysates with WGA beads and immunoblotting with antibodies against Flag (to detect ChREBP) or RL2 (to detect global O-GlcNAcylated proteins). One representative sample is shown. **(D).** ChoRE *Lpk*-driven luciferase reporter and plasmids expressing ChREBP, with OGT<sup>WT</sup> or OGT<sup>H498A</sup>. Dual luciferase reporter assays were performed 24 h post transfection. Data is shown as mean  $\pm$  SEM. Four independent experiments were done in triplicates. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001 by unpaired Student t-test (Mann-Whitney).



**Figure 4. Mutation of Ser839 does not impair O-GlcNAcylation or transcriptional activity of ChREBP.** **A.** Experimental setting is described. **B.** *Lpk*-driven luciferase reporter in cells transfected with plasmids expressing ChREBP<sup>WT</sup> or ChREBP<sup>S839A</sup> and cultured under either low or high glucose concentrations for 24h. Dual luciferase reporter assays were performed 24 h post transfection. Data is shown as mean  $\pm$  SEM. One independent experiment was done in 3-5 replicates. **C.** A representative Western blot of OGT and Flagged proteins (ChREBP) is shown. One representative sample in each condition is presented. Actin was used as loading control. **D.** Immunoprecipitation of cell lysates with WGA beads and immunoblotting with antibodies against RL2 (to detect global O-GlcNAcylation of proteins) and Flag (to detect O-GlcNAcylation of ChREBP). Total amount of transfected ChREBP is shown with immunoblotting with Flag (Input). Actin was used as loading control. One representative sample is shown. Cut membranes were indicated with continuous light grey lines.

## Discussion

In the current work, we provide evidence for the importance of the OGT-ChREBP axis in ChREBP transcriptional activity in liver cells *in vitro* and *in vivo*. Indeed, we show direct interaction of ChREBP with OGT, and that O-GlcNAcylation of ChREBP increases its transcriptional activity. However, despite the identification of Ser839 as a potential major O-GlcNAcylation site for ChREBP function (Yang et al., 2017), we report here that its mutation does not impair ChREBP O-GlcNAcylation and/or transcriptional activity. Our results suggest that residues important for the control of ChREBP activity by O-GlcNAcylation still remain to be identified.

Over the past years, ChREBP has emerged as a major player in the development of metabolic syndrome, but the molecular mechanisms regulating its glucose-dependent activation remain largely unknown. The regulation of ChREBP activity occurs at the level of subcellular localization, DNA binding and transcriptional activity. Since its discovery in 2000, intensive efforts have been employed to identify the precise mechanism by which glucose stimulates ChREBP activity. A metabolite of glucose, rather than glucose itself was found to be responsible for the glucose-activating signal (Abdul-Wahed et al, 2017). The first candidate proposed was xylulose 5-phosphate (X5P), which derives from glucose metabolism through the pentose phosphate pathway (Kabashima et al., 2003; Kawaguchi et al., 2001). This model was challenged by recent research showing that the first intermediate of glucose metabolism Glucose 6-phosphate (G6P) rather than X5P was required for glucose mediated ChREBP activation, mainly through adenoviral modulation of Glucose 6 phosphate dehydrogenase (G6PDH) expression (Dentin et al., 2012; Li et al., 2010). The possible role of G6P was further reinforced by the identification by McFerrin and Atchley of a putative G6P recognition motif in the GRACE domain that is highly conserved within the mondo family of proteins, raising the possibility of an allosteric regulation of ChREBP through direct binding of G6P to this motif (McFerrin and Atchley, 2012).

Besides nucleo-cytoplasmic translocation, high glucose boosts ChREBP transcriptional activity through acetylation and/or O-GlcNAcylation (Bricambert et al., 2010; Guinez et al., 2011). High glucose leads to ChREBP acetylation on Lys672 located in the DNA binding domain by the histone acetyl transferase (HAT) transcriptional coactivator p300, thereby increasing its transcriptional activity by enhancing its binding to promoters of target genes. Accordingly, targeted mutation of Lys672 totally abrogates glucose mediated ChREBP transcription (Bricambert et al., 2010). High glucose concentrations also induce OGT-mediated ChREBP O-

GlcNAcylation, which enhances ChREBP DNA binding and protein stability (Guinez et al., 2011; Ido-Kitamura et al., 2012; Park et al., 2014; Sakiyama et al., 2010). O-GlcNAcylation was also suggested to decrease ubiquitin-mediated degradation of ChREBP (Guinez et al., 2011; Ido-Kitamura et al., 2012). This finding seems puzzling considering that O-GlcNAcylation and ubiquitination target different amino acid residues. The identification of respective sites of ChREBP O-GlcNAcylation and ubiquitination would help unravel the underlying mechanisms involved in such rescue. Meanwhile, the relevance of these post-transcriptional modifications was reinforced by studies showing increased acetylation and O-GlcNAcylation of ChREBP in livers of hyperglycemic mouse models, whereas targeting of either modifications through p300 inhibition or by overexpression of O-GlcNAcylation reversing enzyme O-GlcNAcase (OGA) prevents the development of hepatic steatosis (Bricambert et al., 2010; Guinez et al., 2011).

Because of limited specific detection and lack of relevant identification methods for O-GlcNAcylation sites on ChREBP, the question of how O-GlcNAcylation regulates ChREBP activity remained unresolved until recently. The work of Yang and coworkers not only validated ChREBP O-GlcNAcylation using chemical biology methods but also identified potential ChREBP residues targeted by O-GlcNAcylation. The authors reported that deficient O-GlcNAcylation at Ser839 nearly abolished ChREBP transcriptional activity, attenuated interaction between ChREBP and Mlx and disrupted DNA binding activity. Surprisingly, while remaking the exact same ChREBP mutant (ChREBP<sup>S839A</sup>) we could not reproduce their results and did not detect any change in ChREBP O-GlcNAcylation levels and/or transcriptional activity. While we have no clear explanation for this discrepancy, this is not the first time that O-GlcNAcylation sites identified by a group are not later validated in a follow-up study (Fardini et al., 2015). This was the case for the transcription factor Forkhead box protein O1 (FoxO1) for which several O-GlcNAcylation sites were identified in the human isoform. However, directed mutagenesis of each site individually had modest or no effect on FoxO1 O-GlcNAcylation status and transcriptional activity. Moreover, mutation of all four sites in mouse FoxO1 did not decrease FoxO1 O-GlcNAcylation nor its transcriptional activity (Fardini et al., 2015). Altogether, this suggests that additional crucial ChREBP and FoxO1 O-GlcNAcylation sites are yet to be identified. Alternatively, it is possible that when the identified O-GlcNAcylation sites are mutated on a given protein, OGT glycosylates other adjacent sites. Indeed, promiscuity of OGT for adjacent sites has been described in numerous proteins (Zhao P et al., 2011) and

persistence of high global O-GlcNAc levels after mutagenesis of O-GlcNAcylation sites have also been observed in other proteins (Yang et al., 2008).

We previously reported that reducing ChREBP O-GlcNAcylation levels *via* OGA overexpression prevented hepatic steatosis of obese *db/db* mice, suggesting that O-GlcNAcylation of ChREBP may represent an important regulatory mechanism of hepatic metabolism under physiological and pathological situations. In the current study, we confirmed that the OGT-ChREBP axis is important for the regulation of lipogenic gene expression and triglyceride synthesis in liver. Other key lipogenic transcription factors were reported to be regulated by O-GlcNAcylation, such as Liver X receptors (LXRs), nuclear hormone receptors activated by oxysterols (Anthonisen *et al.*, 2010). Since LXR $\alpha$  is also an upstream regulator of ChREBP, its regulation by O-GlcNAcylation probably reinforces the stimulatory effect on lipogenic gene expression, acting in both a direct manner on lipogenic genes such as FAS and through an indirect manner (through its control of ChREBP activity) (Anthonisen *et al.*, 2010; Bindsbøll *et al.*, 2015). Nevertheless, we observed that, when OGT was overexpressed in liver of *Chrebp*<sup>-/-</sup> mice, the potentiating effect of OGT on lipogenic gene expression is totally blunted. Interestingly, a recent study identified a novel ChREBP-interacting protein regulated by O-GlcNAcylation and required for the control of hepatic lipogenesis. Indeed, O-GlcNAcylation of this factor, called Host cell factor 1 (HCF-1), is a prerequisite for its binding to ChREBP and subsequent recruitment of OGT, ChREBP O-GlcNAcylation and activation. The HCF-1-ChREBP complex resides at lipogenic gene promoters, where HCF-1 regulates H3K4 trimethylation to prime recruitment of the Jumonji C domain-containing histone demethylase PHF2 for epigenetic activation of these promoters (Lane et al., 2019). Interestingly, our team previously identified PHF2 as an important transcriptional co-activator of ChREBP in hepatocytes (Bricambert et al., 2018).

Altogether, our study confirms that the OGT-ChREBP axis acts as an important pathway to control of lipogenic gene expression and fatty acid synthesis. Our study also reports that ChREBP and OGT directly interact, and that catalytic activity of OGT is required for ChREBP O-GlcNAcylation and transcriptional activity. This information may accelerate the design of biological experiments to identify functional ChREBP O-GlcNAcylation sites and the design of inhibitors of the OGT-ChREBP interaction for the prevention of hepatic steatosis onset.

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## References

1. Abdul-Wahed A, Guilmeau S, Postic C. Sweet Sixteenth for ChREBP: Established Roles and Future Goals. *Cell Metab.* 2017 Aug 1;26(2):324-341. doi: 10.1016/j.cmet.2017.07.004. PMID: 28768172.
2. Anthonisen EH, Berven L, Holm S, Nygård M, Nebb HI, Grønning-Wang LM. Nuclear receptor liver X receptor is O-GlcNAc-modified in response to glucose. *J Biol Chem.* 2010 Jan 15;285(3):1607-15. doi: 10.1074/jbc.M109.082685. Epub 2009 Nov 20. PMID: 19933273; PMCID: PMC2804318.
3. Bindsbøll C, Fan Q, Nørgaard RC, MacPherson L, Ruan HB, Wu J, Pedersen TÅ, Steffensen KR, Yang X, Matthews J, Mandrup S, Nebb HI, Grønning-Wang LM. Liver X receptor regulates hepatic nuclear O-GlcNAc signaling and carbohydrate responsive element-binding protein activity. *J Lipid Res.* 2015 Apr;56(4):771-85. doi: 10.1194/jlr.M049130. Epub 2015 Feb 27. PMID: 25724563; PMCID: PMC4373736.
4. Bricambert J, Miranda J, Benhamed F, Girard J, Postic C, Dentin R. Salt-inducible kinase 2 links transcriptional coactivator p300 phosphorylation to the prevention of ChREBP-dependent hepatic steatosis in mice. *J Clin Invest.* 2010 Dec;120(12):4316-31. doi: 10.1172/JCI41624. Epub 2010 Nov 15. PMID: 21084751; PMCID: PMC2993582.
5. Bricambert J, Alves-Guerra MC, Esteves P, Prip-Buus C, Bertrand-Michel J, Guillou H, Chang CJ, Vander Wal MN, Canonne-Hergaux F, Mathurin P, Raverdy V, Pattou F, Girard J, Postic C, Dentin R. The histone demethylase Phf2 acts as a molecular checkpoint to prevent NAFLD progression during obesity. *Nat Commun.* 2018 May 29;9(1):2092. doi: 10.1038/s41467-018-04361-y. PMID: 29844386; PMCID: PMC5974278.
6. Dentin R, Hedrick S, Xie J, Yates J 3rd, Montminy M. Hepatic glucose sensing via the CREB coactivator CRTC2. *Science.* 2008 Mar 7;319(5868):1402-5. doi: 10.1126/science.1151363. PMID: 18323454.
7. Dentin R, Tomas-Cobos L, Fougelle F, Leopold J, Girard J, Postic C, Ferré P. Glucose 6-phosphate, rather than xylulose 5-phosphate, is required for the activation of ChREBP in response to glucose in the liver. *J Hepatol.* 2012

- Jan;56(1):199-209. doi: 10.1016/j.jhep.2011.07.019. Epub 2011 Aug 9. PMID: 21835137.
8. Fardini Y, Perez-Cervera Y, Camoin L, Pagesy P, Lefebvre T, Issad T. Regulatory O-GlcNAcylation sites on FoxO1 are yet to be identified. *Biochem Biophys Res Commun.* 2015 Jun 26;462(2):151-8. doi: 10.1016/j.bbrc.2015.04.114. Epub 2015 May 2. PMID: 25944660.
  9. Guinez C, Filhoulaud G, Rayah-Benhamed F, Marmier S, Dubuquoy C, Dentin R, Moldes M, Burnol AF, Yang X, Lefebvre T, Girard J, Postic C. O-GlcNAcylation increases ChREBP protein content and transcriptional activity in the liver. *Diabetes.* 2011 May;60(5):1399-413. doi: 10.2337/db10-0452. Epub 2011 Apr 6. PMID: 21471514; PMCID: PMC3292313.
  10. Herman MA, Peroni OD, Villoria J, Schön MR, Abumrad NA, Blüher M, Klein S, Kahn BB. A novel ChREBP isoform in adipose tissue regulates systemic glucose metabolism. *Nature.* 2012 Apr 19;484(7394):333-8. doi: 10.1038/nature10986. PMID: 22466288; PMCID: PMC3341994.
  11. Housley MP, Rodgers JT, Udeshi ND, Kelly TJ, Shabanowitz J, Hunt DF, Puigserver P, Hart GW. O-GlcNAc regulates FoxO activation in response to glucose. *J Biol Chem.* 2008 Jun 13;283(24):16283-92. doi: 10.1074/jbc.M802240200. Epub 2008 Apr 17. PMID: 18420577; PMCID: PMC2423255.
  12. Ido-Kitamura Y, Sasaki T, Kobayashi M, Kim HJ, Lee YS, Kikuchi O, Yokota-Hashimoto H, Iizuka K, Accili D, Kitamura T. Hepatic FoxO1 integrates glucose utilization and lipid synthesis through regulation of ChREBP O-glycosylation. *PLoS One.* 2012;7(10):e47231. doi: 10.1371/journal.pone.0047231. Epub 2012 Oct 8. PMID: 23056614; PMCID: PMC3466224.
  13. Iroz A, Montagner A, Benhamed F, Levavasseur F, Polizzi A, Anthony E, Régnier M, Fouché E, Lukowicz C, Cauzac M, Tournier E, Do-Cruzeiro M, Dajjat-Chavanieu M, Gerbal-Chalouin S, Fauveau V, Marmier S, Burnol AF, Guilmeau S, Lippi Y, Girard J, Wahli W, Dentin R, Guillou H, Postic C. A

- Specific ChREBP and PPAR $\alpha$  Cross-Talk Is Required for the Glucose-Mediated FGF21 Response. *Cell Rep.* 2017 Oct 10;21(2):403-416. doi: 10.1016/j.celrep.2017.09.065. PMID: 29020627; PMCID: PMC5643524.
14. Issad T, Kuo M. O-GlcNAc modification of transcription factors, glucose sensing and glucotoxicity. *Trends Endocrinol Metab.* 2008 Dec;19(10):380-9. doi: 10.1016/j.tem.2008.09.001. Epub 2008 Oct 17. PMID: 18929495.
  15. Kabashima T, Kawaguchi T, Wadzinski BE, Uyeda K. Xylulose 5-phosphate mediates glucose-induced lipogenesis by xylulose 5-phosphate-activated protein phosphatase in rat liver. *Proc Natl Acad Sci U S A.* 2003 Apr 29;100(9):5107-12. doi: 10.1073/pnas.0730817100. Epub 2003 Apr 8. PMID: 12684532; PMCID: PMC154306.
  16. Kawaguchi T, Takenoshita M, Kabashima T, Uyeda K. Glucose and cAMP regulate the L-type pyruvate kinase gene by phosphorylation/dephosphorylation of the carbohydrate response element binding protein. *Proc Natl Acad Sci U S A.* 2001 Nov 20;98(24):13710-5. doi: 10.1073/pnas.231370798. Epub 2001 Nov 6. PMID: 11698644; PMCID: PMC61106.
  17. Lane EA, Choi DW, Garcia-Haro L, Levine ZG, Tedoldi M, Walker S, Danial NN. HCF-1 Regulates De Novo Lipogenesis through a Nutrient-Sensitive Complex with ChREBP. *Mol Cell.* 2019 Jul 25;75(2):357-371.e7. doi: 10.1016/j.molcel.2019.05.019. Epub 2019 Jun 18. PMID: 31227231; PMCID: PMC6744259.
  18. Lazarus MB, Nam Y, Jiang J, Sliz P, Walker S. Structure of human O-GlcNAc transferase and its complex with a peptide substrate. *Nature.* 2011 Jan 27;469(7331):564-7. doi: 10.1038/nature09638. Epub 2011 Jan 16. PMID: 21240259; PMCID: PMC3064491.
  19. Li MV, Chen W, Harmancey RN, Nuotio-Antar AM, Imamura M, Saha P, Taegtmeier H, Chan L. Glucose-6-phosphate mediates activation of the carbohydrate responsive binding protein (ChREBP). *Biochem Biophys Res Commun.* 2010 May 7;395(3):395-400. doi: 10.1016/j.bbrc.2010.04.028. Epub 2010 Apr 9. PMID: 20382127; PMCID: PMC2874883.

20. Lou DQ, Tannour M, Selig L, Thomas D, Kahn A, Vasseur-Cognet M. Chicken ovalbumin upstream promoter-transcription factor II, a new partner of the glucose response element of the L-type pyruvate kinase gene, acts as an inhibitor of the glucose response. *J Biol Chem.* 1999 Oct 1;274(40):28385-94. doi: 10.1074/jbc.274.40.28385. PMID: 10497199.
21. Ma L, Tsatsos NG, Towle HC. Direct role of ChREBP.Mlx in regulating hepatic glucose-responsive genes. *J Biol Chem.* 2005 Mar 25;280(12):12019-27. doi: 10.1074/jbc.M413063200. Epub 2005 Jan 20. PMID: 15664996.
22. McFerrin LG, Atchley WR. A novel N-terminal domain may dictate the glucose response of Mondo proteins. *PLoS One.* 2012;7(4):e34803. doi: 10.1371/journal.pone.0034803. Epub 2012 Apr 10. PMID: 22506051; PMCID: PMC3323566.
23. Ortega-Prieto P, Postic C. Carbohydrate Sensing Through the Transcription Factor ChREBP. *Front Genet.* 2019 Jun 4;10:472. doi: 10.3389/fgene.2019.00472. PMID: 31275349; PMCID: PMC6593282.
24. Park MJ, Kim DI, Lim SK, Choi JH, Han HJ, Yoon KC, Park SH. High glucose-induced O-GlcNAcylated carbohydrate response element-binding protein (ChREBP) mediates mesangial cell lipogenesis and fibrosis: the possible role in the development of diabetic nephropathy. *J Biol Chem.* 2014 May 9;289(19):13519-30. doi: 10.1074/jbc.M113.530139. Epub 2014 Mar 10. PMID: 24616092; PMCID: PMC4036358.
25. Sakiyama H, Fujiwara N, Noguchi T, Eguchi H, Yoshihara D, Uyeda K, Suzuki K. The role of O-linked GlcNAc modification on the glucose response of ChREBP. *Biochem Biophys Res Commun.* 2010 Nov 26;402(4):784-9. doi: 10.1016/j.bbrc.2010.10.113. Epub 2010 Oct 29. PMID: 21036147.
26. Yang AQ, Li D, Chi L, Ye XS. Validation, Identification, and Biological Consequences of the Site-specific O-GlcNAcylation Dynamics of Carbohydrate-responsive Element-binding Protein (ChREBP). *Mol Cell Proteomics.* 2017 Jul;16(7):1233-1243. doi: 10.1074/mcp.M116.061416. Epub 2017 Apr 27. PMID: 28450420; PMCID: PMC5500757.

27. Yang WH, Park SY, Nam HW, Kim DH, Kang JG, Kang ES, Kim YS, Lee HC, Kim KS, Cho JW. NFkappaB activation is associated with its O-GlcNAcylation state under hyperglycemic conditions. *Proc Natl Acad Sci U S A*. 2008 Nov 11;105(45):17345-50. doi: 10.1073/pnas.0806198105. Epub 2008 Nov 6. PMID: 18988733; PMCID: PMC2582288.
28. Zhao P, Viner R, Teo CF, Boons GJ, Horn D, Wells L. Combining high-energy C-trap dissociation and electron transfer dissociation for protein O-GlcNAc modification site assignment. *J Proteome Res*. 2011 Sep 2;10(9):4088-104. doi: 10.1021/pr2002726. Epub 2011 Jul 25. PMID: 21740066; PMCID: PMC3172619.

## Summary of research project 1

My first objective was to study of the contribution of the OGT-ChREBP axis in the control of hepatic lipogenesis and to gain further insights into ChREBP regulation by OGT. Although this study will need to be completed by additional experiments, we were able to draw some interesting conclusions. First, our study confirms that the OGT-ChREBP axis is important for ChREBP transcriptional activity in liver cells *in vitro* and *in vivo*. We observed that when OGT is overexpressed in liver of *Chrebp*<sup>-/-</sup> mice, the potentiating effect of OGT on lipogenic gene expression is blunted (**Figure 1**). In addition, our results underline the significant contribution of OGT to the glucose-mediated effect of ChREBP on its target genes. Indeed, in **Figure 2**, we describe that the stimulatory effect of high glucose concentrations (25 mM) on ChREBP target genes (*Chrebp* $\beta$ , *Pklr*, *Scd1*, and *Elvol6*) is significantly reduced under shOGT conditions in primary hepatocytes. Our work also confirms that ChREBP directly interacts with OGT and that O-GlcNAcylation of ChREBP increases its transcriptional activity (**Figure 3**). Using a catalytic mutant of OGT (OGT<sup>H498A</sup>) we demonstrated that interaction between ChREBP and OGT<sup>H498A</sup> is stronger than with OGT<sup>WT</sup>, that O-GlcNAcylation levels of ChREBP were decreased as well as the reporter activity of the *Lpk* promoter. Finally, our study reveals that, despite the identification of Ser839 as a potential O-GlcNAcylation site for ChREBP function, mutation of that residue does not impair ChREBP O-GlcNAcylation and/or transcriptional activity in our experimental conditions (**Figure 4**).

In conclusion, our work suggests that the OGT-ChREBP axis is important for the control of hepatic lipogenic genes but also reports that the residues important for the control of ChREBP activity by O-GlcNAcylation still remain to be validated.

**Research subject 2: Hepatocyte-specific ablation  
of OGT leads to inflammation, oxidative stress  
and rescued OGT expression**

# **Hepatocyte-specific ablation of OGT leads to inflammation, oxidative stress and rescued OGT expression**

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**Abbreviations:** Acc, acetyl-CoA carboxylase; ALT, alanine transferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; ARE, antioxidant response element; BA, bile acid; CCL5, C-C motif chemokine ligand 5; CHOP, CCAAT-enhancer-binding protein homologous protein; ChREBP, carbohydrate responsive element binding protein; ChoRE, carbohydrate responsive element; CK7/19, keratin 7/19; Col3a1, collagen type III alpha 1 chain; Col6a1, collagen type VI alpha 1 chain; Cyc, cycling; Elovl6, Elongation of long chain fatty acids family member 6; Fasn, fatty acid synthase; FoxO1, forkhead box protein O1; FXR, farnesoid X receptor; G6PDH, glucose-6-phosphate dehydrogenase; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; Gclc, glutamate-cysteine ligase catalytic subunit; Gk, glucokinase; Gpx, glutathione peroxidase; GS, glutamine synthetase; GSH, reduced glutathione; GSSG, oxidized glutathione; Gsta, glutathione S-transferase alpha; Gstm, glutathione S-transferase mu; H2A.X, H2A.X variant histone; HBP, hexosamine biosynthesis pathway; HES, hematoxylin eosin saffron; HK, hexokinase; HO1, Heme oxygenase 1; HNF4 $\alpha$ , hepatocyte nuclear factor 4 $\alpha$ ; IL-1 $\beta$ , interleukin-1 $\beta$ ; KEAP1, Kelch-like ECH-associated protein 1; LDH, lactate dehydrogenase; Lpk, liver pyruvate kinase; MCP1, monocyte chemoattractant protein-1; NqO1, NAD(P)H quinone dehydrogenase 1; NRF2, erythroid 2-related factor 2; OGA, O-GlcNAcase; OGT, O-GlcNAc transferase; Phgdh, phosphoglycerate dehydrogenase; Pkm2, pyruvate kinase M2; PTMs, post-translational modifications; qPCR, quantitative real-time PCR; SCD1, steroyl-CoA desaturase; Shp, small heterodimer partner;  $\alpha$ SMA, alpha-smooth muscle actin; SOD2, Lower Superoxide Dismutase 2; SOX9, sex determining region Y-box 2; TGF $\beta$ , transforming growth factor  $\beta$ ; TNF $\alpha$ , tumor necrosis factor alpha

## Abstract

O-GlcNAcylation is a dynamic and reversible post-translational modification mediated through the activity of only two enzymes, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA). Disruption of physiological levels of O-GlcNAcylation is related to abnormal cellular functions and pathologies including glucotoxicity, metabolic syndrome and cancer. To determine the metabolic consequences of a targeted deletion of the OGT enzyme in hepatocytes *in vivo*, we developed a model of mice with a hepatocyte-specific ablation of OGT (OGT<sup>Δ</sup>) by crossing OGT<sup>lox/lox</sup> with Alb-Cre mice. Surprisingly, these mice did not exhibit major disruption of metabolic homeostasis but showed a severe liver phenotype with the presence of numerous regeneration nodules visible after weaning. RT-qPCR and western blot analyzes showed that the expression of cyclins A2, B1 and D1 was significantly increased in the liver of OGT<sup>Δ</sup> mice compared to controls suggesting an exacerbated proliferative state at eight-weeks. Histological analyzes revealed the presence of pro-inflammatory cells and signs of fibrosis in the spans surrounding the regeneration nodules. This was associated with a significant increase in markers of inflammation (TNF $\alpha$ ) and fibrosis (Col3a1 and Col6a1), suggesting significant liver injury in OGT<sup>Δ</sup> mice. Interestingly, while OGT was significantly reduced in livers of young mice (4 week-old), we observed re-expression of OGT in livers of older mice (8 week-old) suggesting a counter selection against OGT deficient cells. To better understand the mechanisms driving rescued expression of OGT as well as the phenotype of liver injury, we performed a microarray analysis comparing four- and eight-week-old mice. RT-qPCR and western blot validation demonstrated an increase in ER stress (CHOP), antioxidant response (Nrf2) and DNA damage ( $\gamma$ H2AX) markers. Furthermore, several REACTOME pathways were enriched in liver of 8 week-old OGT<sup>Δ</sup> mice compared to controls such as extracellular matrix formation, innate immune system and oncogenes suggesting the potential development of tumors in the liver of older OGT<sup>Δ</sup> mice. Follow up of these mice over one year revealed a sexual dimorphism with females developing several tumor-like structures while males showed a normal liver phenotype. Altogether, our results suggest an essential role for OGT in liver physiology.

**Key words:** OGT, NRF2, liver injury, oxidative stress, ER stress, fibrosis

## Introduction

Glucotoxicity is a cellular phenomenon that initiates a vicious circle in which chronic hyperglycemia leads to the development of type 2 diabetes (Luo *et al.*, 2016). Among the mechanisms involved, it is described that excess glucose stimulates the hexosamine biosynthesis pathway (HBP) and thus increases O-GlcNAcylation, a dynamic post-translational modification. Only two enzymes regulate O-GlcNAcylation cycling, the glycosyltransferase OGT (O-GlcNAc transferase) and the glycoside hydrolase OGA (O-GlcNAcase) that add and remove the GlcNAc moiety to and from acceptor proteins, respectively. These two enzymes mediate the dynamic cycling of O-GlcNAcylation on a wide variety of cytosolic, nuclear and mitochondrial proteins in a nutrient- and stress-responsive manner. As a result, O-GlcNAcylation has been proposed to function as a nutrient and stress sensor regulating cellular processes ranging from transcription and translation to signal transduction and metabolism. Physiologically, disruption of O-GlcNAc homeostasis has been implicated in the pathogenesis of several human diseases, including cancer, diabetes, and neurodegeneration (Nie and Yi, 2019). Our laboratory has previously shown that O-GlcNAcylation of key effectors of metabolism contributes significantly to gluco-lipototoxicity in the liver (Kuo *et al.*, 2008; Guinez *et al.*, 2011) and in pancreatic  $\beta$  cells (Fardini *et al.*, 2014; Filhoulaud *et al.*, 2019).

While cellular functions of O-GlcNAcylation are emerging, little is known regarding the precise mechanisms how the OGT enzyme senses the environmental cues to elicit molecular and physiological changes. While a recent study suggests that OGT may act as a suppressor of hepatocyte necroptosis (a programmed form of cell death) and that deletion of OGT in liver of mice may trigger liver fibrosis (B. Zhang *et al.*, 2019), this study did not provide a global map of the OGT targets in liver in response to different nutritional stimuli. In this context the objectives of our study were to determine the consequences of a targeted deletion of the OGT enzyme in hepatocytes *in vivo*, and to identify novel OGT targets in the liver to provide novel mechanistic insights.

Through the use of mice constitutively deficient for OGT in liver (OGT<sup>Δ</sup>), our study reveals that OGT is essential for liver homeostasis at early stages after birth. We demonstrate that lack of OGT causes spontaneous inflammation at 4 weeks leading to liver fibrosis associated to ductal reaction and proliferation. Unexpectedly, we observed that OGT expression was fully rescued in liver of 8 week-old mice. Micro-array analyses revealed important changes in global mRNA expression occurring between 4

and 8 weeks, with particular marked activation of the NRF2 antioxidant response and DNA repair pathway in liver of 4 week-old OGT<sup>Δ</sup> mice. We also observed a sexual dimorphism at later stages associated with the development of hepatic tumors in females OGT<sup>Δ</sup> mice but not in males. Altogether our findings suggest that hepatocyte OGT expression needs to be tightly controlled for correct liver function.

## Material and methods

### Generation of OGT<sup>Δ</sup> mice

Conditional hepatic-specific OGT knock-out mice (OGT<sup>Δ</sup>) and control floxed littermates (OGT<sup>F</sup>) were generated by crossing *Albumin-Cre; Ogt<sup>F/Y</sup>* with *Ogt<sup>F/F</sup>* mice. OGT<sup>fllox</sup> mice are floxed on either side of the exons encoding amino acids 206 and 232 corresponding to exon 6-7. These mice were obtained from Jackson Laboratories (JAX stock #00486). Loss of OGT expression was confirmed by PCR with the following primers: forward 5'-CAT CTC TCC AGC CCC ACA AAC TG-3' and reverse 5'- GAC GAA GCA GGA GGG GAG AGC AC-3'.

### Animals and physiological tests

All animal procedures were carried out according to the French guidelines for the care and use of experimental animals (Animal authorization agreement n° CEEA34.AFB/CP.082.12, Paris Descartes Ethical Committee). All mice were housed in colony housing in a 12 hr light/dark cycle. All mice were given free access to water and control chow diet (61.3% carbohydrate, 13.5% fat, 25.2% protein ; SAFE A03). Both genders were included for this study. Gender and age of mice are specified in the text and Figure legends. Glucose tolerance tests were performed in OGT<sup>Δ</sup> and control floxed littermates OGT<sup>F</sup> at 4 weeks of age. Mice received a oral glucose gavage (1g/kg body weight) and blood glucose was measured. Blood glucose was measured in total blood using an Accu-Check glucometer (Roche). Serum cytokines were analysed using the V-PLEX proinflammatory panel 1 mouse kit (Mesoscale, K15048D).

### RNA isolation, reverse transcription and qPCR

Snap frozen liver tissue was powdered and approximately 15mg of liver powder was used for RNA isolation using the SV Total RNA isolation system (Promega, Z3101). RNA samples (2μg) were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA was measured by qPCR (LightCycler480 SYBR Green I Master, Roche) using the primers described in the **Supplementary Table 4**. Gene expression was normalized over expression of the TATA-box binding protein (TBP) mRNA levels.

### Protein extraction, western blot and WGA-binding assay

Whole liver was lysed in lysis buffer (20mM Tris HCl [pH 7.5], 150mM NaCl, 5mM EDTA, 50mM NaF, 30mM NaP, 1% Triton X-100, EDTA free protease inhibitor

cocktail [Roche, 4693132001], orthovanadate 1mM, Thiamet-G 10 $\mu$ M [Sigma, SML0244]). Western blot was performed using 30 $\mu$ g of lysate. For lectin-based precipitation assay, the lysate (1mg of proteins) was incubated overnight with 25 $\mu$ L of Wheat Germ Agglutinin agarose beads (Sigma, L1882) at 4°C. The precipitate was resolved by SDS-PAGE and immunoblotted with specific antibodies. Antibodies used are the following: anti-OGT (Sigma, SAB4200311), anti-O-linked N-acetylglucosamine antibody (RL2, ab2739), anti-GAPDH (Santa Cruz, sc-25778), anti- $\beta$ -actin (CST #4970), anti-CycA2 (Santa Cruz, sc-596), anti-CycD1 (CST #2978), anti-PCNA (CST #2586), anti-KEAP1 (Proteintech, 10503-2-AP), anti-SOD2 (Sigma, HPA001814), anti-NQO1 (CST #3187), anti-CHOP (CST #5554), anti- $\gamma$ H2AX (CST #2577), anti-H2AX total (CST #7631). Semi-quantitative analysis was performed using ChemiDoc software (Bio-rad).

### **Tissue preparation and staining**

Fixed livers (4% PFA, 24h at 4°C) were embedded in paraffin and slide at 4 $\mu$ m. After deparaffinization and rehydration, slides were stained with hematoxylin-eosin-saffron (HES) and Masson's trichrome, for fibrosis staining, using standard procedure. In the case of immunolabeling, antigen retrieval was performed in citrate buffer for 10 min at 95°C and treated with peroxidase blocking reagent (Sigma, H1009). Slides were permeabilized with 0.01% Tween20 and unspecific antigenicity was blocked with 2% BSA and 2% serum, from the specie in which the secondary antibody was raised. Primary antibodies used are the following: rabbit anti-OGT (ab96718), Ki67 (ThermoFisher, MA5-14520), HNF4 $\alpha$  (Santa Cruz, sc-6556), SOX9 (EMD millipore, ab5535), CK19 (Abcam, ab52625) and glutamine synthetase (GS) (BD Bioscience, #610518). Secondary antibodies HRP-conjugated were revealed by incubation with DAB substrate (Dako), co-stained with hematoxylin (Vector laboratories, H-3404) and mounted with VectaMount™ mounting medium (Vector laboratories, H-5000). Images analysis was processed with ImageJ software.

### **Microarray based transcriptome profiling**

RNA extraction was performed as mentioned previously and checked for integrity using Bioanalyser. Microarray experiment and data normalization was performed by the transcriptomic core facility at Cochin Institute. Briefly, gene expression profiling was carried out on 5 biological replicates per condition using the Affymetrix GeneChip Mouse Gene 2.0 ST array, which interrogates 25.000 gene sequences. Normalization of raw data was performed using the Robust Multichip Algorithm (RMA) in Bioconductor

R software. Partek GS software was used for quality controls and statistics. Downstream analysis was done to identify differentially expressed (DE) genes based in comparison with control conditions. The DE genes were selected based on the corrected p-value for False Discovery Rate (FDR p-value < 0.05) and the fold change from OGT<sup>A</sup> compared to controls (up-regulated genes : fold change  $\geq 2$ , down-regulated genes : fold change  $\leq -2$ ). Venn diagram was performed using the interactive tool 'Venny 2.1.0' (Oliveros et al. 2007-2015). Up-regulated and down-regulated genes were annotated using Reactome gene set. Functional enrichment of specific pathways in the gene set was performed using Fisher's exact test and FDR correction. Pathways were considered significantly enriched at an FDR < 0.05. R statistical software v.3.6.2 and R packages were used for bioinformatic analysis and graph representation. Volcano plot were represented using the "Enhanced Volcano" package (Blighe et al. 2019). Heatmap were represented using 'pheatmap' package (R. Kolde, 2019), where correlation clustering distance row was applied.

### **Cell culture**

Mice at 4 weeks of age consuming a control diet were used for primary hepatocytes culture. Mice were anesthetized with a 10:1 ketamine-xylazine solution intra-peritoneally. Liver was perfused through the portal vein with Hank's balanced salt solution followed by collagenase perfusion. Cell viability was calculated by trypan blue exclusion test using a Malassez chamber and seeded in 6-well plates at a concentration of 600.000 cells per well. Cells were seeded in medium M199 5mM of glucose (Invitrogen, #11150059) supplemented with 100  $\mu$ g/mL streptomycin, 100 units/mL of penicillin, L-glutamine (2 mM), 0.1% Bovine serum albumin, 2.5% Nu-serum (BD Bioscience, Cat#355104) and dexamethasone (100nM, Novo Nordisk). Following a 4 h incubation (37°C, 5% CO<sub>2</sub>), M199 medium was replaced and cells were transfected with a 3xARE-Luciferase reporter and a  $\beta$ -galactosidase plasmid using Lipofectamine 2000 and OptiMEM media. After overnight incubation, medium was changed to low glucose (5mM) for 24 hours. Luciferase assay was performed after cell lysis.  $\beta$ -galactosidase assays were performed for normalization of 3xARE-luciferase activity.

For GSH/GSSG assay, hepatocytes were seeded in 96-well plates at a concentration of 15000 cells per well. Twenty-four hours after seeding, GSH/GSSG ratio were measured using GSH/GSSG-Glo assay kit (Promega, Madison, WI) in accordance with the manufacturer's instructions.

### **Statistical analysis**

All statistical analyses were performed in Prism GraphPad Software Inc, using one-way ANOVA followed by Bonferroni port-test. When comparing groups of only two data sets, unpaired two-tailed t-test was performed. The number of independent experiments performed and the statistical test used are indicated in each figure legend.

## Results

### Hepatocyte-specific ablation of OGT leads to liver inflammation at 4 weeks.

To investigate the metabolic consequences of a targeted depletion of *Ogt* in mouse liver, we generated hepatocytes-specific OGT knock-out mice (OGT<sup>Δ</sup>) and control floxed littermates (OGT<sup>F</sup>) by crossing *Albumin-Cre; Ogt<sup>F/Y</sup>* with *Ogt<sup>F/F</sup>* mice. Expression and protein levels of OGT were significantly reduced in liver 1 week and 4 weeks after birth, together with a parallel decrease in O-GlcNAcylated proteins (**Figure 1A and B, Supplementary Figure 3A**) confirming recombination efficiency. In addition, OGT<sup>Δ</sup> mice presented a significant decrease in OGA expression in liver (**Supplementary Figure 1, Supplementary Figure 3A**). OGT<sup>F</sup> and OGT<sup>Δ</sup> mice showed comparable blood glucose, body and liver weight (**Figure 1C**). Interestingly, we observed a significant increase in spleen weight in OGT<sup>Δ</sup> compared to OGT<sup>F</sup> mice (**Figure 1C**). Mice presented normal glucose tolerance as demonstrated by comparable oral glucose tolerance test (OGTT) between OGT<sup>F</sup> and OGT<sup>Δ</sup> mice (**Supplementary Figure 1B**). To investigate possible changes in glucose metabolism we measured expression of enzymes and transcription factors involved in glycolysis, lipogenesis and gluconeogenesis by qPCR (**Figure 1D**). We observed that the expression of *FoxO1* target gene *G6Pase* was significantly decreased in liver of OGT<sup>Δ</sup> compared to OGT<sup>F</sup> mice. Surprisingly, while *Chrebp* expression was significantly decreased, only two of its targets (*Lpk* and *Scd1*) showed a significant reduction in liver of OGT<sup>Δ</sup> compared to OGT<sup>F</sup> mice (**Figure 1D**). Histological analysis of liver structure by HES and Massons' Trichrome staining showed not major defects in OGT<sup>Δ</sup> mice compared to OGT<sup>F</sup> (**Figure 1E**). Staining with the proliferation marker Ki67 suggested an increase in the number of proliferative cells in OGT<sup>Δ</sup> compared to OGT<sup>F</sup> mice, together with increase in inflammatory infiltration (**Figure 1E**, yellow arrow). However, no significant changes were observed for proliferation or fibrosis markers by qPCR (**Supplementary Figure 1C and 1D, Supplementary Figure 3C**). In contrast, analysis of inflammatory markers expression by qPCR showed significant induction of *Tnfa*, *Mcp1* and *Ccl5* in OGT<sup>Δ</sup> compared to OGT<sup>F</sup> mice (**Figure 1F, Supplementary Figure 3D**). Surprisingly, this was not associated with a significant change in liver injury markers as shown by alanine aminotransferase (ALT), aspartate aminotransferase (AST) or lactate dehydrogenase (LDH) (**Supplementary Figure 1E**). Altogether, our results report that OGT<sup>Δ</sup> mice develop spontaneous inflammation at 4 weeks of age.

## **OGT<sup>Δ</sup> mice exhibit fibrosis, hepatocyte proliferation and rescued OGT expression 8 weeks after birth.**

To study the consequences of the spontaneous liver inflammation occurring in 4 week-old OGT<sup>Δ</sup> mice, we analysed the phenotype of OGT<sup>Δ</sup> mice 8 weeks after birth. Similar blood glucose and body weight were observed in 8 week-old OGT<sup>F</sup> and OGT<sup>Δ</sup> mice (**Figure 2A, Supplementary Figure 3E**). However, both spleen and liver were bigger in OGT<sup>Δ</sup> compared OGT<sup>F</sup> mice (**Figure 2A, Supplementary Figure 3E**). Analysis of OGT<sup>Δ</sup> livers by echography highlighted the presence of nodules reaching a size of up to 1.5 cm<sup>2</sup> (**Figure 2B**). These nodules were also observed macroscopically at sacrifice (**Figure 2C**). Panoramic view of histological liver section of OGT<sup>Δ</sup> mice from Massons' Trichrome staining showed homogeneous distribution of these nodules identified as nodules of regeneration typical from highly injured livers (**Figure 2D**). Surprisingly, analysis of OGT expression and protein levels revealed rescued OGT expression in liver of OGT<sup>Δ</sup> mice reaching similar levels than in OGT<sup>F</sup> mouse liver (**Figure 2E and 2F, Supplementary Figure 3F**). Expression levels of OGA remained significantly decreased in liver of OGT<sup>Δ</sup> mice compared to OGT<sup>F</sup> (**Supplementary Figure 2A**). Staining of liver sections with HES and Massons' trichrome, showed the presence of multiple regeneration nodules with high levels of bridging fibrosis (**Figure 2G**). Interestingly, OGT immunolabelling showed OGT positive cells in the regenerative nodules suggesting a counter selection against OGT deficient cells (Baker, 2020)(**Figure 2G**). Ki67 immunostaining revealed an increase in proliferative cells in the fibrotic tissue surrounding the nodules of regeneration (**Figure 2G**). In agreement, expression levels of proliferation markers were significantly induced in liver of OGT<sup>Δ</sup> mice compared to OGT<sup>F</sup> at the mRNA (*ki67, CycA2, CycB1, CycD1*) and protein levels (Cyclin A, Cyclin D1, PCNA) (**Figure 2H, Supplementary Figure 3G**). The expression of inflammatory markers (*Tnfa, Mcp1* and *Ccl5*) and circulating cytokine concentrations (TNF $\alpha$ , IL-2, IL-6) were also up-regulated in liver of OGT<sup>Δ</sup> mice compared to OGT<sup>F</sup> (**Figure 2I, Supplementary Figure 2B, Supplementary Figure 3I and 3J**), together with a significant increase in some fibrosis markers (*Col3a1, Col6a1*) (**Figure 2J**). These data correlate with the increase in fibrosis shown upon Massons' trichrome staining (**Figure 2G**). OGT<sup>Δ</sup> mice also exhibited a significant elevation of circulating ALT and LDH levels suggesting liver injury (**Figure 2K**). These data suggest a rapid deterioration of liver status in OGT<sup>Δ</sup> mice that may be compensated by counter selection of OGT expressing cells.

### **Microarray analysis reveals liver global transcriptional changes at 4 and 8 weeks in OGT<sup>Δ</sup> compared to OGT<sup>F</sup> mice.**

Given the marked phenotype developed between 4 and 8 weeks in OGT<sup>Δ</sup> mice, we performed a transcriptomic analysis with the purpose of elucidating possible mechanisms controlling OGT rescue. Volcano plot of 4- and 8-week-old mice were generated using a total of 22206 hepatic genes comparing OGT<sup>F</sup> to OGT<sup>Δ</sup> mice (**Figure 3A and 3B**). Venn diagram represents the differentially expressed (DE) genes (fold change  $\geq$  [2], false discovery rate (FDR) p-value  $<$  0.05) at 4 weeks (652 genes) and 8 weeks (588 genes), with 95 common to 4 and 8 weeks (**Figure 3C, Supplementary Table 1, 2, 3**). Heatmap represents the DE genes at 4 and 8 weeks between OGT<sup>Δ</sup> and OGT<sup>F</sup> mice (**Figure 3D**). Analysis of REACTOME pathways significantly upregulated in liver of 4 week-old mice showed an increase in immune signalling pathways while general metabolic pathways were significantly down-regulated at this stage (**Figure 4E and Supplementary Figure 4A**). Analysis of DE genes at 8 weeks mostly reported upregulated genes related to immune system activation but also to formation and degradation of extracellular matrix (ECM) and, interestingly, O-linked glycosylation (**Figure 3F**). Altogether, the results indicate that important changes in global mRNA expression are occurring between 4 and 8 weeks leading to OGT rescue and development of liver fibrosis.

### **Hepatic OGT deficiency leads to increase bile duct development and bilirubin secretion.**

To better understand the hepatic phenotype of OGT<sup>Δ</sup> mice, we examined markers of cell identity in the microarray analysis performed at 4 and 8 week-old mice. A significant increase in makers of stem cells and cholangiocytes was observed in 8 week-old OGT<sup>Δ</sup> mice compared to OGT<sup>F</sup> (**Figure 4A**). These results suggested a switch in the differentiation path from hepatoblasts to cholangiocytes (**Figure 4B**). To validate this hypothesis, differentiation markers were analysed by qPCR in 4 and 8 week-old mice. A slight decrease in the marker of differentiated hepatocytes *Hnf4a* was observed in liver of 4 week-old OGT<sup>Δ</sup> mice. These results correlated with the up-regulation of cholangiocyte markers, Ck7 and Ck19 in 4 week-old OGT<sup>Δ</sup> versus OGT<sup>F</sup> mice. A further increase was observed at 8 weeks (**Figure 4C**). Liver sections immunolabeled with HNF4 $\alpha$ , SOX9 and CK19 confirmed an increase in bile duct formation in the fibrosis septa (see square on **Figure 4D**). Analysis of *Fxr* expression and of its target *Shp*, known to control bile acids (BAs) homeostasis, showed a downregulation in liver

of 4 week-old OGT<sup>Δ</sup> mice (**Figure 4E**). Bilirubin serum concentrations tended to increase in 8 week-old OGT<sup>Δ</sup> mice compared to OGT<sup>F</sup> (**Figure 4F**). OGT<sup>Δ</sup> mice also presented a significant increase in serum alkaline phosphatase (ALP) traditionally associated to biliary obstruction (Toda *et al.*, 1980) (**Figure 4G**). Taken together, our results suggest that OGT plays an important role in cell differentiation and potentially affects BAs/bilirubin production and secretion.

### **Hepatic-specific OGT ablation activates NRF2 antioxidant response and DNA repair pathways in 4 week-old OGT<sup>Δ</sup> mice.**

Given that nuclear factor erythroid 2-related factor 2 (*Nrf2*) was recently suggested to be associated to FXR repression and dysregulation of BAs metabolism in the context of autophagy-deficiency (Khambu *et al.*, 2019), we examined the expression of *Nrf2* targets in our microarray data performed at both 4 and 8 weeks. Microarray analysis showed a significant upregulation of *Nrf2* targets in liver of 4 week-old OGT<sup>Δ</sup> mice (**Figure 5A**). NRF2 western blot analysis showed an increase of protein levels in liver of 4 week-old OGT<sup>Δ</sup> mice compared to OGT<sup>F</sup> (**Figure 5B**). Interestingly, a significant increase in O-GlcNAcylated KEAP1 was observed in liver of 8 week-old OGT<sup>Δ</sup> mice, which paralleled with the decrease in NRF2 protein (**Figure 5B**). These data correlate with the proposed mechanism in which O-GlcNAcylation of KEAP1 is required for NRF2 ubiquitination and degradation (Chen *et al.*, 2017). These results were validated by qPCR and western blot analyses of NRF2 targets at 4 and 8 weeks (**Figure 5C, 5D, 5E**). A significant upregulation of NRF2 targets *Gstm3*, *Gsta1* and *Phgdh* genes involved in the production of reduced glutathione (GSH), was observed in liver of 4 week-old OGT<sup>Δ</sup> mice (**Figure 5C**). The expression of *Gstm3* and *Gsta1* was also significantly upregulated at 8 weeks, together with *Gpx3* suggesting sustained activation of the NRF2 at 8 weeks (**Figure 5D**). Analysis of protein level showed not only an increase in antioxidant response (SOD2 and NQO1) but also an increase in the ER stress marker CCAAT-enhancer-binding protein homologous protein (CHOP) (**Figure 5E**). Enhanced NRF2 activity was validated in primary cultures of 4 week-old OGT<sup>Δ</sup> hepatocytes (**Figure 5F**). Cells were transfected with an ARE-luciferase reporter (**Figure 5F**) and the GSH/GSSG ratio, a reliable indicator of oxidative stress was measured (**Figure 5G**). A marked increase in ARE-luciferase activity was observed in OGT<sup>Δ</sup> hepatocytes together with a significant decrease in the GSH/GSSG ratio, suggesting enhanced oxidative stress at 4 weeks (**Figure 5F and G**). Given that oxidative stress is associated with DNA damage (Van Houten, Santa-Gonzalez and

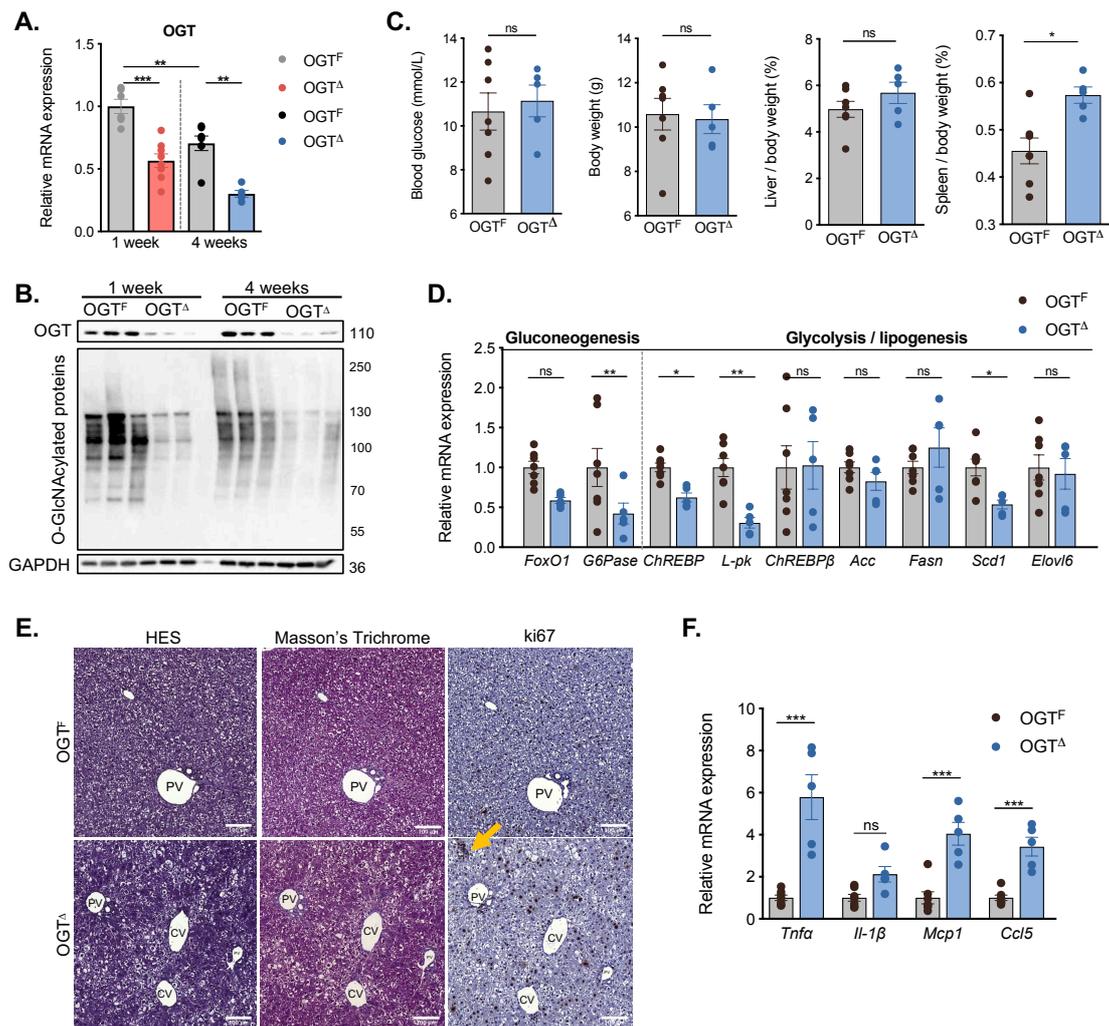
Camargo, 2018), we measured phosphorylation of histone H2A.X ( $\gamma$ H2A.X), a known marker of DNA damage.  $\gamma$ H2AX expression was elevated in liver of 4 week-old OGT<sup>Δ</sup> compared to OGT<sup>F</sup> mice (**Figure 5H**), correlating with an increase in expression levels of DNA repair machinery genes as shown in the microarray (**Figure 5I**). Our results suggest an increased oxidative stress and DNA damage in liver of 4 week-old OGT<sup>Δ</sup> mice through a NRF2-KEAP1-related mechanism (**Figure 5J**).

### **Sexual dimorphism in 1 year-old OGT<sup>Δ</sup> mice with generation of tumors in females but not in males.**

The microarray performed at 4 and 8 weeks in OGT<sup>Δ</sup> mice revealed the up-regulation of a set of genes recognized as oncogenes (**Figure 6A**). Interestingly, the expression of *Hk2* and *Pkm2*, two glycolytic genes associated with the development of liver cancer (Feng *et al.*, 2020) were found increased in liver of 8 week-old OGT<sup>Δ</sup> mice. *G6pdh*, the rate limiting enzyme of the pentose phosphate pathway (PPP), was also found up-regulated in liver of OGT<sup>Δ</sup> compared to OGT<sup>F</sup> mice, suggesting cellular reprogramming commonly set up by cancer cells to support high proliferation rates (Yang, Stern and Chiu, 2020) (**Figure 6B**). To investigate the long-term effect of OGT deficiency in the liver, OGT<sup>F</sup> and OGT<sup>Δ</sup> mice were followed-up up to 1 year after birth. Physiological parameters were comparable in both males and females OGT<sup>F</sup> and OGT<sup>Δ</sup> mice except for an increase in spleen weight in OGT<sup>Δ</sup> female mice compared to controls (**Supplementary figure 5A**). OGT and OGA relative mRNA expression levels were also similar in all groups, underlining the fact that rescued OGT expression was maintained to similar levels than control in OGT<sup>Δ</sup> males and females even after one year of life (**Supplementary Figure 5B**). ALT concentrations were increased in the serum of OGT<sup>Δ</sup> mice (males and females) compared to OGT<sup>F</sup> mice (**Figure 6C**). However, no differences in AST (**Figure 6C**), LDH (**Supplementary Figure 5C**) or ALP concentrations were observed (**Supplementary Figure 5D**). Total bilirubin concentrations were significantly induced in OGT<sup>Δ</sup> male mice but with no difference in direct bilirubin concentrations (**Figure 6D**). Liver histology analyses in 1 year-old males showed no differences in liver structure or Ki67<sup>+</sup> staining between OGT<sup>F</sup> and OGT<sup>Δ</sup> mice (**Figure 6E**). OGT staining also confirmed the lack of difference in hepatic OGT protein content between 1 year-old OGT<sup>F</sup> and OGT<sup>Δ</sup> mice (**Figure 6E**). Relative mRNA expression of proliferation, inflammation and fibrosis markers in OGT<sup>Δ</sup> compared to OGT<sup>F</sup> males demonstrated comparable levels of expression validating the

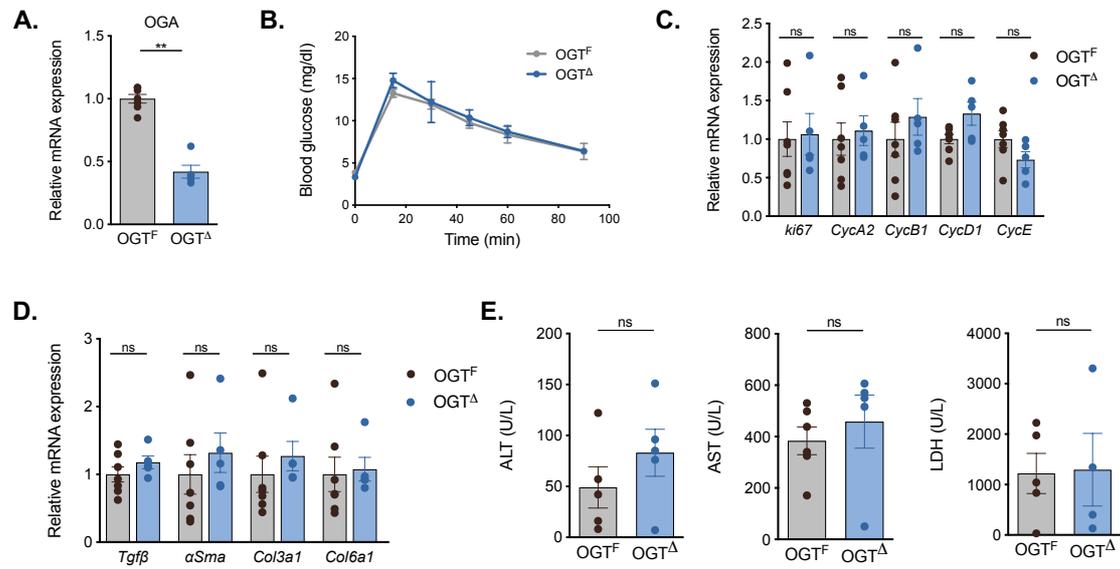
recovered phenotype (**Supplementary Figure 5 E-G**). Interestingly, OGT<sup>Δ</sup> females develop tumor structures identified by cell morphology (increased hepatocyte size (orange arrow), proliferation (Ki67, black arrows) and presence of numerous binuclear cells (red and blue arrows)) (**Figure 6F**). Glutamine synthetase (GS) staining was negative within the cells of the tumor structure (**Figure 6F**) suggesting that the Wnt/ $\beta$ -catenin pathway is not up-regulated in those cells. All together these data suggest a role for OGT in the sexual dimorphism associated to the development of liver cancer.

# Figures



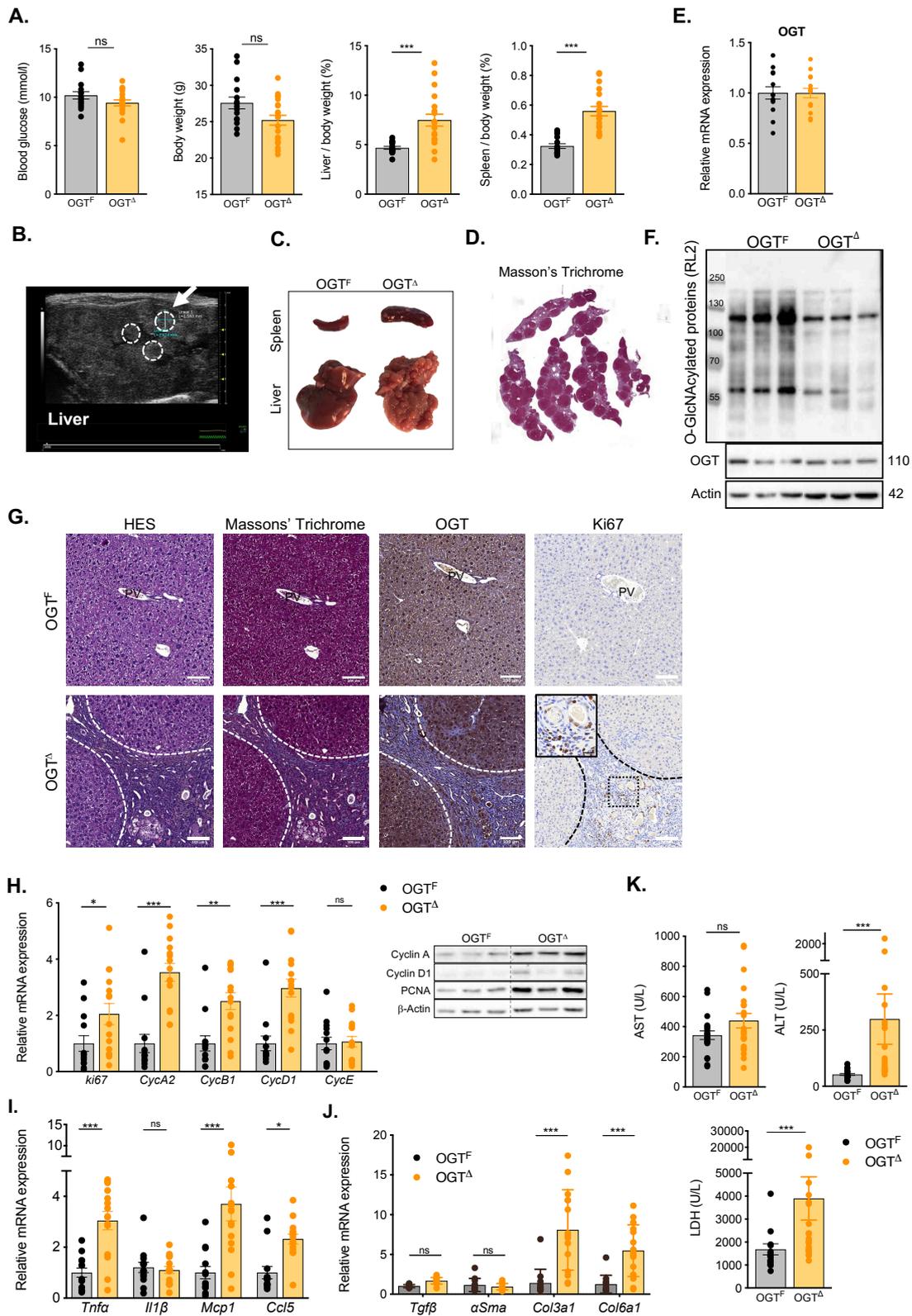
**Figure 1. Hepatocyte-specific ablation of OGT leads to liver inflammation 4 weeks after birth.**

Mutant (OGT<sup>A</sup>) and control floxed littermates (OGT<sup>F</sup>) mice were studied 1 and 4 weeks after birth in the fed stage. This figure corresponds to male samples. **A.** Relative expression of OGT normalized to TBP. **B.** Western blot analysis of OGT and O-GlcNAcylation levels in whole liver lysate of OGT<sup>F</sup> and OGT<sup>A</sup> mice at 1 and 4 weeks. GAPDH was used as loading control. Three representative samples are presented per condition. **C.** Physiological parameters including blood glucose (mmol/L), body (g), liver and spleen weight (% of body weight) in 4 week-old mice. **D.** Relative expression levels of key glucose metabolism genes measured by qPCR from liver at 4 weeks. **E.** Liver sections stained with hematoxylin-eosin-saffron (HES), Massons' trichrome and Ki67 (proliferation) are shown for 4 week-old mice. Scale bars = 100  $\mu$ m. **F.** Relative expression levels of inflammatory markers measured by qPCR from liver at 4 weeks. Data is shown as Mean  $\pm$  SEM of 4-7 individual mice. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001 by unpaired Student t-test (Mann-Whitney).



### Supplementary Figure 1.

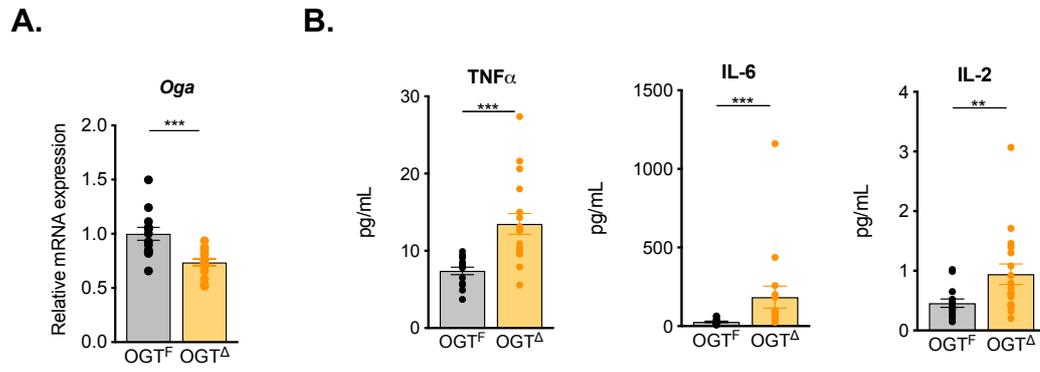
All results correspond to mice at 4 weeks. **A.** Relative mRNA expression of OGA measured by qPCR. **B.** Oral glucose tolerance test (OGTT) measured over a time course of 90 minutes after glucose gavage (20% glucose solution) **C.** Relative mRNA expression of proliferation markers. **D.** Relative expression of fibrosis markers. **E.** Serum analysis of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH). All mRNA expression analysis are normalized to TBP. Data is shown as Mean  $\pm$  SEM of 4-7 individual mice. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  by unpaired Student t-test (Mann-Whitney).



**Figure 2. OGT<sup>Δ</sup> mice exhibit fibrosis, hepatocyte proliferation and rescued OGT expression 8 weeks after birth.**

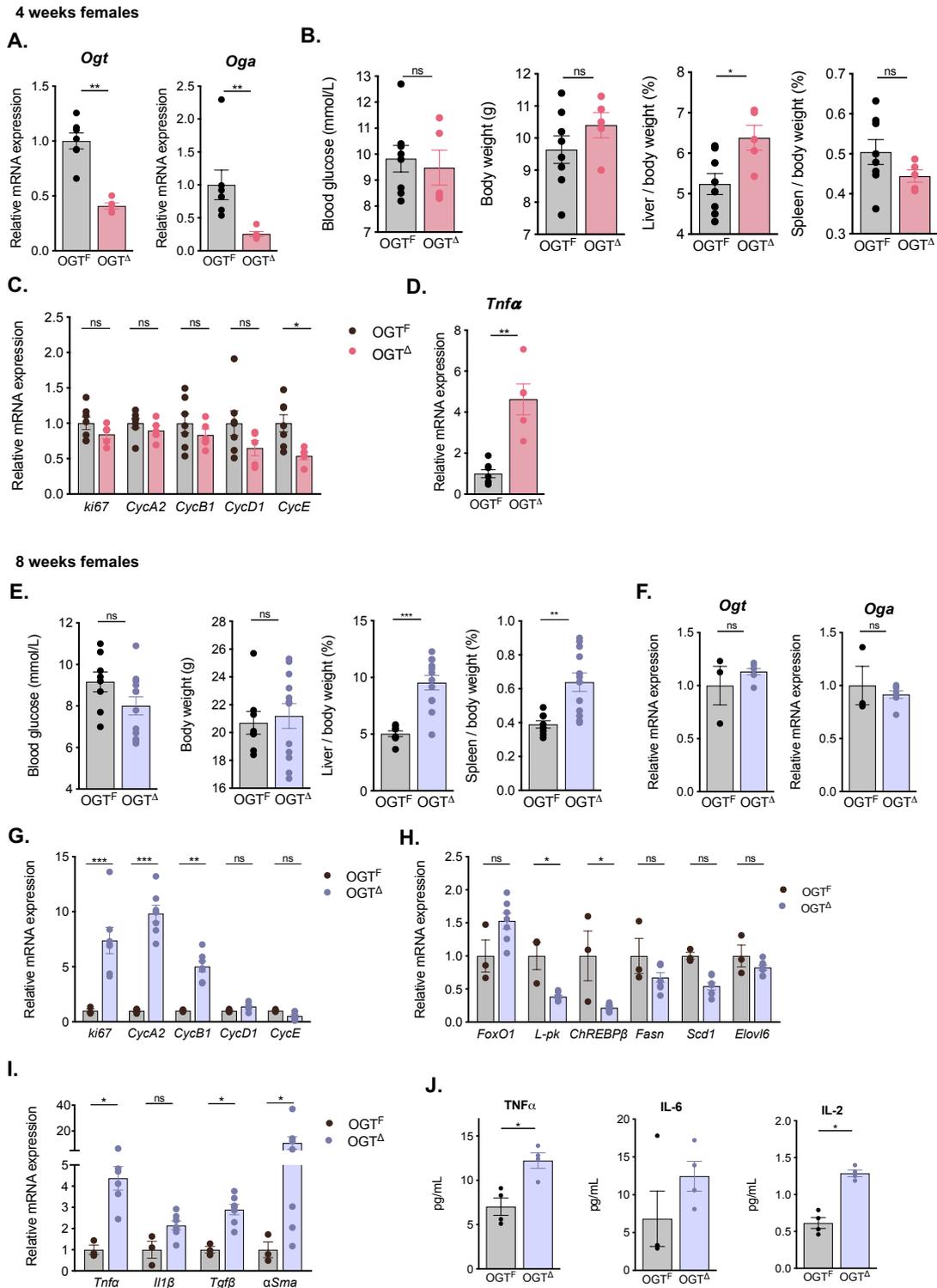
This figure corresponds to male samples. **A.** Physiological parameters including blood glucose (mmol/L), body (g) and liver and spleen weight are shown. Liver and spleen weight are represented as % of body weight. **B.** Echography of liver from OGT<sup>Δ</sup> mice showing nodules of regeneration of up to 1.5 cm<sup>2</sup>. **C.** Gross appearance of spleen and liver from OGT<sup>F</sup> and OGT<sup>Δ</sup> mice. **D.** Panoramic view of histological liver section of

OGT<sup>Δ</sup> mice from Massons' Trichrome staining. **E.** Expression levels of OGT normalized to TBP. **F.** Western blots showing OGT content and O-GlcNAc levels at 8 weeks in whole liver lysates. Actin was used for normalization. **G.** Liver sections stained with hematoxylin-eosin-saffron (HES), Massons' trichrome, OGT and Ki67 (proliferation) are shown. Scale bars = 100 μm. **H.** Relative expression and protein levels of proliferation markers. Actin was used for normalization. **I.** Relative expression levels of inflammatory markers measured by qPCR. **J.** Relative mRNA levels of fibrosis markers measured by qPCR. **K.** Markers of liver injury parameters, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) in blood serum. Data is shown as Mean ± SEM of 13-15 mice per condition. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001 by unpaired Student t-test (Mann-Whitney).



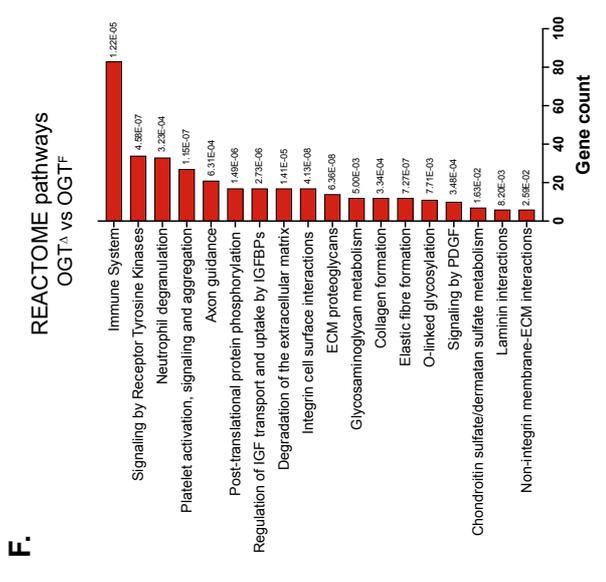
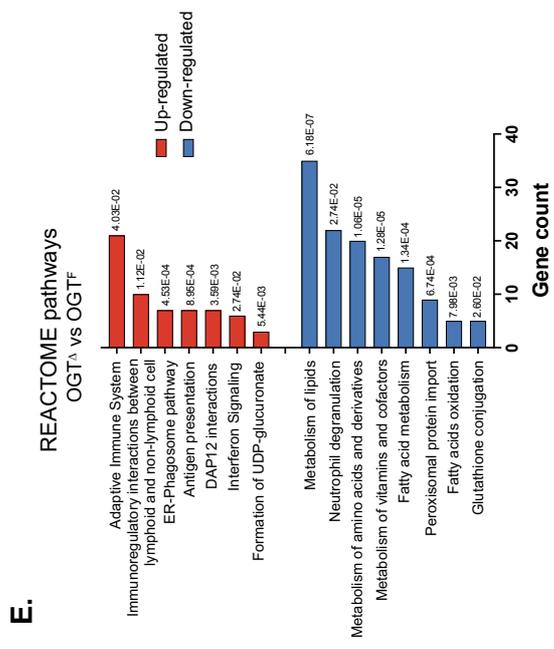
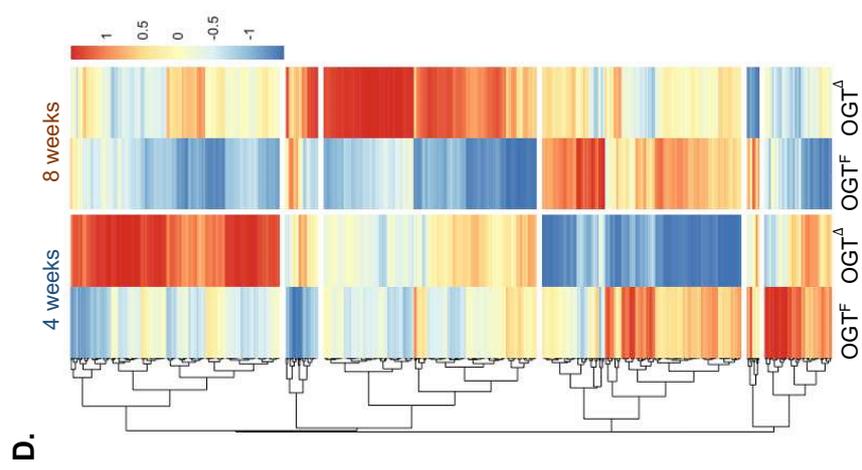
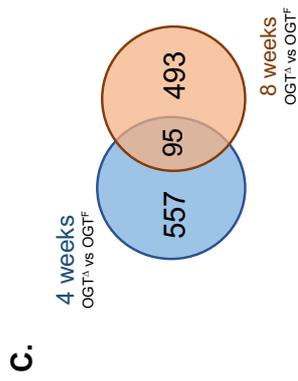
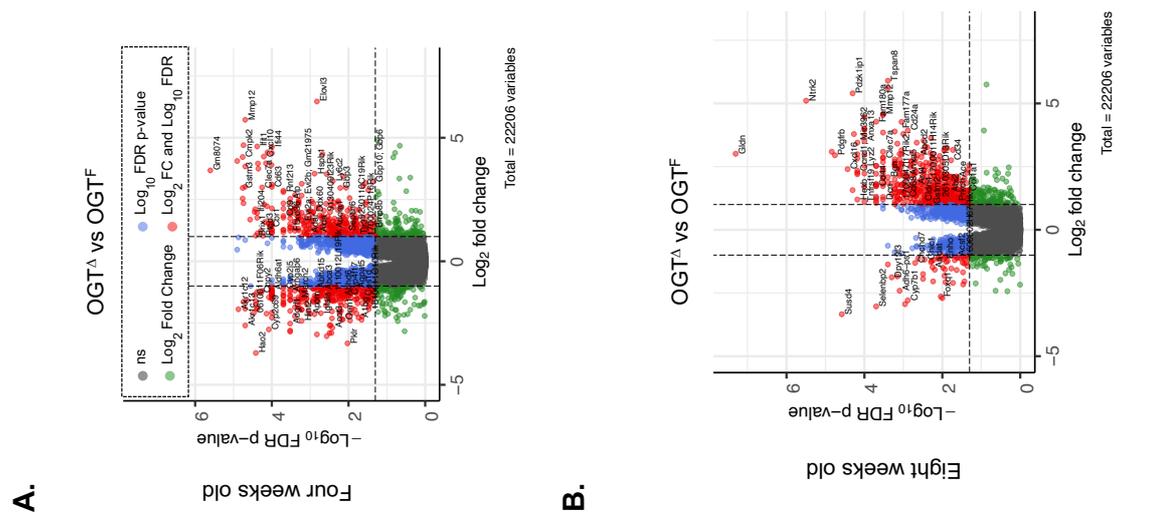
**Supplementary Figure 2.**

Males at 8 weeks are represented in this figure. **A.** Relative mRNA expression of OGA measure by qPCR. TBP was used for normalization. **B.** Serum levels of the cytokines TNF $\alpha$ , IL-6 and IL-2 (pg/mL). Data is shown as Mean  $\pm$  SEM of 13-14 individual mice. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001 by unpaired Student t-test (Mann-Whitney).



### Supplementary Figure 3.

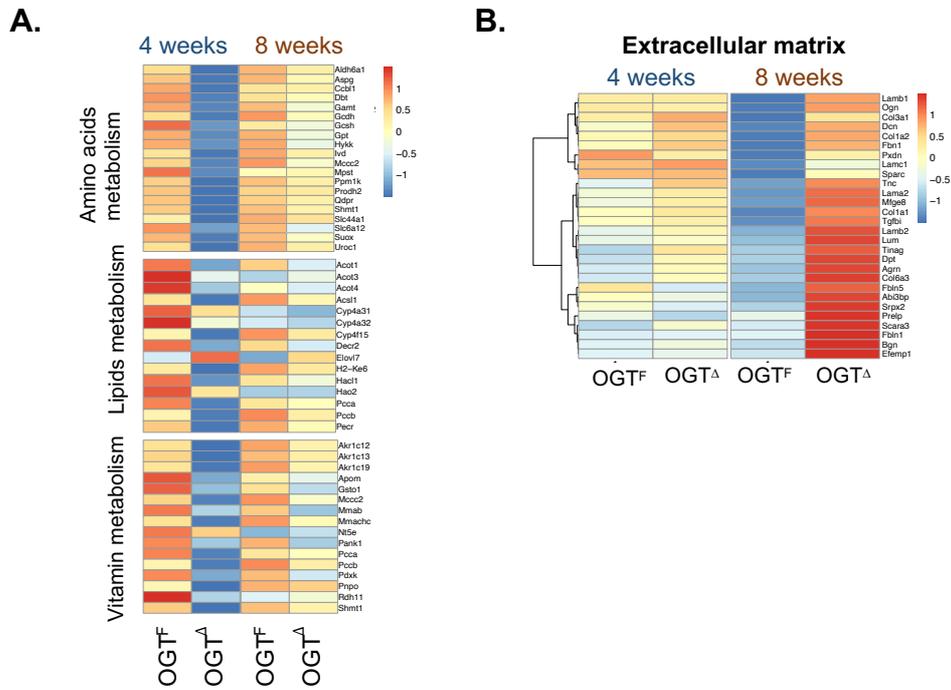
Females at 4 (A-D) and 8 (E-I) weeks are represented. A and F. Relative mRNA expression of OGT and OGA measured by qPCR. B and E. Physiological parameters including blood glucose (mmol/L), body (g), liver and spleen weight (% of body weight). C and G. mRNA expression levels of proliferation markers. D. Relative mRNA expression of *Tnfa*. H. Relative expression of gluconeogenic, glycolytic and lipogenic genes. I. Expression levels of inflammatory markers measured by qPCR. J. Serum levels of the cytokines  $TNF\alpha$ , IL-6 and IL-2 (pg/mL). Data is shown as Mean  $\pm$  SEM of 4-7 individual mice. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  by unpaired Student t-test (Mann-Whitney).



**Figure 3. Microarray analysis reveals global transcriptional changes in OGT<sup>Δ</sup> compared to OGT<sup>F</sup> mice.**

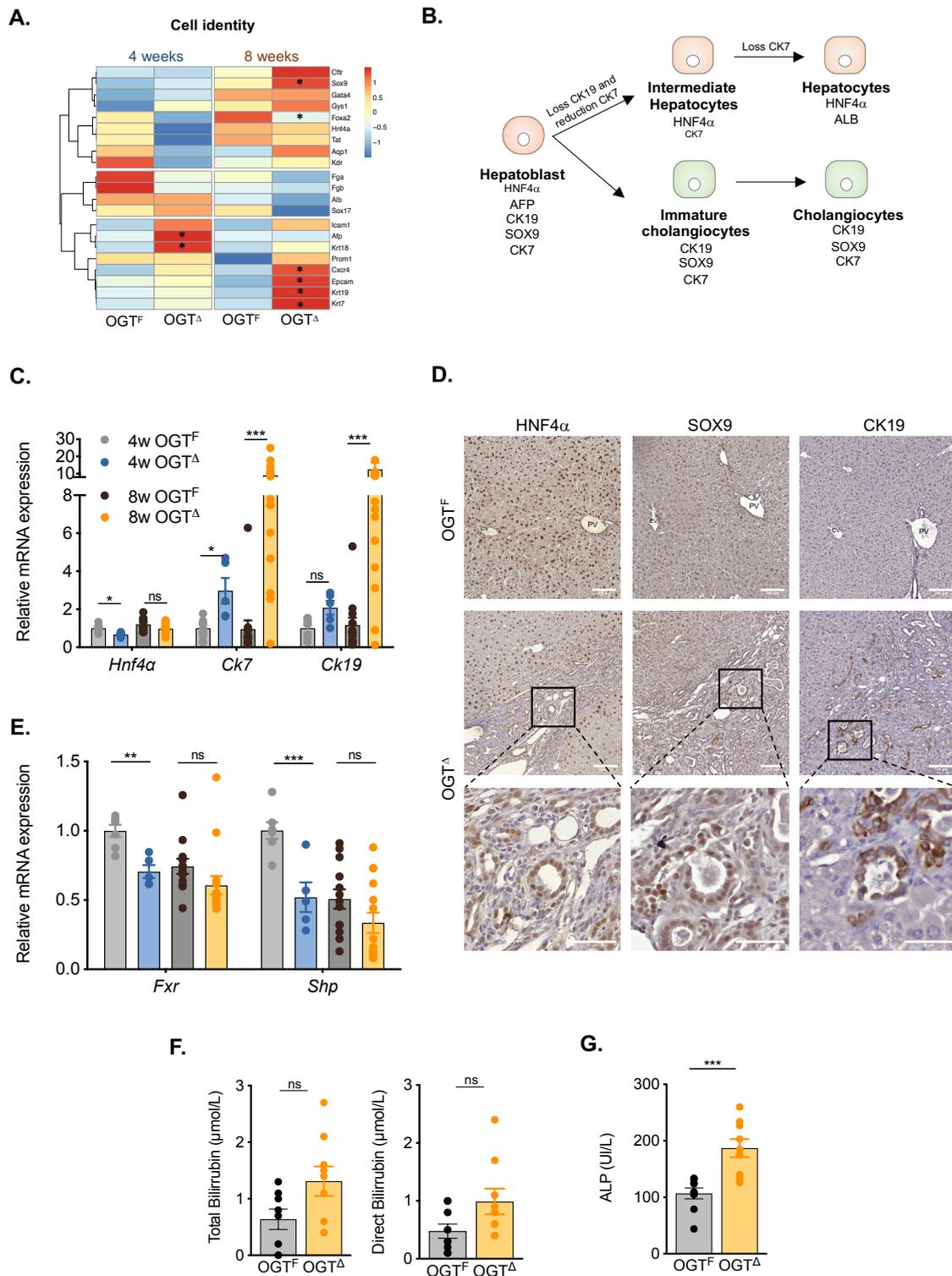
Results were generated using microarray data from 5 replicates per condition.

**A-B.** Volcano plot representing differential gene expression of 22206 genes comparing livers from OGT<sup>A</sup> and OGT<sup>F</sup> mice 4 (**A**) and 8 weeks after birth (**B**). Log<sub>2</sub> fold change is represented in the X-axis and log<sub>10</sub> False Discovery Rate (FDR) p-value in the Y-axis. Fold change  $\geq [2]$  and FDR p-value  $< 0.05$  was selected as cut off parameters for selection of differentially expressed (DE) genes, represented in red. **C.** Venn diagram of DE genes in the liver of 4 and 8 week-old mice. **D.** Hierarchical clustering showing differential expression of genes in liver of OGT<sup>A</sup> mice compared to OGT<sup>F</sup>. **E-F.** Top REACTOME pathways significantly up- or down-regulated using the DE genes between OGT<sup>A</sup> and OGT<sup>F</sup> mice at 4 (**E**) or 8 weeks (**F**).



**Supplementary Figure 4.**

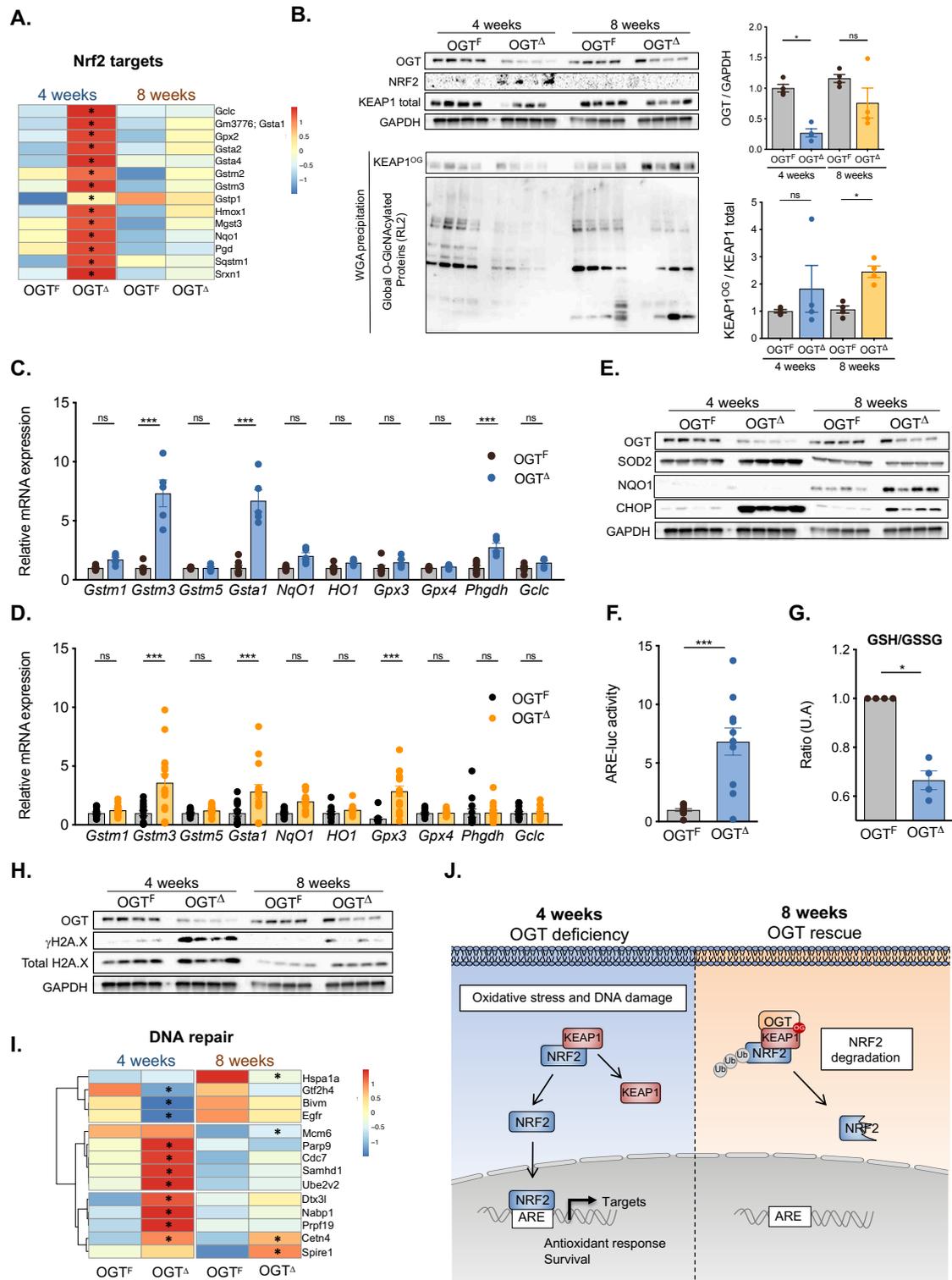
**A.** Microarray results of differentially expressed (DE) genes at four weeks related to metabolism including amino acids, lipids and vitamin metabolism. **B.** Microarray results of DE genes at eight weeks related to extracellular matrix formation. DE genes cut-off was fold change  $\geq [2]$  and FDR p-value  $< 0.05$ .



**Figure 4. Hepatic OGT deficiency leads to increase bile duct development and bilirubin secretion.**

**A.** Heatmap showing differences in the expression of cell identity markers in liver of  $OGT^F$  and  $OGT^{\Delta}$  mice at 4 and 8 weeks. Asterisk (\*) indicates DE genes. **B.** Schematic representation of hepatoblast differentiation into hepatocytes or cholangiocytes. Markers used for liver characterization are represented under each cellular type. **C.** Relative expression of cell identity markers in livers from  $OGT^F$  compared to  $OGT^{\Delta}$  mice at 4 and 8 weeks. Normalization gene used was TBP. **D.** Liver sections of 8 week-old mice stained with HNF4 $\alpha$ , SOX9 and CK19 antibodies. Scale bars = 100  $\mu$ m on the two upper sections. Detailed zoom is shown in the bottom sections with a scale bar =

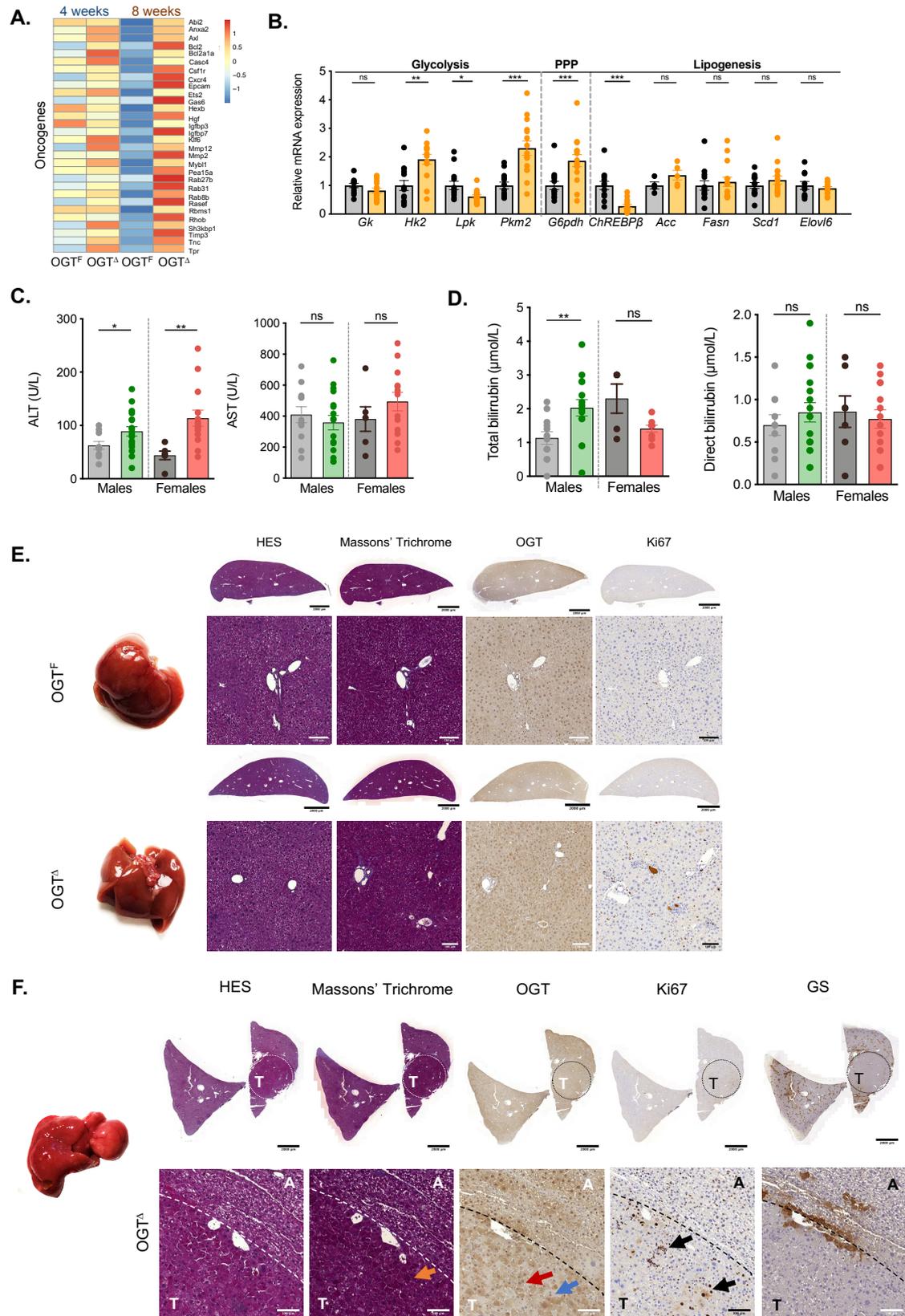
50µm. **E.** Relative expression of the bile acid-activated nuclear receptor *Fxr* and its target gene *Shp*. TBP was used as housekeeping gene. **F.** Serum total and direct bilirubin (µmol/L) and alkaline phosphatase (ALP, U/L) in 8-week-old mice. Values are represented as Mean ± SEM of 4-7 mice at 4 weeks and 13-15 mice at 8 weeks. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001 by unpaired Student t-test (Mann-Whitney).



**Figure 5. Hepatic OGT deficiency activates NRF2 antioxidant response and DNA repair pathway at 4 weeks.**

**A.** Heatmap represents increased expression of NRF2 targets in 4 week-old OGT<sup>A</sup> mice compared to OGT<sup>F</sup>. Asterisk (\*) indicates DE genes. **B.** Western blots showing OGT, NRF2 and total KEAP1 in liver lysate normalized to GAPDH. WGA assay is used for precipitation of O-GlcNAcylated proteins revealed with RL2 antibody. KEAP1 O-GlcNAcylation level was revealed after WGA assay. Semi-quantification of OGT protein content normalized to GAPDH and O-GlcNAcylated KEAP1 (KEAP1<sup>OG</sup>) normalized to total KEAP1. **C-D.** qPCR analysis of NRF2 targets in liver of OGT<sup>F</sup>

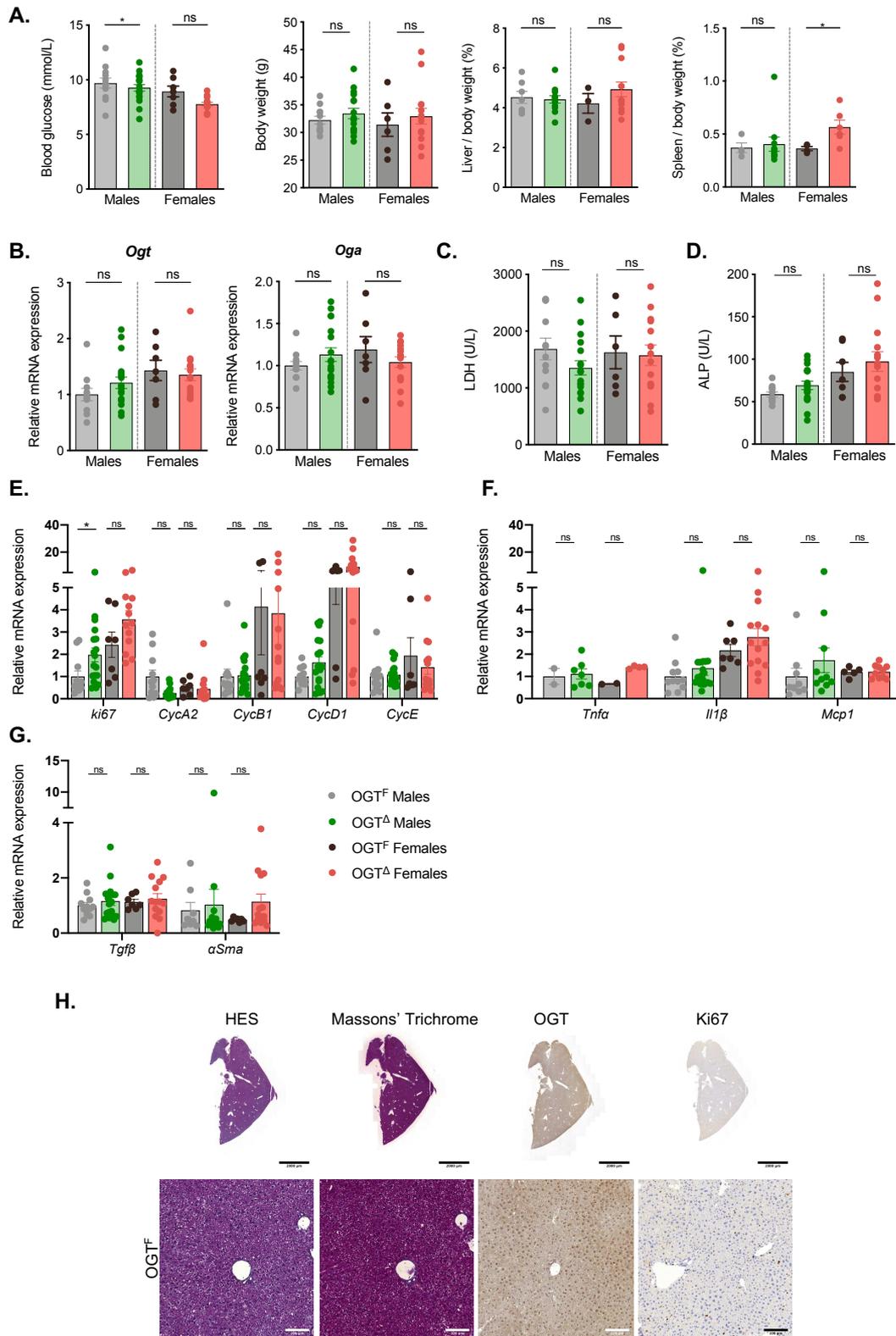
compared to OGT<sup>Δ</sup> mice at 4 weeks (C) and 8 weeks (D). Normalization gene used was TBP. E. Western blot analysis of SOD2 and NQO1, and the ER stress marker CHOP normalized to GAPDH. F. ARE-luciferase reporter activity measured in primary hepatocytes from 4 week-old mice. G. Ratio of reduced to oxidized glutathione (GSH/GSSG) measured in primary culture hepatocytes from 4 week-old mice. Data are expressed as arbitrary units and represent 2 independent cultures done in triplicates H. Western blot analysis of the DNA damage marker  $\gamma$ H2A.X normalized to the total expression of the H2A.X histone. Four representative samples are shown. GAPDH was used for loading control. I. Heatmap representing significant changes in DNA repair machinery in 4 week-old OGT<sup>Δ</sup> mice compared to controls. Values are represented as Mean  $\pm$  SEM of 4-7 mice at 4-weeks and 13-15 mice at 8-weeks mice. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001 by unpaired Student t-test (Mann-Whitney). Samples used for western blots are the same in all the blots. J. Mechanistic hypothesis based on data obtained comparing 4 and 8 weeks stages. In 4 week-old mice, the deficiency in OGT causes a liberation of NRF2 by KEAP1 allowing its translocation to the nucleus and activation of its targets genes. In 8 week-old, rescued OGT expression promotes the O-GlcNAcylation of KEAP1 favouring the ubiquitination and degradation of NRF2, reducing the antioxidant response.



**Figure 6. Sexual dimorphism at 1 year with generation of tumors in OGT<sup>A</sup> females but not in males.**

**A.** Heatmap represents males at 8 weeks with significant increased in a set of oncogenes identified from the database GeneSetDB:CancerGenes. **B.** Expression levels of glucose metabolism genes involved in glycolysis, pentose phosphate pathway (PPP) and lipogenesis. TBP was used for normalization. **C.** Measure of liver injury parameters

alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) on blood serum. **D.** Serum total and direct bilirubin ( $\mu\text{mol/L}$ ) and alkaline phosphatase (U/L). **E.** Liver sections from 1 year-old OGT<sup>F</sup> and OGT<sup>A</sup> males stained with hematoxylin-eosin-saffron (HES), Massons' trichrome, OGT and ki67 (proliferation). Scale bars = 100  $\mu\text{m}$ . **F.** Liver section from 1 year-old OGT<sup>A</sup> females stained by hematoxylin-eosin-saffron (HES), Massons' trichrome, OGT, ki67 (proliferation) and glutamine synthetase (GS). Scale bars = 100  $\mu\text{m}$ . T: tumor. A: adjacent tissue. Orange arrow: bigger hepatocyte. Red arrow: cell positive for OGT staining. Blue arrow: cell negative for OGT staining. Black arrow: Ki67+ cells. Values are represented as Mean  $\pm$  SEM of 13-15 mice at 8-weeks and 7-18 mice at 1 year. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001 by unpaired Student t-test (Mann-Whitney).



### Supplementary Figure 5.

Results correspond to mice sacrificed 1 year after birth.

**A.** Measure of physiological parameters including blood glucose (mmol/L), body (g) and liver and spleen weight. Liver and spleen weight are represented as % of body weight. **B.** Relative mRNA expression of OGT and OGA. TBP was used for qPCR normalization. **C.** Serum measurement of lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) concentrations in arbitrary units/L (U/L). **E-G.** Relative expression

of proliferation, inflammation and fibrosis markers by qPCR. **H.** Liver section staining from OGT<sup>F</sup> females at 1 year with hematoxylin-eosin-saffron (HES), Massons' trichrome, OGT and Ki67. Scale bars = 100  $\mu$ m.

**Supplementary Table 1.**

DE genes at 4w					
Qsox1	Hao2	Zfp729a	Rtk2	Ccl2	Samhd1
Fbxo4	Pknox1	Etfhdh	Rhod	Nop56; Snord57	Cyp2u1
Prr14	Sulf3a1	Slc44a1	Prune	Dnllp2	Ifit1b1
Aldh6a1	Fabp5	Marchf2	Trim34b; Trim34a	Tor3a	Lilf4b
Vps28	Hsd3b1	Nr1f3	Hspbp1	Gdgd1	Gabrb3
Gm10768; Abcc2	Ug3a1	Fzd8	Bcl2a1a	Ddx58	Gm10639
Vwa8	Ces1f	Pdxk	Cox19	H2-Q4	Fgf21
Tprkb	Cyp2c69	Gpr155	Slc35c2	Ifih1	Slc20a1
Lmf1	Aco3	Pde9a	Nup133	Bmp8b	Pydc4
Tesk2	Wfdc21	Cyp2a22	Hear1	Atp2b2	Gstp1
Rpn2	Akr1c13	Mccc2	Ccl19	Gpr35	Gstp2
Abca3	Gm11437	Ogt	Rock2	Parp14	Saa3
Naglu	Adgrf1	Ulkk2	Gclc	Lrp11	Psmb8
H2-Ke6	Hmt2	Tox	Ptdc3	Sell	Zbp1
Cnmm2	Plk3c2g	Cisd1	Mild1	Eif6	Gbp9
Decr2	Dnaic1	Gm4794	Ftsj3	Cdc7	Apol9a
Cipb	Tmem256	Pex7	Brix1	Rhoc	Pyhin1
Gstk1	Orm1	Hykk	Gpi1	Pak1ip1	Afp
Fhit	Sulf2a8	Pccb	Dnajc2	Gm18853	Trmt10a
Guf1	A1bg	Uqcrl10	Ugp2	Trmprs2	lsg15
Fetub	Ces1e	Pdk1	1700024P16Rik	Cbr1	Herc6
Tmem53	Fgf4	Qdpr	Tor1b	Eif2ak2	Tlr2
Borcs8	Cyp4a32	Mprl34	H2-T10	Ear12; Ear2; Ear3	Rnf213
Rbbp9	Prir	Slc35b4	Wfdc2	Pwp2	Usp18
Irs2	Apom	Ifih1	Acad12	Gbp2b; Gbp5	Gbp11
Mgll1	Inmt	Tm2d2	H2-T22; H2-T9	Gpx2	Gbp3
Suox	Scnn1a	Abhd6	Mb21d1	Lsg1	Krt20
Epas1	Igfals	Hacl1	Gde1	Mndal	Gp4
Myo6	Cyp2c40	Mmachc	Bcl2a1d	Cd5l	Srxn1
Cryz	0610011F06Rik	Ces1d	Eif2d	Fam60a	Oasi2
Gaint4	Hes6	Gtf2ird2	Hn1	Cidec	Ly6c1
Al464131	Rdh11	Ugt3a2	Ifi35	H2-Q5	Gbp10; Gbp6
N4bp2	Prodh2	Gtf2h4	Cdk11b	Nudt19	Ly6c2
Abcb8	Slc47a1	Gstt3	Stb2	Xaf1	Gipc2
Kptn	Gstt3	Polr3g	Tlr3	Zpr1	Tsku
Mpst	Lin7a	Tmem97	Eda2r	Oas1a	Hspb1
Rnf34	Aspdh	Cyp4f15	Dynll1	Nqo1	Gm8074
Atp5e	Tlcd2	Odf3b	Psmd5	Calml4	Ifit3
Vps51; Tm7sf2	1810011O10Rik	Pxmp4	Urm1	Serpine2	Gm3776; Gsta1
Echdc1	Akr1c12	Akr1c19	Slc15a3	Cenpp	Ifit3b
Sp4	Stard4	Slc25a23	Pfdn2	Tlr1	Rsad2
Slc25a51	Slc22a30	Sesn3	Gzmd; Gzme	Kitl	Irf7
Cyp2j5	Pxmp2	Cyp4f17	Cyp2a5; Cyp2a4	Rars	Mmp13
Timm21	Pnpo	Klk1b4	Prpsap1	Prss8	Cmpk2
Hps4	Bivm	Slc16a12	Prpf19	Slpi; Mir7678	Cxcl10
Slc4a7	Ccbl1	Sox6	Il2rg	Ms4a6b	Cxcl9
Thtpa; Zfhx2os	Ces3b	Sqle	Nampt	Ptgr1	Mup20
Fam210b	Paqr9	Cmb1	Slc35d1	Phf11b	Mup-ps19; Mup20
Elp4	Phkg2	Tfdp2	Gm7120	Fam131c	Ifi44
Lrsam1	Ppm1k	Dbt	Pvr	Zcwpw1	Ifit1
Zfp729b	Slc22a28	Arrdc4	Parp9	Ephx1	Gsta2
Abhd15	Aco1t1	Fam98a	Fam98a	Gsta4	Elov3
Cyp2j2	Pbid1	Epb4114b	Epst1	Gm4955	
Rab11fp2	Paflah2	Scap	Dhx37	Kif3a	
Slc29a1	Tsc22d3	Gm2a	Psmd8	Oas1g	
Bnip3	Gm4952	Gamt	Ube2v2	Ms4a4b	
Oaf	Ivd	Cnpy2	Entpd5	Lgals3bp	
Yeats4	Aco1t4	Slc16a2	Ptprc	Wfdc3	
Ugt2a3	Sfxn5	Selo	Adar	Nup62cl	
Agpat5	Ndufa3	Gpt	Phf11a	Trim30a	
Coa3	Acs2	Map2k6	Lyar	Sstr2	
Ttc36	Mgea5	Bphl	Aida	Lomp1	
Pecr	BC021614	Klfl12	Stat2	2010003K11Rik	
Abhd14b	Cyp4a31	Isoc2a	Dusp10	Peg10	
Hs3st3b1	Hsd17b7	Sfxn1	Ccdc120	Gm13251	
Pygo1	Pmvk	Klf9	Ccrl2	Nabp1	
Zfp65	F11	Ndufa3	Hmox1	Ddit3	
Stab2	Acs1	Ogfr1	Atp6v1d	Pydc3	
Sgsm1	St6galnac6; St6galnac4	Cyp2d37-ps	Fastkd2	Serf1	
Aspg	BC089597	Grb7	Mx2	Uap111	
Apon	Dlg3	Dcxr	Cops7b	Defb1	
Kdelc1	Sec11c	Mest	Gm1966	Impact	
Hgfac	Aph1c	Mgam	Dhx38	C6	
Mlxipl	Mprl24	Dnajc4	Sqstm1	H2-Q8; H2-Q6	
Nt5dc1	Prkra	Zfand4	Dhx33	Pkd2l2	
Mmab	Nudl2	Irak1; Mir718; Mir5132	Man2b2	Tllf7	
Hsbp111	B4galnt1	Nl5e	Bax	Krt8	
Atat1	Rabac1	Mpv17l2	Dtx3l	Neto2	
Reep6	Pm20d1	Grem2	Cd200r1	Myc	
Cdk5	Yif1a	Lage3	Htra2	Gm13139; Znfx41-ps	
Gcdh	Nxl2	Gcsh	Slc11a1	Ifit2	
Mut	Dpyd	Papss2	Fbxo36	Nrg1	
Fam20a	Shb	Slc6a12	H2-L; H2-D1	Slnf8	
Ttc38	Ma1b	Arhgap6	Pcid2	H2-Q7; H2-Q9	
Crip2	Car5a	Pcca	Slnf5	Ugdh	
Mterf3	Car5b	Hcfc1r1	Sp100	Plek	
Enpep	Slc25a42	Sema4g	Ppfbp2	Il22ra1	
Hpn	Zcchc24	Tmem184c	Slc35e3	Gm13248	
Serpinf2	Srd5a2	Dixdc1	Atf3	Ddx60	
Nduf3	Ncald; Gm15941	1700001C19Rik	Snrpa1	Dhx58	
Hsd11b1	Msmo1	1110012L19Rik	Tmem43	Mklj	
Pank1	Klkb1; Cyp4v3	Cldn12	Xdh	9130409I23Rik	
Pter	Dapk1	0610010F05Rik	Gaint10	Tnfrsf12a	
Fcgrt	Adra1b	Gstt1	Thoc6	Cbr3	
Tcp112	Uroc1	Nli2	Pgd	Mx1	
Gsto1	Foxo1	Phyhd1	Asrg1	Apol9b	
Emc10	Rdh16	Lrrc3	Parp12	Krt18	
Dnpep	Shmt1	Cmtm6	Tnfsf10	Rtp4	
Bmp1	Enox2	Slc10a5	H2-Q1	Gvin1	
Dpm3	Serpinf1	Nr5a2	Spats2	Rgs1	
Slc16a10	Clec2d	Foxp1	Myo5c	Ly6a	

Supplementary Table 2.

DE genes at 8w				
Mybl1	Susd4	Fzd1	Ecam	Timp3
Pkd2	Selenbp2	Adgrg1	H2-M2	Pde1a
Tgfb3	Hsd3b4; Gm10681	Rragd	Sico3a1	Nfyc3b
Pbk	Cyp7b1	Gpc3	Lamc1	Sestd1
Tnc	Slc22a7	Dcdc2a	Igf1r	Cd68
Gja1	Foxq1	Clic6	Pawr	Dpys2
Tcf4	Adh6-ps1	Loxl1	Tnfrsf19	Sema6a
Add1; Mir7036b	Rarres1	Ccnd1; Mir3962	Sod3	Vgll3
App	Phlda1	Flrt2	Cxcr4	Tns1
Itpr3; Mir7677	Nudt7	Cd36	Gem	Plat
Rab8b	Slc15a5	Tmem45a	G2e3	Adamts2
Robo1	Cyp8b1	Klf5	Rbl1	Ptprs
Nedd9	Sult5a1	Lbh	Ets2	Fut10
Cdk14	Dpy193	Cygb	Ddx26b	2610305D13Rik
Crtap	Tiam2	Adamts5	Ngfrap1	C1ca3a2; C1ca2
Chst15	Slc17a3	Rbp1	Tns3	Tgfb2
Zeb2; Mir5129	Cd163	Gpm6b	Mmp14	Fbln5
Wis	Obp2a	Gpm6a	C1qc	Casp12
Wbp5	Sdr9c7	Tagln2	Myo7a	Tpm4
Fmo2	Aadat	Ccdc80	Slain1	Prex2
Bcl2	Moxd1	C1qb	Selplg	Igfb8
Cib3	Slc13a2	Lhfp	AI506816	Ltbp4
Slc43a3	Chchd7	Gm609	Prkch	Igfa3
Plod2	Ugt2b38	Lyz2	Ldb1	Tmem229b
Gucy1a3; Mir7010	Slc22a3	Pdgfra; Mir7025	Mark1	Clec4n
Igfbp3	Nox4	Rassf9	Rgs2	Cstn1
Map4k4	Fam47e	Gsn	Itm2c	Cic1
Tmem173	Srgap3	Rbms3	C1qtnf7	Hist1h2ak
Ucp2	Ldhd	D17H6S56E-5	Cd300lh; Gm11711	Emp3
Gabra3	Enho	Efemp1	Ms4a4a	Cacnb3
Fgl2	Fcna	Emp2	Pld4	Prep
Tinag	Cox18	Ehf	Ifi30	Tir7
Ly86	Cadm4	Sparc	Macc1	Arg2
Tbc1d19	Trib1	Thbs2	Cldn4	Ddr2
Heph	Npr2	Ptgifn	Gm11710; Gm11711; Cd300lh	Adamts9
Rab27b	1600002H07Rik	Ncam1	Igf1	Bambi
Slc25a24	Tmem189	H2-Ab1	Hexa	Rbm3
Dab2	Capn8	Sl8sia4	E1f4	Krtf9
Ndrg1	Nmnat1	Cd74; Mir5107	Wfdc15b	Sy7
Axl	Ctp	Scara3	Cd52	E14
Tgfb1	Acs2	Timp2	Kalm	Pxdn
Epha7	Ttc3	Anbr1	Ddah2	Tuba1a
Ano6	Tgfb1	Cdh6	Rasef	Cmtm3
Art4	Nek5	Cd34	Myadm	Selm
Epha3	Ralgds	Col1a2	Tmem106a	Ptpn13
Ddit4l	Scar2	Anxa2	Csf1r	Slc16a7
Rhob	Tpr	Ano1	Scppdh	Cldn7
Fam107a	Eng	Syl5	Sema3c	Pea15a
Arhgef6	Fcgr3	Igfa8	G6pdx	Map3k1
Tmem47	Creb3l1	Rab31	SrpX2	Plk2
Igfb1	Egflam	Pdgfd	Dcn	Ikbip
Spint1	Ncoa7	Pdgfrb	Adamts1	Gpr65
Ppic	Rbms1	Abcd2	Slc16a9	Igfa9
Nipa1	Tox3	Gdf10	Rnase1	Ifi57
Parp8	Golm1	Ifi2712b	Agpat4	Lrrcc1
Tspan3	Mgat4a	Gas6	Nav1	Fhl2
Scd2; Mir5114	Ehd2	Mxra8	Syt2	Phactr2
Angpt1	Sema3f	Gldn	Hexb	Septin8
Adgrg6	Mamdc2	Islr	1700047I17Rik2; Fam177a	Lg12
Tf2	Serpinb6a	Plet1	ldh2	Car2
Col6a3	Slc44a2	Csrp1	2210013O21Rik	Lpar1
Ctgf	Trove2	Slc5a1	Fermt1	Ccdc3
Ahnak; Mir6367	Kifc3	Tbx20	Ptpn14	Adamts2
Dkk3	Sla	Mfge8	Cerk	Sox9
Cldn8	Gstm2	H2-DMb1	Myo1d	Fgfr3
Pam	Fam105a	Ptxdc2	Emb	Khl13
Slc16a5	Enkur	Vim	Fam205a2; Gm10600	Septin11
Tmsb4x	Cysl1r1	Dpl	Parm1	Aldh1a2
Icosl	Myh9	Htra1	Vep1	Il10b
Lpl	Erich5	Lama2	Lamb2	Plekha2
Krt7	Ccdc88a	Col3a1	Jchain	Mcm6
Cd14	Lgmn	Anxa13	Arhgdib	Pear1
Tm4sf1	6720489N17Rik	S100a11	Mamld1	Rhoj
Alp6v0d2	Lox12; Mir6950	Gabrp	Abi3bp	Ogn
Bgn	Spry1	Pkhd1	Ilfgr1	Abi2
Fbn1	Mboat1	Prom1	St14	C1ca3a1; C1ca1
Serpinh1	Plekho1	Emp1	Sh2d4a	Ephx4
Pdgfc	Dnal1	Epcam	Tead1	Ms4a4d
Tir4	Ifi2712a	Cd24a	Spire1	Agm
Thbs1	Jag1	Olfml3	Apobec3	Frzb
C3ar1	Mbnl3	Bicc1	Clmp	Osmr
Ace	Sfppd	Spp1	Ppp1r9b	Prr15l
Pamr1	Phldb2	Fam180a	Gfp2	Pisd-ps3
1700011H14Rik	Hnf4g	Lum	Igfb5	Plxna4
Anxa3	Itpr1p2	Mmp2	Meis2	Cav2
Efnb2	Atp2b4; Mir6903	Pdzk1p1	Sh3kbp1	Prrg1
Ezr	Dock10	Sparcl1	Ehd2	Sema5a
Slc15a2	Aco9	Tspan8	Adgre5; Mir1668	Pcdhgb1;
Plp2	B4gal6	Actg1; Mir6935	Atp8a1	Pcdhgb2;
Tmsb10	Pirb	Galnt3	Aoah	Pcdhgb4;
Ankrd1	Npcd; Cbx6; Npctr	Col1a1	Aebp1	Pcdhgb5;
Lamb1	Sor1	Robo2	Marcks	Pcdhgb6;
Gpx3	Fbln1	Rcn1	Shroom3	Pcdhgb7;
Igfbp7	Gng2	Slc35f2	Cttnbp2nl	Pcdhgb8;
Slc22a27	Arpc1b	Fam19a2	Hpgds	Pcdhgc3;
Rtn4	Wwc2	Slc25a36	Cln6	Pcdhgc4;
Soat1	Pacs1	Klf6	Unc5b	
Stmn1	Ptpm	Mrc2	Mid1	
Tcf21	Igfb6	Fxyd5; Mir7050	Clsk	
Abhd2	Ptger4	Abr	Hnmpa1; Gm10052	
Muc1	Nipal2	Casc4	Kcnma1	

### Supplementary Table 3.

DE genes at 4 and 8w
Slc22a26
Ido2
Retsat
Mpv17l
Fgf1
Plekhh1
P2ry4
Lifr
Chic1
Slc25a45
Tmem19
Lenep; Flad1
Tcf24
Fn3krp
Myl12a
Epb4114a
Lapm5
Csprs
Pla2g7
Iqgap1
Fads3
Tmem176b
Tlr13
Pafah1b3
Cxcl16
Cd44
Gm13212
Myo1
Hcls1
Aebp2
Flna
Ai607673
Tnfai3
Tceal8
Septin6
Spi1
H2-DMb2
Ms4a7
Ms4a6d
Capsl
Ajuba
Slamf9
Adora1
Ly6e
Cd53
Ccr2
Gm9844
Bcl2a1b; Bcl2a1a
Pygb
Vcam1
Clss
Ms4a6c
Cetn4
Alcam
Sfn2
Sirpa
Nid1
Abcb1a
Slamf7
H2-Eb1
9930111J21Rik1; 9930111J21Rik2
9930111J21Rik2
Gm5431
Gm2399
Ifi203
Evi2a; Evi2b; Gm21975
Ccl22
Phf11d; Phf11c
Glpr1
Cybb
9230110C19Rik
Gpnmb
Mgst3
Cd180
Anxa5
Cd9
Clec12a
F2r1
Ifi204
Samd9l
Ifi27
Plac8
Mnda
Lilrb4a
Haus8
Gm3934
H2-Aa
Npnt
Ntrk2
Elovl7
Gstm3
Clec7a
Cd63
Ms4a4c
Mmp12

## Supplementary Table 4.

Name	Sequence
Acc F	GAGGTGGCTAAGAGGAGGCTCT
Acc R	CAGCACCGAGACTGAACTGTAAGG
Cel5 F	CTG ACC CTG TAT AGC TTC CCT
Cel5 R	GGG ATT ACT GAG TGG CAT CC
Chrebp F	ATGACCCTCACTCAGGGAATA
Chrebp R	GATCCAAGGTCCAGAGCAG
Chrebp $\beta$ F	TCT GCA GAT CGC GTG GAG
Chrebp $\beta$ R	CTT GTC CCG GCA TAG CAA C
Col3a1 F	CTGTAACATGGAACTGGGGAAA
Col3a1 R	CCATAGCTGAACTGAAAACCACC
Col6a1 F	CTGCTGTACAAGCTGTCT
Col6a1 R	CCCCATAAGGTTTCAGCCTCA
CycA2 F	TGC CTT CAC TCA TTG CTG GA
CycA2 R	TGT GGC GCT TTG AGG TAG GT
CycB1 F	TGC CTT TGT CAC GGC CTT AG
CycB1 R	GGA AAT TCT TGA CAA CGG TG
CycD F	GCG TAC CCT GAC ACC AAT CTC
CycD R	CTC CTC TTC GCA CTT CTG CTC
CycE F	CAGAGCAGCGAGCAGGAGA
CycE R	CAGCTGTTCCACCACTG
Elovl6 F	ACAATGGACCTGTAGCAAA
Elovl6 R	GTACCAGTCAGGAAGATCAGT
Fas F	TTC CAA GAC GAA AAT GAT GC
Fas R	AAT TGT GGG ATC AGG AGA GC
Fxr F	GCT TGA TGT GCT ACA AAA GCT G
Fxr R	CGT GGT GAT GGT TGA ATG TCC
FoxO1 F	TGTTACTTAGCTCTCCCTCG
FoxO1 R	AGACGAGCAGTGGCTCAAT
G6pase F	TTACCAGCCTCTGTCCG
G6pase R	GACACAACGAAAGCCGGTTAG
Gk F	CCCTGAGTGGCTTACAGTTC
Gk R	ACGGATGTGAGTGTGAAGC
Gpx3 F	GCC ATT TGG CTT GGT CAT TC
Gpx3 R	TGG GGA GTA TCT CCG AGT TC
Gpx4 F	GCA CGA ATT CTC AGC CAA GG
Gpx4 R	CAA ACT GGT TGC AGG GGA AG
Gele F	CTG CAC ATC TAC CAC GCA GT
Gele R	TTC ATG ATC GAA GGA CAC CA
Gss F	GAAGCAGCTCGAAGAAGCTGG
Gss R	AGCACTGGGTACTGGTGAGG
Gsta1 F	CCA GAG CCA TTC TCA ACT A
Gsta1 R	TGC CCA ATC ATT TCA GTC AG
Gstm1 F	CTACCTGCCCCGAAAGCAC
Gstm1 R	ATGTCTGCACGGATCCTCTC
Gstm3 R	TGA AGG CCA TCC CTG AGA AA
Gstm3 F	CTT GGG AGG AAG CGG CTA CT
Gstm5 R	AGA TAC ATC GCA CGC AAG CA
Gstm5 F	CCA TGT GAA TTT CCC CAG GA
Hk2 F	CTGCCACCTTTGTGAGGTC
Hk2 R	TCGGCAAATGGTCAAACAG
Hnf4 $\alpha$ F	TGCCTGCCTCAAAGCCAT
Hnf4 $\alpha$ R	CACTCAGCCCCCTGGCAT
HO-1 F	GTCAAGCACAGGGTGACAGA
HO-1 R	ATCACCTGCAGCTCCTCAAA
Il-1 $\beta$ F	GGCCCTCAAAGGAAAGAATC
Il-1 $\beta$ R	TACCAGTIGGGGAACCTCTGC
Kl67 F	AGG ATG GAA GCA AGC CAA CA
Kl67 R	GGC CCT TGG CAT ACA CAA AA
Krt7 F	CACCCGGAATGAGATTGCG
Krt7 R	GCACGCTGGTCTTCAAGGT
Krt19 F	TGCTGGATGAGCTGACTCTG
Krt19 R	AATCCACTCCACACTGACC
L-pk F	CTTGCTTACCGTGAGCCTC
L-pk R	ACCACAATCACCAGATCACC
Nq01 F	AGC GTT CGG TAT TAC GAT CC
Nq01 R	AGT ACA ATC AGG GCT CTT CTC G
Nrf2 F	TTC TTT CAG CAG CAT CCT CTC CAC
Nrf2 R	ACA GCC TTC AAT AGT CCC GTC CAG
Mep1 F	TGAATGTGAAGTTGACCCGT
Mep1 R	AGAAATGCTTGAGGTGGTTG
Oga F	TTCACTGAAGGCTAATGGCTCCCG
Oga R	TGTCACAGGCTCCGACCAAGT
Ogt F	TCGCACAGCTCTGTCAAAA
Ogt R	GCCCTGGGTCGCTTGAAGA
Phgdh F	GGAGGAGATCGGCCTCTCT
Phgdh R	GCACACCTTCTTGCACTGA
Pkm2 F	CTGAGGTGAAGGAGAAAGG
Pkm2 R	AGATGCAAAACACCATGTCCA
Sed1 F	CCGGAGACCCCTTAGATCGA
Sed1 R	TAGCCTGTAAAAGATTTCTGCAAA
Shp F	CAG GCA CCC TTC TGG TAG ATC T
Shp R	GTC TTC AAG GAG TTC AGT GAT GTC A
$\alpha$ Sma F	TGA CCC AGA TTA TGT TTG AGA CC
$\alpha$ Sma R	CCA GAG TCC AGC ACA ATA CCA
Tbp F	CCCCACAACCTTCCATCT
Tbp R	GCAGGAGTGATAGGGTTCAT
Tgf $\beta$ F	TGAGTGGCTGTCTTTTGACG
Tgf $\beta$ R	AGTGAGCGCTGAATCGAAAG
Tnfr $\alpha$ F	TGGGAGTAGACAAGGTACAACC
Tnfr $\alpha$ R	CATCTTCAAAAATTGGAGTGAC

## Discussion

The study of OGT function in *Ogt* global knock-out mice has been limited since it leads to embryonic lethal phenotype demonstrating that the OGT protein is essential for embryo and early life development (Shafi *et al.*, 2000). Generation of tissue/cell-specific *Ogt* knock-out mice appeared therefore essential to elucidate the specific role of OGT in different context and locations. We generated hepatocyte-specific *Ogt* knock-out mice (OGT<sup>Δ</sup>) to better understand the role of O-GlcNAcylation and of OGT in the regulation of liver homeostasis, specifically their roles in the regulation of enzymes involved in glucose and lipid metabolism.

Early life study of OGT<sup>Δ</sup> mice validated the Cre-dependent recombinant efficiency. Surprisingly, comparison of blood glucose levels, body weight and liver weight revealed no major metabolic alterations, hypothesis further validated by similar glucose tolerance between OGT<sup>Δ</sup> and OGT<sup>F</sup> mice at 4-weeks. Interestingly, while no clear metabolic phenotype could be observed, splenomegaly was evidenced in OGT<sup>Δ</sup> compared to OGT<sup>F</sup> mice. Splenomegaly, which is associated with liver portal hypertension and progression to cirrhosis (Li *et al.*, 2017), gave us the first clue for the potential progressive development of hepatic fibrosis in OGT<sup>Δ</sup> mice. However, at 4 weeks of age, HES staining demonstrated normal liver structure. Inflammation was demonstrated by inflammatory cells infiltration and increase in mRNA expression of *Tnfa*, *Mcp1* and *Ccl5*. Follow-up analysis of OGT<sup>Δ</sup> mice demonstrated the development of hepatomegaly (abnormal liver growth) 8 weeks after birth. Hepatomegaly at 8 weeks was accompanied by extensive cirrhosis and cholestasis, features commonly seen in bile duct ligation models one week after ligation (Geerts *et al.*, 2008). Standard histological analysis with HES and Massons' trichrome staining reveal fibrosis, dilatation of bile canaliculi, ductular proliferation (evidenced by Ki67 staining) and development of hepatic nodules of regeneration. Surprisingly, these nodules of regeneration were positive for OGT staining, and paralleled with global rescued OGT expression at the level of mRNA and protein in livers of OGT<sup>Δ</sup> mice. While we were puzzled at first with rescued expression of OGT in livers of OGT<sup>Δ</sup> mice, we hypothesize that “cell competition” could explain this rescue (Baker, 2020). Indeed, this hypothesis can be explained as follows: hepatocytes deficient in OGT encounter difficulties to survive and enter the process of cell death; while, interestingly, hepatocytes escaping to the recombinant process will repopulate the liver with cells expressing OGT protein. This hypothesis was already proposed in several animal models (Caldwell *et al.*, 2010; Yamaji *et al.*, 2010; Zhang *et al.*, 2010; Grindheim *et al.*, 2019).

Our study also documents that OGT<sup>Δ</sup> mice exhibit a proliferative phenotype associated with ductular and fibrotic regions. Proliferation was confirmed with up-regulation of proliferative markers at the mRNA expression and protein levels. Correlating with the severe phenotype observed, concentrations of circulating cytokines (TNF $\alpha$ , IL-2 and IL-6) were increased in OGT<sup>Δ</sup> mice at 8 weeks. Markers of liver injury (ALT, LDH and ALP) were also increased in circulation at 8 but not at 4 weeks. Microarray data show a significant amount of genes up- and down-regulated at 4 weeks involved in immune signalling and metabolism respectively, suggesting the necessity of liver to adapt to the inflammatory phenotype of OGT<sup>Δ</sup> mice at this stage. One of the features appreciated was the change in cell identity profile favouring the differentiation of hepatoblast to cholangiocytes typical of the ductular reaction produced in the initial steps of liver regeneration (Roskams *et al.*, 2004). Ductular reaction was validated by cholangiocytes staining with SOX9 and CK19 antibodies, a staining strongly increased in the inter-nodular fibrotic area of OGT<sup>Δ</sup> mouse liver sections. This phenotype could be partially explained by the decrease in *Fxr* and *Shp* expression. Indeed, *Fxr* knock-out mice exhibit increased BAs pool and show a hyperproliferative and inflammatory phenotype (Cariello *et al.*, 2018) similar to the one we observed for OGT<sup>Δ</sup> mice. In this context, it would be of potential interest to treat OGT<sup>Δ</sup> with a FXR agonist, such as INT-767 or GW4064, known to reduce liver injury and cholestasis (Han, 2018), to determine whether their severe liver phenotype can be partially reversed.

To attempt to provide mechanistic leads to the phenotype of the OGT<sup>Δ</sup> mice, we examined some of gene clusters dysregulated at 4 and/or 8 weeks. We focused on a set of NRF2 target genes, found significantly increased in liver of 4 week-old OGT<sup>Δ</sup> mice. Western blot analysis confirmed that NRF2 protein content was elevated at 4 but not at 8 weeks in liver of OGT<sup>Δ</sup> mice. Considering that KEAP1 regulates the cytosolic retention of NRF2 and that KEAP1 is O-GlcNAcylated, we followed this interesting lead. Indeed, rescued OGT expression at 8 weeks correlated with significant increased KEAP1 O-GlcNAcylation and decreased NRF2 protein content. NRF2 targets measured by qPCR were found significantly increased at both 4 weeks and more modestly at 8 weeks in OGT<sup>Δ</sup> mice, suggesting enhanced NRF2 transcriptional activity. ARE-luciferase reporter activity in cultured hepatocytes from 4 week-old OGT<sup>Δ</sup> mice validated a clear increased in NRF2 activity. In addition, pathways of ER stress and oxidative stress were also induced in 4 week-old OGT<sup>Δ</sup> mice as evidenced by the increase in CHOP and SOD2 expression, respectively. Further validation of oxidative stress was shown in cultured hepatocytes from OGT<sup>Δ</sup> mice at 4 weeks, which presented

a significant decreased in GSH/GSSH ratio, a well-accepted marker oxidative stress (Ballatori *et al.*, 2009). Given the relationship between oxidative stress and DNA damage (Van Houten, Santa-Gonzalez and Camargo, 2018), we observed increased DNA damage at 4-weeks evidenced by increased phosphorylation of H2AX. Microarray heatmap also revealed significant changes in expression of DNA repair machinery especially at 4 weeks in OGT<sup>Δ</sup> compared to OGT<sup>F</sup> mice. Because NRF2 was recently suggested to repress FXR expression in conditions of autophagy deficiency (Khambu *et al.*, 2019), it would be important to elucidate the levels of expression of proteins involved in liver autophagy such as *Agt7* and *Agt5*.

Importantly, an oncogene signature was observed in liver of 8-week-old OGT<sup>Δ</sup> mice suggesting the potential development of liver cancer in older mice. These data correlated with up-regulation of *Hk2* and *Pkm2*, rate-limiting enzymes of glycolytic pathway, only expressed in adult liver in the context of liver cancer (Feng *et al.*, 2020) and *G6pdh*, the rate-limiting enzyme of the pentose phosphate pathway (PPP), associated with proliferation and cancer development (Yang, Stern and Chiu, 2020). Altogether, our observations support the hypothesis of liver cancer development in OGT<sup>Δ</sup> mice at latest life stages. To validate the hypothesis, we followed up OGT<sup>Δ</sup> and OGT<sup>F</sup> mice up to 1 year of life. Physiological parameters and relative expression of OGT were found similar in liver of males and females OGT<sup>Δ</sup> compared to their respective controls, validating the fact that OGT rescue is maintained over time. Serum concentrations of ALT were increased in males and females OGT<sup>Δ</sup> compared to OGT<sup>F</sup> mice, yet no differences were found in AST, LDH or ALP, suggesting a moderate residual liver injury in these mice compared to 8-week-old OGT<sup>Δ</sup> mice. Interestingly, liver tumor development was only observed in OGT<sup>Δ</sup> females at 1 year. OGT staining showed expression of the OGT protein both in the tumor and the adjacent tissue; however, the distribution did not seem to be homogenous in all cells. Recombination by *Alb-Cre* in some hepatocytes could explain the lack of OGT in some hepatocytes (Yamaji *et al.*, 2010). However, some cells express high levels of OGT both in the cytoplasm and the nucleus. Over-expression of OGT due to re-activation of the inactivated X chromosome, largely associated to cancer development (Kaneko and Li, 2018), could be a potential explanation for this heterogenic expression. Staining of proliferation markers also showed significant increase in Ki67<sup>+</sup> cells in the tumor area but no major expression of GS was evidenced suggesting that OGT<sup>Δ</sup> female mice develop hepatocellular adenomas (HCA) rather than HCC. It would be interesting to have access to human samples to elucidate the potential implications of OGT in the

progression and prognosis of HCA. Taken all together these results suggest that female mice are more sensitive to develop liver cancer after OGT rescue than males.

In conclusion, our study reveals that *Ogt*-deficient mouse is an interesting tool for the study of liver cirrhosis and cholestasis since OGT<sup>Δ</sup> mice develop in less than 8 weeks a severe liver phenotype. The OGT<sup>Δ</sup> mouse model is also a fascinating tool to better understand the phenotype of sexual dimorphism in the development of HCA, highly associated to females over males since it is promoted by hormonal exposure. In addition, the classification of HCA types is extremely important to understand the risk complications associated such as hemorrhage and malignant transformation into HCC (Védie *et al.*, 2018).

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## References

1. Abdul-Wahed A, Guilmeau S, Postic C. Sweet Sixteenth for ChREBP: Established Roles and Future Goals. *Cell Metab.* 2017 Aug 1;26(2):324-341. doi: 10.1016/j.cmet.2017.07.004. PMID: 28768172.
2. Anthonisen EH, Berven L, Holm S, Nygård M, Nebb HI, Grønning-Wang LM. Nuclear receptor liver X receptor is O-GlcNAc-modified in response to glucose. *J Biol Chem.* 2010 Jan 15;285(3):1607-15. doi: 10.1074/jbc.M109.082685. Epub 2009 Nov 20. PMID: 19933273; PMCID: PMC2804318.
3. Bindsbøll C, Fan Q, Nørgaard RC, MacPherson L, Ruan HB, Wu J, Pedersen TÅ, Steffensen KR, Yang X, Matthews J, Mandrup S,
4. Nebb HI, Grønning-Wang LM. Liver X receptor regulates hepatic nuclear O-GlcNAc signaling and carbohydrate responsive element-binding protein activity. *J Lipid Res.* 2015 Apr;56(4):771-85. doi: 10.1194/jlr.M049130. Epub 2015 Feb 27. PMID: 25724563; PMCID: PMC4373736.
5. Bricambert J, Miranda J, Benhamed F, Girard J, Postic C, Dentin R. Salt-inducible kinase 2 links transcriptional coactivator p300 phosphorylation to the prevention of ChREBP-dependent hepatic steatosis in mice. *J Clin Invest.* 2010 Dec;120(12):4316-31. doi: 10.1172/JCI41624. Epub 2010 Nov 15. PMID: 21084751; PMCID: PMC2993582.
6. Bricambert J, Alves-Guerra MC, Esteves P, Prip-Buus C, Bertrand-Michel J, Guillou H, Chang CJ, Vander Wal MN, Canonne-Hergaux F, Mathurin P, Raverdy V, Pattou F, Girard J, Postic C, Dentin R. The histone demethylase Phf2 acts as a molecular checkpoint to prevent NAFLD progression during obesity. *Nat Commun.* 2018 May 29;9(1):2092. doi: 10.1038/s41467-018-04361-y. PMID: 29844386; PMCID: PMC5974278.
7. Dentin R, Hedrick S, Xie J, Yates J 3rd, Montminy M. Hepatic glucose sensing via the CREB coactivator CRTC2. *Science.* 2008 Mar 7;319(5868):1402-5. doi: 10.1126/science.1151363. PMID: 18323454.
8. Dentin R, Tomas-Cobos L, Fougelle F, Leopold J, Girard J, Postic C, Ferré P. Glucose 6-phosphate, rather than xylulose 5-phosphate, is required for the

- activation of ChREBP in response to glucose in the liver. *J Hepatol.* 2012 Jan;56(1):199-209. doi: 10.1016/j.jhep.2011.07.019. Epub 2011 Aug 9. PMID: 21835137.
9. Fardini Y, Perez-Cervera Y, Camoin L, Pagesy P, Lefebvre T, Issad T. Regulatory O
  10. GlcNAcylation sites on FoxO1 are yet to be identified. *Biochem Biophys Res Commun.* 2015 Jun 26;462(2):151-8. doi: 10.1016/j.bbrc.2015.04.114. Epub 2015 May 2. PMID: 25944660.
  11. Guinez C, Filhoulaud G, Rayah-Benhamed F, Marmier S, Dubuquoy C, Dentin R, Moldes M, Burnol AF, Yang X, Lefebvre T, Girard J, Postic C. O-GlcNAcylation increases ChREBP protein content and transcriptional activity in the liver. *Diabetes.* 2011 May;60(5):1399-413. doi: 10.2337/db10-0452. Epub 2011 Apr 6. PMID: 21471514; PMCID: PMC3292313.
  12. Herman MA, Peroni OD, Villoria J, Schön MR, Abumrad NA, Blüher M, Klein S, Kahn BB. A novel ChREBP isoform in adipose tissue regulates systemic glucose metabolism. *Nature.* 2012 Apr 19;484(7394):333-8. doi: 10.1038/nature10986. PMID: 22466288; PMCID: PMC3341994.
  13. Housley MP, Rodgers JT, Udeshi ND, Kelly TJ, Shabanowitz J, Hunt DF, Puigserver P, Hart GW. O-GlcNAc regulates FoxO activation in response to glucose. *J Biol Chem.* 2008 Jun 13;283(24):16283-92. doi: 10.1074/jbc.M802240200. Epub 2008 Apr 17. PMID: 18420577; PMCID: PMC2423255.
  14. Ido-Kitamura Y, Sasaki T, Kobayashi M, Kim HJ, Lee YS, Kikuchi O, Yokota-Hashimoto H, Iizuka K, Accili D, Kitamura T. Hepatic FoxO1 integrates glucose utilization and lipid synthesis through regulation of ChREBP O-glycosylation. *PLoS One.* 2012;7(10):e47231. doi: 10.1371/journal.pone.0047231. Epub 2012 Oct 8. PMID: 23056614; PMCID: PMC3466224.
  15. Iroz A, Montagner A, Benhamed F, Levavasseur F, Polizzi A, Anthony E, Régnier M, Fouché E, Lukowicz C, Cauzac M, Tournier E, Do-Cruzeiro M,

- Daujat-Chavanieu M, Gerbal-Chalouin S, Fauveau V, Marmier S, Burnol AF, Guilmeau S, Lippi Y, Girard J, Wahli W, Dentin R, Guillou H, Postic C. A Specific ChREBP and PPAR $\alpha$  Cross-Talk Is Required for the Glucose-Mediated FGF21 Response. *Cell Rep.* 2017 Oct 10;21(2):403-416. doi: 10.1016/j.celrep.2017.09.065. PMID: 29020627; PMCID: PMC5643524.
16. Issad T, Kuo M. O-GlcNAc modification of transcription factors, glucose sensing and glucotoxicity. *Trends Endocrinol Metab.* 2008 Dec;19(10):380-9. doi: 10.1016/j.tem.2008.09.001. Epub 2008 Oct 17. PMID: 18929495.
  17. Kabashima T, Kawaguchi T, Wadzinski BE, Uyeda K. Xylulose 5-phosphate mediates glucose-induced lipogenesis by xylulose 5-phosphate-activated protein phosphatase in rat liver. *Proc Natl Acad Sci U S A.* 2003 Apr 29;100(9):5107-12. doi: 10.1073/pnas.0730817100. Epub 2003 Apr 8. PMID: 12684532; PMCID: PMC154306.
  18. Kawaguchi T, Takenoshita M, Kabashima T, Uyeda K. Glucose and cAMP regulate the L-type pyruvate kinase gene by phosphorylation/dephosphorylation of the carbohydrate response element binding protein. *Proc Natl Acad Sci U S A.* 2001 Nov 20;98(24):13710-5. doi: 10.1073/pnas.231370798. Epub 2001 Nov 6. PMID: 11698644; PMCID: PMC61106.
  19. Lane EA, Choi DW, Garcia-Haro L, Levine ZG, Tedoldi M, Walker S, Danial NN. HCF-1 Regulates De Novo Lipogenesis through a Nutrient-Sensitive Complex with ChREBP. *Mol Cell.* 2019 Jul 25;75(2):357-371.e7. doi: 10.1016/j.molcel.2019.05.019. Epub 2019 Jun 18. PMID: 31227231; PMCID: PMC6744259.
  20. Lazarus MB, Nam Y, Jiang J, Sliz P, Walker S. Structure of human O-GlcNAc transferase and its complex with a peptide substrate. *Nature.* 2011 Jan 27;469(7331):564-7. doi: 10.1038/nature09638. Epub 2011 Jan 16. PMID: 21240259; PMCID: PMC3064491.
  21. Li MV, Chen W, Harmancey RN, Nuotio-Antar AM, Imamura M, Saha P, Taegtmeyer H, Chan L. Glucose-6-phosphate mediates activation of the carbohydrate responsive binding protein (ChREBP). *Biochem Biophys Res*

- Commun. 2010 May 7;395(3):395-400. doi: 10.1016/j.bbrc.2010.04.028. Epub 2010 Apr 9. PMID: 20382127; PMCID: PMC2874883.
22. Lou DQ, Tannour M, Selig L, Thomas D, Kahn A, Vasseur-Cognet M. Chicken ovalbumin upstream promoter-transcription factor II, a new partner of the glucose response element of the L-type pyruvate kinase gene, acts as an inhibitor of the glucose response. *J Biol Chem*. 1999 Oct 1;274(40):28385-94. doi: 10.1074/jbc.274.40.28385. PMID: 10497199.
  23. Ma L, Tsatsos NG, Towle HC. Direct role of ChREBP.Mlx in regulating hepatic glucose-responsive genes. *J Biol Chem*. 2005 Mar 25;280(12):12019-27. doi: 10.1074/jbc.M413063200. Epub 2005 Jan 20. PMID: 15664996.
  24. McFerrin LG, Atchley WR. A novel N-terminal domain may dictate the glucose response of Mondo proteins. *PLoS One*. 2012;7(4):e34803. doi: 10.1371/journal.pone.0034803. Epub 2012 Apr 10. PMID: 22506051; PMCID: PMC3323566.
  25. Ortega-Prieto P, Postic C. Carbohydrate Sensing Through the Transcription Factor ChREBP. *Front Genet*. 2019 Jun 4;10:472. doi: 10.3389/fgene.2019.00472. PMID: 31275349; PMCID: PMC6593282.
  26. Park MJ, Kim DI, Lim SK, Choi JH, Han HJ, Yoon KC, Park SH. High glucose-induced O-GlcNAcylated carbohydrate response element-binding protein (ChREBP) mediates mesangial cell lipogenesis and fibrosis: the possible role in the development of diabetic nephropathy. *J Biol Chem*. 2014 May 9;289(19):13519-30. doi: 10.1074/jbc.M113.530139. Epub 2014 Mar 10. PMID: 24616092; PMCID: PMC4036358.
  27. Sakiyama H, Fujiwara N, Noguchi T, Eguchi H, Yoshihara D, Uyeda K, Suzuki K. The role of O-linked GlcNAc modification on the glucose response of ChREBP. *Biochem Biophys Res Commun*. 2010 Nov 26;402(4):784-9. doi: 10.1016/j.bbrc.2010.10.113. Epub 2010 Oct 29. PMID: 21036147.
  28. Yang AQ, Li D, Chi L, Ye XS. Validation, Identification, and Biological Consequences of the Site-specific O-GlcNAcylation Dynamics of Carbohydrate-responsive Element-binding Protein (ChREBP). *Mol Cell Proteomics*. 2017

Jul;16(7):1233-1243. doi: 10.1074/mcp.M116.061416. Epub 2017 Apr 27. PMID: 28450420; PMCID: PMC5500757.

29. Yang WH, Park SY, Nam HW, Kim DH, Kang JG, Kang ES, Kim YS, Lee HC, Kim KS, Cho JW. NFkappaB activation is associated with its O-GlcNAcylation state under hyperglycemic conditions. *Proc Natl Acad Sci U S A*. 2008 Nov 11;105(45):17345-50. doi: 10.1073/pnas.0806198105. Epub 2008 Nov 6. PMID: 18988733; PMCID: PMC2582288.
30. Zhao P, Viner R, Teo CF, Boons GJ, Horn D, Wells L. Combining high-energy C-trap dissociation and electron transfer dissociation for protein O-GlcNAc modification site assignment. *J Proteome Res*. 2011 Sep 2;10(9):4088-104. doi: 10.1021/pr2002726. Epub 2011 Jul 25. PMID: 21740066; PMCID: PMC3172619.

## Summary of research project 2

The research objective 2 consisted in the metabolic characterization of hepatocyte-specific *Ogt* knock-out mice ( $OGT^{\Delta}$ ) to better understand the role of O-GlcNAcylation and OGT in the regulation of liver homeostasis. To determine the metabolic consequences of a targeted deletion of the OGT enzyme in hepatocytes *in vivo*, we developed a mouse model with liver-specific ablation of OGT ( $OGT^{\Delta}$ ) by crossing  $OGT^{lox/lox}$  with Alb-Cre mice. While these mice did not exhibit major disruption of metabolic homeostasis (**Figure 1C and D, Supplementary Figure 1B**) they exhibited a severe liver phenotype with the presence of numerous regeneration nodules visible after weaning (**Figure 2B, 2C, 2D**). RT-qPCR and western blot analyzes showed that the expression of cyclins A2, B1 and D1 was significantly increased in the liver of  $OGT^{\Delta}$  mice compared to controls suggesting an exacerbated proliferative state at eight-weeks (**Figure 2H**). Histological analyzes revealed the presence of pro-inflammatory cells and signs of fibrosis in the spans surrounding the regeneration nodules (**Figure 2G**). This was associated with a significant increase in markers of inflammation (Tnf $\alpha$ , Mcp1 and Ccl5), circulating cytokines (TNF $\alpha$ , IL-2 and IL-6) and fibrosis (Col3a1 and Col6a1), suggesting significant liver injury in  $OGT^{\Delta}$  mice (**Figure 2I, 2J and Supplementary Figure 2B**). Interestingly, while OGT was significantly reduced in livers of young mice (4 week-old) (**Figure 1A and B**), we observed re-expression of OGT in livers of older mice (8 week-old) (**Figure 2E**) suggesting a counter selection against OGT deficient cells. To better understand the mechanisms driving rescued expression of OGT as well as the phenotype of liver injury, we performed a microarray analysis comparing 4- and 8-week-old mice (**Figure 3**). RT-qPCR and western blot validation demonstrated an increase in ER stress (CHOP), antioxidant response (Nrf2) and DNA damage ( $\gamma$ H2AX) markers (**Figure 5**). Furthermore, several REACTOME pathways were enriched in liver of 8 week-old  $OGT^{\Delta}$  mice compared to controls such as extracellular matrix formation, innate immune system and oncogenes suggesting the potential development of tumors in the liver of older  $OGT^{\Delta}$  mice (**Figure 3, 5A and 5I, 6A and Supplementary Figure 4**). Follow up of these mice over one year revealed a sexual dimorphism with females developing several tumor-like structures while males showed a normal liver phenotype (**Figure 6**).

In conclusion, our study reveals that lack of hepatic OGT is associated with a severe liver phenotype and suggests an essential role for OGT / O-GlcNAcylation in maintaining liver homeostasis.

# **General discussion and conclusions**

## Article 1: Importance of the OGT-ChREBP axis in the control of hepatic lipogenesis

My first PhD objective was to address the importance of the OGT-ChREBP axis in the control of hepatic lipogenesis. Indeed, over the past years, ChREBP has emerged as a major player of hepatic steatosis and Non Alcoholic Fatty Liver Disease (NAFLD) (Abdul-Wahed, Guilmeau and Postic, 2017), but the molecular mechanisms regulating its glucose-dependent activation is complex and remains partially understood. The regulation of ChREBP activity takes place at the level of subcellular localization, DNA binding and transcriptional activity. Since its discovery in 2000, intensive efforts have been employed to identify the precise mechanisms by which glucose stimulates ChREBP activity (Ortega-Prieto and Postic, 2019). Our team and other groups revealed that high glucose induces OGT-mediated O-GlcNAcylation of ChREBP in liver cells which enhances its DNA binding and protein stability (Sakiyama *et al.*, 2010; Guinez *et al.*, 2011; Ido-Kitamura *et al.*, 2012; Park *et al.*, 2014).

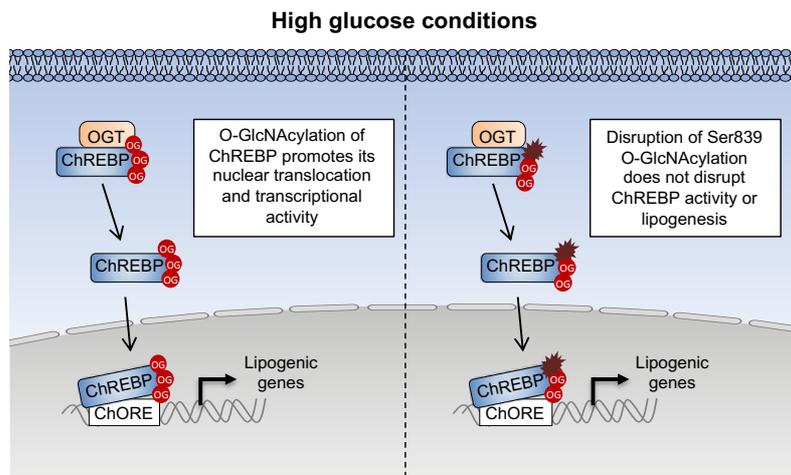
My PhD work further confirms that ChREBP directly interacts with OGT and that O-GlcNAcylation of ChREBP increases its transcriptional activity. Interestingly, when we used a catalytic mutant of OGT (OGT<sup>H498A</sup>), we observed that, while the interaction between ChREBP and OGT<sup>H498A</sup> was stronger than with OGT<sup>WT</sup>, O-GlcNAcylation levels of ChREBP were decreased as well as reporter activity of the *Lpk* promoter. These results underline the fact that the catalytic activity of OGT is required for ChREBP transcriptional activity. Further characterization of this OGT mutant could help us to discriminate the catalytic role of OGT from its role as a co-factor in transcriptional complexes. Indeed, several lines of evidence suggest that gene transcription is a major process regulated by O-GlcNAcylation (Hart, 2015; Wu, Cai and Jin, 2017). Genome-wide profiling of O-GlcNAc or OGT by chromatin immunoprecipitation (ChIP) assays found O-GlcNAcylated proteins or OGT binding at specific chromosomal sites and proteins acting at all steps of gene transcription, including histones (Leturcq, Lefebvre and Vercoutter-Edouart, 2017). We cannot exclude that the OGT<sup>H498A</sup> failed to recruit and/or limited O-GlcNAcylation of partners and/co factors of ChREBP. Indeed, a recent study identified a novel ChREBP-interacting protein regulated by O-GlcNAcylation required for the control of hepatic lipogenesis. O-GlcNAcylation of HCF-1 is a prerequisite for its binding to ChREBP and subsequent recruitment of OGT, ChREBP O-GlcNAcylation and activation. The HCF-1-ChREBP complex resides at lipogenic gene promoters, where HCF-1 regulates

H3K4 trimethylation to prime recruitment of the Jumonji C-domain-containing histone demethylase PHF2 for epigenetic activation of these promoters (Lane *et al.*, 2019). Interestingly, our team previously identified PHF2 as an important transcriptional co-activator of ChREBP in hepatocytes (Bricambert *et al.*, 2018), whether O-GlcNAcylation favors PHF2 and ChREBP interaction is not known.

Our study also reveals that, despite the identification of Ser839 as a potential major O-GlcNAcylation site for ChREBP function (Yang *et al.*, 2017), we could not unfortunately confirm that this mutation impairs ChREBP O-GlcNAcylation and/or transcriptional activity. Our results suggest that residues important for the control of ChREBP activity by O-GlcNAcylation still remain to be identified. In this context, robust methods to study O-GlcNAcylation appear essential to elucidate its key roles in the regulation of individual proteins, complex cellular processes, and/or diseases. A set of chemoenzymatic labeling methods to detect O-GlcNAcylation on proteins of interest, monitor changes in both the total levels of O-GlcNAcylation and its stoichiometry on proteins of interest, and enable mapping of O-GlcNAc to specific serine/threonine residues within proteins have been recently described (Thompson, Griffin and Hsieh-Wilson, 2018). In their study, Yang and coworkers used a cell-free coupled transcription/translation system and chemo-enzymatic and metabolic labeling in cells to validate direct modification of ChREBP by O-GlcNAcylation (Yang *et al.*, 2017). Importantly, the authors identified several ChREBP O-GlcNAcylation sites by mass spectrometry. In particular, O-GlcNAcylation of ChREBP on serine 839 (Ser839) was reported essential under high glucose concentrations for heterodimerization of ChREBP with its obligatory transcriptional partner Max like Protein X (Mlx) and for enhanced DNA-binding activity. Surprisingly, while remaking the exact same ChREBP mutant (ChREBP<sup>S839A</sup>) we could not reproduce their results and did not detect any change in ChREBP O-GlcNAcylation levels and/or transcriptional activity. While we have no clear explanation for this discrepancy, this is not the first time that O-GlcNAcylation sites identified by a group are not later validated in a follow-up study (Fardini *et al.*, 2015). This was the case for the transcription factor FoxO1 for which several O-GlcNAcylation sites were identified in the human isoform. However, directed mutagenesis of each site individually had modest or no effect on FoxO1 O-GlcNAcylation status and transcriptional activity. Moreover, mutation of the four identified O-GlcNAcylation sites did not decrease FoxO1 O-GlcNAcylation nor its transcriptional activity (Fardini *et al.*, 2015). Alternatively, it is possible that when the identified O-GlcNAcylation sites are mutated on a given protein, OGT glycosylates

other adjacent sites. Indeed, promiscuity of OGT for adjacent sites has been described in several proteins (Zhao *et al.*, 2011) and persistence of high global O-GlcNAc levels after mutagenesis of O-GlcNAcylation sites were observed in other proteins (W. H. Yang *et al.*, 2008). Altogether, this suggests that additional crucial ChREBP O-GlcNAcylation sites are yet to be identified.

Importantly, our study confirms *in vivo* and *in vitro* that the OGT-ChREBP axis is important for the regulation of lipogenic gene expression and triglyceride synthesis in the liver. We took advantage of mice globally deficient for *Chrebp* (*Chrebp*<sup>-/-</sup>) (Iroz *et al.*, 2017) to determine the specific contribution of ChREBP to the stimulatory effect of OGT on lipogenic gene expression. It would be important in this particular experiment to measure O-GlcNAcylation levels of ChREBP and LXR $\alpha$  (Anthonisen *et al.*, 2010) in response to OGT overexpression in livers of *Chrebp*<sup>+/+</sup> mice. Indeed, the nuclear receptor LXR $\alpha$  was previously shown to be O-GlcNAcylated in response to high glucose concentrations. Nevertheless, we observed that when OGT was overexpressed in liver of *Chrebp*<sup>-/-</sup> mice, the potentiating effect of OGT on lipogenic gene expression was prevented.



*In conclusion, our study confirms that the OGT-ChREBP axis acts as an important pathway to control lipogenic gene expression and fatty acid synthesis. Our study also reports that ChREBP and OGT directly interact, and that catalytic activity of OGT is required for ChREBP O-GlcNAcylation and transcriptional activity. This information may accelerate the design of biological experiments to identify functional ChREBP O-GlcNAcylation sites and the design of inhibitors of the OGT-ChREBP interaction for the prevention of hepatic steatosis onset.*

## Article 2: Hepatocyte-specific ablation of OGT leads inflammation, oxidative stress and rescued OGT expression

My second PhD objective was to address the *in vivo* role of OGT in hepatic function. The study of OGT function in *Ogt* global knock-out mice has been limited since it leads to embryonic or early postnatal lethal phenotype demonstrating that the OGT protein is essential for embryo and early life development (Shafi *et al.*, 2000). Generation of tissue/cell-specific *Ogt* knock-out mice appeared therefore crucial to elucidate the specific role of OGT in different context and locations. Although liver-specific *Ogt* knock-out mice, inducible and/or constitutive model, have been previously published (McGreal *et al.*, 2018; Zhang *et al.*, 2019), none of the studies clearly investigated the role of OGT in glucose and lipid homeostasis.

The team initially generated hepatocyte-specific *Ogt* knock-out mice (OGT<sup>Δ</sup>) to better understand the role of O-GlcNAcylation and of OGT in the regulation of liver homeostasis, specifically their roles in the regulation of enzymes involved in glucose and lipid metabolism. As mentioned, previous work from our team and other groups demonstrated that O-GlcNAcylation of two key factors of gluconeogenesis and *de novo* lipid synthesis (lipogenesis), namely FoxO1 and ChREBP, increased their transcriptional activity in liver and pancreatic  $\beta$  cells, thereby contributing to the gluco-lipototoxicity phenomenon (Guinez *et al.*, 2011; Fardini *et al.*, 2014). We first observed that *Ogt* deficient mice survived for at least one year, allowing the study of long-term effect of *Ogt* ablation in the liver of young and adult mice. Although both male and female mice were analysed during the course of the experiments, no major differences were appreciated during the first 8 weeks of life. Reproduction and breeding was also similar to wild-type mice.

Surprisingly, comparable levels of blood glucose, body weight and liver weight revealed no major metabolic alterations in terms of glucose metabolism, hypothesis further validated by similar glucose tolerance between OGT<sup>Δ</sup> and OGT<sup>F</sup> mice at 4-weeks. While mRNA expression of enzymes controlling glucose homeostasis suggested a decrease in the generation and utilization of glucose, no major impact on gene expression involved key metabolic pathways could be observed (i.e. gluconeogenesis and lipogenesis). Although the expression of FoxO1 and ChREBP was found decreased (and of some of their specific targets (*G6pase* and *Scd1*), we did not measure O-GlcNAcylation levels of ChREBP and/or FoxO1 in livers of OGT<sup>Δ</sup> mice.

Interestingly, while no clear metabolic phenotype could be observed, splenomegaly was evidenced at 4 weeks in OGT<sup>Δ</sup> compared to OGT<sup>F</sup> mice. Splenomegaly is associated to liver portal hypertension and progression to cirrhosis (Li *et al.*, 2017), giving the first clue for the potential progressive development of fibrosis in the liver of OGT<sup>Δ</sup> mice. However, HES staining demonstrated normal liver structure, without any periportal and/or significant central vein expansion nor fibrosis. Massons' trichrome staining confirmed lack of fibrosis, which correlated with normal expression of fibrotic markers. Inflammation was evidenced by inflammatory cell infiltration observed in the Ki67 staining and significant increase in mRNA expression of *Tnfa*, *Mcp1* and *Ccl5*. Follow-up analysis of OGT<sup>Δ</sup> mice demonstrated the development of hepatomegaly, abnormal liver growth, 8 weeks after birth. Hepatomegaly at 8 weeks was accompanied by extensive cirrhosis and cholestasis, features commonly seen in bile duct ligation models one week after ligation (Geerts *et al.*, 2008). Standard histological analysis with HES and Massons' trichrome staining show fibrosis, dilatation of bile canaliculi, ductular proliferation (seen in Ki67 staining) and development of hepatic nodules of regeneration. Surprisingly, these nodules of regeneration expressed the OGT protein, which explained rescued OGT expression evidenced at the level of mRNA and protein in livers of OGT<sup>Δ</sup> mice. Interestingly, western blot analysis of O-GlcNAcylation levels in whole liver lysate did not reveal comparable levels of global O-GlcNAcylated proteins in livers of OGT<sup>Δ</sup> compared to OGT<sup>F</sup> mice, and this despite full recovery of OGT protein content. This discrepancy could be explained by the fact that the inter-nodular tissue does not express significant levels of OGT.

We were puzzled at first with the rescued expression of OGT in livers of OGT<sup>Δ</sup> mice. A possible explanation for OGT rescued expression is the phenomenon of “cell competition” (Baker, 2020). The hypothesis could be explained as follow: hepatocytes deficient in OGT encounter difficulties to survive and enter the process of cell death; while, interestingly, hepatocytes escaping to the recombinant process will repopulate the liver with cells expressing OGT protein. This hypothesis was already proposed in several animal models (Caldwell *et al.*, 2010; Yamaji *et al.*, 2010; Zhang *et al.*, 2010; Grindheim *et al.*, 2019). In 2010, breast cancer xenografts in nude mice were inactivated for OGT using an shRNA strategy (Caldwell *et al.*, 2010). The authors showed a decreased in proliferation and tumor regression when OGT expression was abolished. However, some tumors were eventually able to grow associated to a re-expression of OGT (Caldwell *et al.*, 2010). Another example is the model of

hepatocyte-specific deletion of the DNA binding protein 1 (DDB1), a protein involved in DNA repair, and essential for self-renewal hepatocytes capacity (Yamaji *et al.*, 2010). It was reported that the constitutive model of DDB1 deficiency (using the Alb-Cre transgenic mice) leads to development of hepatocellular carcinoma (HCC), but surprisingly with a full rescue in DDB1 protein concentrations in hepatocytes (Yamaji *et al.*, 2010). Interestingly, the liver-specific deletion of YAP protein in mice, causes not only the re-expression of YAP in hepatocytes supposedly explained by a cell competition process but also, presents a phenotype similar to OGT<sup>Δ</sup> mice (Zhang *et al.*, 2010). Indeed, hepatocyte-specific deletion of YAP causes hepatocyte apoptosis but also a defect in bile duct development (Zhang *et al.*, 2010). Another recent mouse model that exhibited rescued expression of the protein initially targeted and similar phenotype to OGT<sup>Δ</sup> mice is the hepatocyte-specific *Ezh1/2* knock-out mouse (Grindheim *et al.*, 2019). Ablation of *Ezh1* and *Ezh2* in the liver caused premature differentiation of hepatocytes and resulted in liver fibrosis. EZH1/2 proteins maintain repressive chromatin through different mechanisms (Margueron *et al.*, 2008). At the mechanistic level, the authors demonstrated that regeneration nodules observed 2 months after birth present high amount of the repressive mark H3K27me3, a hallmark of EZH2 epigenetic activity (Grindheim *et al.*, 2019).

We also document in our study that OGT<sup>Δ</sup> mice exhibit a proliferative phenotype associated with ductular and fibrotic regions. Proliferation was confirmed with up-regulation of proliferative markers at the mRNA expression and protein levels. Correlating with the severe phenotype observed, circulating cytokines (TNF $\alpha$ , IL-2 and IL-6) were increase in OGT<sup>Δ</sup> mice at 8 weeks. Markers of liver injury (ALT, LDH and ALP) were also increased in circulation at 8 but not at 4 weeks. Microarray data show a significant amount of genes up- and down-regulated at 4 weeks involved in immune signalling and metabolism respectively. These changes suggest the necessity of liver reprogramming to cope with the inflammatory phenotype of OGT<sup>Δ</sup> mice at this stage. One of the features appreciated was the change in cell identity profile favouring the differentiation of hepatoblasts to cholangiocytes, typical of the ductular reaction produced in the initial steps of liver regeneration (Roskams *et al.*, 2004). The mentioned ductular reaction was validated by cholangiocytes staining with SOX9 and CK19 antibodies, a staining strongly increased in the inter-nodular fibrotic area of OGT<sup>Δ</sup> mouse liver sections. This phenotype could be partially explained by the decrease in *Fxr* and *Shp* expression. Indeed, *Fxr* knock-out mice exhibit increased BAs pool and show a

hyperproliferative and inflammatory phenotype (Cariello *et al.*, 2018) similar to the one we observed in OGT<sup>A</sup> mice. In this context, it would be of potential interest to treat OGT<sup>A</sup> mice with a FXR agonist, such as INT-767 or GW4064, known to reduce liver injury and cholestasis (Han, 2018), to determine whether their severe liver phenotype can be partially reversed.

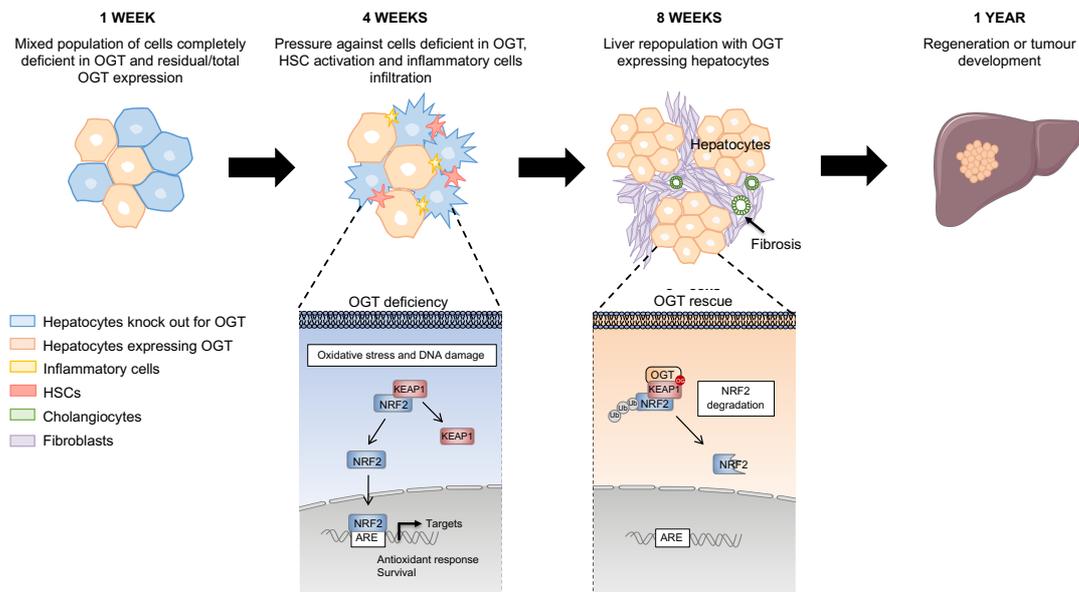
While a recent study suggests that OGT may act as a suppressor of hepatocyte necroptosis (a specific form of apoptosis) and that deletion of OGT in liver of mice may trigger liver fibrosis (Zhang *et al.*, 2019) this study did not provide a global map of the OGT targets in liver in response to different nutritional stimuli. Attempting to provide mechanistic leads to the phenotype of the OGT<sup>A</sup> mice, we examined some of gene clusters dysregulated at 4 and/or 8 weeks. We focused on a set of NRF2 target genes, found significantly increased in liver of 4 week-old OGT<sup>A</sup> mice. Western blot analysis confirmed that NRF2 protein content was elevated at 4 weeks but not at 8 weeks in liver of OGT<sup>A</sup> mice. Considering that KEAP1 regulates the cytosolic retention of NRF2 and that KEAP1 is O-GlcNAcylated, we followed this interesting lead. Indeed, rescued OGT expression at 8 weeks correlated with significant increased KEAP1 O-GlcNAcylation and decreased NRF2 protein content. Although we did not check for the nuclear localization of NRF2, we observed that several NRF2 targets measured by qPCR were found significantly increased at both 4 weeks and more modestly at 8 weeks in OGT<sup>A</sup> mice, suggesting enhanced NRF2 transcriptional activity. ARE-luciferase reporter activity in cultured hepatocytes from 4 week-old OGT<sup>A</sup> mice validated a clear increase in NRF2 activity. In addition, pathways of ER stress and oxidative stress were also induced at 4 weeks in OGT<sup>A</sup> mice as evidenced by the increase in CHOP and SOD2 expression, respectively. Further validation of oxidative stress was obtained in cultured hepatocytes from OGT<sup>A</sup> mice at 4 weeks, which presented a significant decrease in the GSH/GSSH ratio, a well-accepted marker of oxidative stress response (Ballatori *et al.*, 2009). Given the relationship between oxidative stress and DNA damage (Van Houten, Santa-Gonzalez and Camargo, 2018), we observed increased DNA damage at 4-weeks evidenced by increased phosphorylation of H2AX. Microarray heatmap also revealed significant changes in the expression of DNA repair machinery especially at 4 weeks in OGT<sup>A</sup> compared to OGT<sup>F</sup> mice. Because NRF2 was recently suggested to repress FXR expression in conditions of autophagy deficiency (Khambu *et al.*, 2019), it would be important to elucidate the levels of expression of proteins involved in liver autophagy such as *Agt7* and *Agt5*.

Application of antioxidants is an attractive strategy to prevent and cure liver diseases involving oxidative stress. Although conclusions drawn from clinical studies remain uncertain, animal studies have revealed the promising *in vivo* therapeutic effect of antioxidants on liver diseases (Branen *et al.*, 1973; Keum *et al.*, 2006; Dassarma *et al.*, 2018). Butylated hydroxyanisole (BHA) is a common antioxidant used for its antioxidant effect *via* NRF2 activation. In 1973, BHA tested in young monkeys fed with corn oil diet, showed significantly lower levels of cholesterol in plasma and liver (Branen *et al.*, 1973). In human and rat primary cultured hepatocytes, BHA was demonstrated to induce the expression of HO-1 and NQO1, two targets of NRF2 (Keum *et al.*, 2006). More recently, BHA supplementation in CCl<sub>4</sub> treated mice demonstrated a protective effect against CCl<sub>4</sub>-induced hepatotoxicity with decreased in liver injury parameters, restored levels of intracellular glutathione and induced expression of antioxidant enzymes such as SOD, Catalase or GPx (Dassarma *et al.*, 2018). The authors suggested healing of hepatic parenchymal cells and liver regeneration improved by BHA administration (Dassarma *et al.*, 2018). These data suggest that a BHA treatment could ameliorate the liver injury of OGT<sup>Δ</sup> by increasing their regenerative capacities.

Importantly, an oncogene signature was observed in liver of 8-week-old OGT<sup>Δ</sup> mice suggesting the potential development of liver cancer in older mice. These data correlated with up-regulation of *Hk2* and *Pkm2*, two rate-limiting enzymes of glycolytic pathway only expressed in adult liver in the context of liver cancer (Feng *et al.*, 2020). *G6pdh*, the rate-limiting enzyme of the pentose phosphate pathway (PPP), was also significantly upregulated in the liver of 8 week-old OGT<sup>Δ</sup> mice. Induction of G6PDH activity is highly associated to proliferation and cancer development (Yang, Stern and Chiu, 2020), which further comforts the idea of cancer development in OGT<sup>Δ</sup> mice. Considering that G6PDH and PKM2 proteins are O-GlcNAcylated (Rao *et al.*, 2015; Singh *et al.*, 2020), it would be interesting to validate whether their O-GlcNAcylation levels are increased in liver of OGT<sup>Δ</sup> compared to OGT<sup>F</sup> mice. Altogether, our observations support the hypothesis of liver cancer development in OGT<sup>Δ</sup> mice at latest life stages.

To validate this hypothesis, we followed up OGT<sup>Δ</sup> and OGT<sup>F</sup> mice until up to 1 year of life. As previously mentioned, males and females were studied and sacrificed at 1 year-old. Physiological parameters (blood glucose concentrations, body, liver and spleen weights) were comparable between the different genotypes and sexes except for

an increase in spleen weight in OGT<sup>Δ</sup> females, suggesting a residual portal hypertension. Relative expression of OGT was found similar in liver of males and females OGT<sup>Δ</sup> compared to their respective controls, validating that OGT rescue is maintained over time. Serum concentration of ALT was increased in males and females OGT<sup>Δ</sup> compared to OGT<sup>F</sup> mice suggesting a residual liver injury up to 1-year old. Yet, nor AST, LDH or ALP concentrations were different between groups, suggesting that liver injury in these mice is moderate compared to 8 week-old OGT<sup>Δ</sup> mice. Interestingly, liver tumor development was only observed in 1 year-old OGT<sup>Δ</sup> females. Liver section of OGT<sup>Δ</sup> mice show the development of a tumor structure characterized by increased proliferation, bigger hepatocytes and, in some cases, microvascular steatosis (data not shown). OGT staining revealed expression of OGT protein both in the tumor and the adjacent tissue; however, the distribution did not seem to be homogenous in all cells. This heterogenous expression could be due to recombination by *Alb-Cre* in some hepatocytes or increased expression in some cells due to reactivation of the inactivated X chromosome largely associated to development of cancer (Kaneko and Li, 2018). Proliferation staining also showed significant increase in Ki67<sup>+</sup> cells in the tumor area; however, these results did not correlate with significant increased expression levels of proliferation markers. In addition, GS staining showed that the tumor did not express increased levels of the Wnt/β-catenin pathway highly related to HCC development (Wu *et al.*, 2020) suggesting that females OGT<sup>Δ</sup> mice develop hepatocellular adenomas (HCA) rather than HCC. It would be interesting to have access to human samples to elucidate the potential implication of OGT in the progression and prognosis of HCA. Taken all together, our results suggest that females OGT<sup>Δ</sup> mice are more sensitive to develop liver cancer after OGT rescue than males. Future experiments using RNA fluorescence *in situ* hybridization (FISH) approach could help to elucidate if females have greater sensitivity to HCA development due to X chromosome reactivation.



In conclusion, our study reveals that *Ogt*-deficient mouse is an interesting tool for the study of liver fibrosis and cholestasis since  $OGT^{\Delta}$  mice develop these diseases within 8 weeks. In addition, the  $OGT^{\Delta}$  mouse is a fascinating model to better understand the mechanism of sexual dimorphism in the development of liver cancer (HCA), highly associated to females over males since it is promoted by hormonal exposure. In addition, the classification of HCA types is extremely important to understand the risk complications associated such as hemorrhage and malignant transformation into HCC (Védie *et al.*, 2018). The characterization of tumors developed in the liver of  $OGT^{\Delta}$  females could help better understand the mechanisms underlying the subtypes of HCA.

## Future work/ Perspectives

Emerging evidence reveals that OGT plays an important role in the nucleus through its involvement in several epigenetic modulator complexes, such as the mSin3A/HDAC1/OGT or Polycomb repressive complex 2 (PRC2) (Comtesse et al. 2001; Chu et al. 2014). Our hypothesis, based on my PhD results, is that the modulation of epigenetics marks could be part of the mechanism explaining the phenotype observed in OGT<sup>Δ</sup> mice. Further study of the role of OGT as an epigenetic modulator in the liver of OGT<sup>Δ</sup> mice could be performed using an OGT ChIP-seq strategy. Interestingly, performing ChIP-seq of methyl marks, such as H3K27me<sub>3</sub>, direct target of EZH2 (modified by O-GlcNAcylation), could help to correlate expression and repression of specific targets with the absence or presence of OGT.

In order to translate our findings to human disease, it would be interesting to analyse the expression of OGT in RNA-Seq data sets from human liver cancers (HCC or HCA). Altogether, by combining transcriptomic and ChIP Seq analysis, these experiments could shed light in the emerging role of OGT as an epigenetic remodeler together with its potential role in the development of hepatic tumors.

Lastly, given the severe hepatic phenotype observed in OGT<sup>Δ</sup> mice, we have developed a complementary model in which the OGT enzyme can be deleted in a time-controlled manner in adult liver. We generated these mice by crossing OGT<sup>lox/lox</sup> mice with transgenic mice expressing a tamoxifen-inducible Cre recombinase under the control of the transthyretin promoter (TTR-Cre ind) (Tannour-Louet *et al.*, 2002). To induce the deletion, tamoxifen (10 mg/ml solution) will be injected twice a day during 5 consecutive days as previously described (Morzyglod *et al.*, 2017). To better understand the role of OGT in lipid metabolism and NASH, OGT<sup>INDLiverKO</sup> mice (3 weeks after deletion) will be given a western diet (WD), which is high-fat, high-fructose and high-cholesterol, combined with low dose weekly intraperitoneal carbon tetrachloride (CCl<sub>4</sub>) recently described to serve as a tool for rapid progression of extensive fibrosis and HCC (Tsuchida *et al.*, 2018).

# References

1. Abdul-Wahed, A., Guilmeau, S. and Postic, C. (2017) ‘Sweet Sixteenth for ChREBP: Established Roles and Future Goals’, *Cell Metabolism*. Elsevier Inc., 26(2), pp. 324–341. doi: 10.1016/j.cmet.2017.07.004.
2. Adams, C. J. *et al.* (2019) ‘Structure and molecular mechanism of ER stress signaling by the unfolded protein response signal activator IRE1’, *Frontiers in Molecular Biosciences*, 6(MAR), pp. 1–12. doi: 10.3389/fmolb.2019.00011.
3. Adams, G. E., Chandru, A. and Cowley, S. M. (2018) ‘Co-repressor, co-activator and general transcription factor: the many faces of the Sin3 histone deacetylase (HDAC) complex’, *Biochemical Journal*, 475(24), pp. 3921–3932. doi: 10.1042/BCJ20170314.
4. Ahmad, T. R. and Haeusler, R. A. (2019) ‘Bile acids in glucose metabolism and insulin signalling — mechanisms and research needs’, *Nature Reviews Endocrinology*, 15(12), pp. 701–712. doi: 10.1038/s41574-019-0266-7.
5. Ahn, S. B. *et al.* (2014) ‘Expression of Liver X Receptor Correlates with Intrahepatic Inflammation and Fibrosis in Patients with Nonalcoholic Fatty Liver Disease’, *Digestive Diseases and Sciences*, 59(12), pp. 2975–2982. doi: 10.1007/s10620-014-3289-x.
6. Akinrotimi, O. *et al.* (2017) ‘Small heterodimer partner deletion prevents hepatic steatosis and when combined with farnesoid X receptor loss protects against type 2 diabetes in mice’, *Hepatology*, 66(6), pp. 1854–1865. doi: 10.1002/hep.29305.
7. Al-Mukh, H. *et al.* (2020) ‘LPS induces GFAT2 expression to promote O-GlcNAcylation and attenuate inflammation in macrophages’. doi: 10.1101/2020.03.22.002303.
8. Alejandro, E. U. *et al.* (2015) ‘Disruption of O-linked N-Acetylglucosamine Signaling Induces ER Stress and  $\beta$  Cell Failure’, *Cell Reports*, 13(11), pp. 2527–2538. doi: 10.1016/j.celrep.2015.11.020.
9. Alpini, G. *et al.* (1996) ‘Morphological, molecular, and functional heterogeneity of cholangiocytes from normal rat liver’, *Gastroenterology*, 110(5), pp. 1636–1643. doi: 10.1053/gast.1996.v110.pm8613073.
10. Alpini, G. (2002) ‘The pathobiology of biliary epithelia’, *Hepatology*, 35(5), pp. 1256–1268. doi: 10.1053/jhep.2002.33541.
11. Altarejos, J. Y. and Montminy, M. (2011) ‘CREB and the CRTC co-activators: sensors for hormonal and metabolic signals.’, *Nature reviews. Molecular cell biology*, 12(3), pp. 141–51. doi: 10.1038/nrm3072.

12. Altomonte, J. *et al.* (2003) 'Inhibition of Foxo1 function is associated with improved fasting glycemia in diabetic mice.', *American journal of physiology. Endocrinology and metabolism*, 285(4), pp. E718-28. doi: 10.1152/ajpendo.00156.2003.
13. Alves-Bezerra, M. and Cohen, D. E. (2017) 'Triglyceride Metabolism in the Liver.', *Comprehensive Physiology*, 8(1), pp. 1–8. doi: 10.1002/cphy.c170012.
14. Anderson, N. M. *et al.* (2018) 'The emerging role and targetability of the TCA cycle in cancer metabolism.', *Protein & cell*, 9(2), pp. 216–237. doi: 10.1007/s13238-017-0451-1.
15. Anthonisen, E. H. *et al.* (2010) 'Nuclear receptor liver X receptor is O-GlcNAc-modified in response to glucose', *Journal of Biological Chemistry*, 285(3), pp. 1607–1615. doi: 10.1074/jbc.M109.082685.
16. Armstrong, L. E. and Guo, G. L. (2017) 'Role of FXR in Liver Inflammation during Nonalcoholic Steatohepatitis.', *Current pharmacology reports*, 3(2), pp. 92–100. doi: 10.1007/s40495-017-0085-2.
17. Au, S. W. *et al.* (2000) 'Human glucose-6-phosphate dehydrogenase: the crystal structure reveals a structural NADP(+) molecule and provides insights into enzyme deficiency.', *Structure (London, England : 1993)*, 8(3), pp. 293–303. doi: 10.1016/s0969-2126(00)00104-0.
18. Bachman, M. *et al.* (2015) '5-Formylcytosine can be a stable DNA modification in mammals', *Nature Chemical Biology*, 11(8), pp. 555–557. doi: 10.1038/nchembio.1848.
19. Baker, N. E. (2020) 'Emerging mechanisms of cell competition', *Nature Reviews Genetics*, 21(11), pp. 683–697. doi: 10.1038/s41576-020-0262-8.
20. Baldini, S. F., Steenackers, A., *et al.* (2016) 'Glucokinase expression is regulated by glucose through O-GlcNAc glycosylation', *Biochemical and Biophysical Research Communications*, 478(2), pp. 942–948. doi: 10.1016/j.bbrc.2016.08.056.
21. Baldini, S. F., Wavelet, C., *et al.* (2016) 'The Nutrient-Dependent O-GlcNAc Modification Controls the Expression of Liver Fatty Acid Synthase', *Journal of Molecular Biology*, 428(16), pp. 3295–3304. doi: 10.1016/j.jmb.2016.04.035.
22. Ballatori, N. *et al.* (2009) 'Glutathione dysregulation and the etiology and progression of human diseases.', *Biological chemistry*, 390(3), pp. 191–214. doi: 10.1515/BC.2009.033.
23. Banales, J. M. *et al.* (2019) 'Cholangiocyte pathobiology', *Nature Reviews*

- Gastroenterology and Hepatology*, 16(5), pp. 269–281. doi: 10.1038/s41575-019-0125-y.
24. Barkovskaya, A. *et al.* (2019) ‘O-GlcNAc Transferase Inhibition Differentially Affects Breast Cancer Subtypes’, *Scientific Reports*, 9(1), pp. 1–11. doi: 10.1038/s41598-019-42153-6.
  25. Bauer, C. *et al.* (2015) ‘Phosphorylation of TET Proteins Is Regulated via O -GlcNAcylation by the O -Linked N -Acetylglucosamine Transferase (OGT)’, *Journal of Biological Chemistry*, 290(8), pp. 4801–4812. doi: 10.1074/jbc.M114.605881.
  26. Baumann, D. *et al.* (2020) ‘Role of nutrient-driven O-GlcNAc-post-translational modification in pancreatic exocrine and endocrine islet development’, *Development*, 147(7), p. dev186643. doi: 10.1242/dev.186643.
  27. Benhamed, F. *et al.* (2012) ‘The lipogenic transcription factor ChREBP dissociates hepatic steatosis from insulin resistance in mice and humans’, *Journal of Clinical Investigation*. doi: 10.1172/JCI41636.
  28. Berrabah, W. *et al.* (2014) ‘Glucose sensing O-GlcNAcylation pathway regulates the nuclear bile acid receptor farnesoid X receptor (FXR)’, *Hepatology*, 59(5), pp. 2022–2033. doi: 10.1002/hep.26710.
  29. Berthier, A. *et al.* (2018) ‘Combinatorial regulation of hepatic cytoplasmic signaling and nuclear transcriptional events by the OGT/REV-ERB $\alpha$  complex’, *Proceedings of the National Academy of Sciences of the United States of America*, 115(47), pp. E11033–E11042. doi: 10.1073/pnas.1805397115.
  30. Besse-Patin, A. *et al.* (2017) ‘Estrogen Signals Through Peroxisome Proliferator-Activated Receptor- $\gamma$  Coactivator 1 $\alpha$  to Reduce Oxidative Damage Associated With Diet-Induced Fatty Liver Disease.’, *Gastroenterology*, 152(1), pp. 243–256. doi: 10.1053/j.gastro.2016.09.017.
  31. Bian, E.-B. *et al.* (2012) ‘DNMT1-mediated PTEN hypermethylation confers hepatic stellate cell activation and liver fibrogenesis in rats’, *Toxicology and Applied Pharmacology*, 264(1), pp. 13–22. doi: 10.1016/j.taap.2012.06.022.
  32. Bindesbøll, C. *et al.* (2015) ‘Liver X receptor regulates hepatic nuclear O-GlcNAc signaling and carbohydrate responsive element-binding protein activity’, *Journal of Lipid Research*, 56(4), pp. 771–785. doi: 10.1194/jlr.M049130.
  33. Birnbaum, M., Haspel, H. and Rosen, O. (1987) ‘Transformation of rat fibroblasts by FSV rapidly increases glucose transporter gene transcription’, *Science*, 235(4795), pp. 1495–1498. doi: 10.1126/science.3029870.

34. Blackledge, N. P., Rose, N. R. and Klose, R. J. (2015) 'Targeting Polycomb systems to regulate gene expression: modifications to a complex story', *Nature Reviews Molecular Cell Biology*, 16(11), pp. 643–649. doi: 10.1038/nrm4067.
35. Bond, M. R. and Hanover, J. A. (2015) 'A little sugar goes a long way: The cell biology of O-GlcNAc', *Journal of Cell Biology*, 208(7), pp. 869–880. doi: 10.1083/jcb.201501101.
36. Bonnet, J., Devys, D. and Tora, L. (2014) 'Histone H2B ubiquitination: signaling not scrapping', *Drug Discovery Today: Technologies*, 12, pp. e19–e27. doi: 10.1016/j.ddtec.2012.09.002.
37. Branen, A. L. *et al.* (1973) 'Lipid and enzyme changes in the blood and liver of monkeys given butylated hydroxytoluene and butylated hydroxyanisole', *Food and Cosmetics Toxicology*, 11(4), pp. 797–806. doi: 10.1016/S0015-6264(73)80363-3.
38. Bricambert, J. *et al.* (2018) 'The histone demethylase Phf2 acts as a molecular checkpoint to prevent NAFLD progression during obesity', *Nature Communications*. Springer US, 9(1). doi: 10.1038/s41467-018-04361-y.
39. Brown, K. S. *et al.* (1997) 'Glucokinase regulatory protein may interact with glucokinase in the hepatocyte nucleus.', *Diabetes*, 46(2), pp. 179–86. doi: 10.2337/diab.46.2.179.
40. Bugge, A. *et al.* (2012) 'Rev-erb and Rev-erb coordinately protect the circadian clock and normal metabolic function', *Genes & Development*, 26(7), pp. 657–667. doi: 10.1101/gad.186858.112.
41. Bullen, J. W. *et al.* (2014) 'Cross-talk between two essential nutrient-sensitive enzymes O-GlcNAc Transferase (OGT) and Amp-Activated Protein Kinase (AMPK)', *Journal of Biological Chemistry*, 289(15), pp. 10592–10606. doi: 10.1074/jbc.M113.523068.
42. Butkinaree, C. *et al.* (2008) 'Characterization of  $\beta$ -N-acetylglucosaminidase cleavage by caspase-3 during apoptosis', *Journal of Biological Chemistry*, 283(35), pp. 23557–23566. doi: 10.1074/jbc.M804116200.
43. Caldwell, S. A. *et al.* (2010) 'Nutrient sensor O-GlcNAc transferase regulates breast cancer tumorigenesis through targeting of the oncogenic transcription factor FoxM1', *Oncogene*. Nature Publishing Group, 29(19), pp. 2831–2842. doi: 10.1038/onc.2010.41.
44. Calfon, M. *et al.* (2002) 'IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA', *Nature*, 415(6867), pp. 92–96. doi:

10.1038/415092a.

45. Campbell, N. A. and Reece, J. B. (2005) *Biology*. Pearson, Benjamin Cummings (Biology). Available at: <https://books.google.fr/books?id=0jbm8J7VW0AC>.
46. Cao, B. *et al.* (2019) 'O-GlcNAc transferase activates stem-like cell potential in hepatocarcinoma through O-GlcNAcylation of eukaryotic initiation factor 4E', *Journal of Cellular and Molecular Medicine*, 23(4), pp. 2384–2398. doi: 10.1111/jcmm.14043.
47. Cao, L. *et al.* (2014) 'Correlation of low expression of hMOF with clinicopathological features of colorectal carcinoma, gastric cancer and renal cell carcinoma.', *International journal of oncology*, 44(4), pp. 1207–14. doi: 10.3892/ijo.2014.2266.
48. Cao, W. *et al.* (2013) 'Discovery and Confirmation of O-GlcNAcylated Proteins in Rat Liver Mitochondria by Combination of Mass Spectrometry and Immunological Methods', *PLoS ONE*, 8(10). doi: 10.1371/journal.pone.0076399.
49. Capelli, D. *et al.* (2016) 'Structural basis for PPAR partial or full activation revealed by a novel ligand binding mode', *Scientific Reports*, 6(1), p. 34792. doi: 10.1038/srep34792.
50. Cariello, M. *et al.* (2018) 'Nuclear receptor FXR, bile acids and liver damage: Introducing the progressive familial intrahepatic cholestasis with FXR mutations', *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1864(4), pp. 1308–1318. doi: 10.1016/j.bbadis.2017.09.019.
51. Caron, S. *et al.* (2013) 'Farnesoid X receptor inhibits the transcriptional activity of carbohydrate response element binding protein in human hepatocytes.', *Molecular and cellular biology*, 33(11), pp. 2202–11. doi: 10.1128/MCB.01004-12.
52. Cha, J. Y. and Repa, J. J. (2007) 'The Liver X Receptor (LXR) and hepatic lipogenesis: The carbohydrate-response element-binding protein is a target gene of LXR', *Journal of Biological Chemistry*, 282(1), pp. 743–751. doi: 10.1074/jbc.M605023200.
53. Chaiyawat, P. *et al.* (2015) 'Alteration of O-GlcNAcylation affects serine phosphorylation and regulates gene expression and activity of pyruvate kinase M2 in colorectal cancer cells.', *Oncology reports*, 34(4), pp. 1933–42. doi: 10.3892/or.2015.4178.
54. Champattanachai, V. *et al.* (2013) 'Proteomic analysis and abrogated expression of O -GlcNAcylated proteins associated with primary breast cancer', *PROTEOMICS*, 13(14), pp. 2088–2099. doi: 10.1002/pmic.201200126.

55. Chen, H., Liu, H. and Qing, G. (2018) 'Targeting oncogenic Myc as a strategy for cancer treatment', *Signal Transduction and Targeted Therapy*, 3(1), p. 5. doi: 10.1038/s41392-018-0008-7.
56. Chen, P.-H. *et al.* (2017) 'Glycosylation of KEAP1 links nutrient sensing to redox stress signaling.', *The EMBO journal*, 36(15), pp. 2233–2250. doi: 10.15252/emj.201696113.
57. Chen, P. H., Chi, J. T. and Boyce, M. (2017) 'KEAP1 has a sweet spot: A new connection between intracellular glycosylation and redox stress signaling in cancer cells', *Molecular and Cellular Oncology*. Taylor & Francis, 4(6), pp. 1–2. doi: 10.1080/23723556.2017.1361501.
58. Chen, Q. *et al.* (2013) 'TET2 promotes histone O-GlcNAcylation during gene transcription', *Nature*, 493(7433), pp. 561–564. doi: 10.1038/nature11742.
59. Chen, Q. and Yu, X. (2016) 'OGT restrains the expansion of DNA damage signaling', *Nucleic Acids Research*, 44(19), pp. 9266–9278. doi: 10.1093/nar/gkw663.
60. Chen, S. *et al.* (2018) 'EZH2 promotes hepatocellular carcinoma progression through modulating miR-22/galectin-9 axis.', *Journal of experimental & clinical cancer research : CR*, 37(1), p. 3. doi: 10.1186/s13046-017-0670-6.
61. Chen, W. *et al.* (2007) 'Enzymatic Reduction of Oxysterols Impairs LXR Signaling in Cultured Cells and the Livers of Mice', *Cell Metabolism*, 5(1), pp. 73–79. doi: 10.1016/j.cmet.2006.11.012.
62. Cheung, W. D. and Hart, G. W. (2008) 'AMP-activated protein kinase and p38 MAPK activate O-GlcNAcylation of neuronal proteins during glucose deprivation.', *The Journal of biological chemistry*, 283(19), pp. 13009–20. doi: 10.1074/jbc.M801222200.
63. Choi, Y.-K. *et al.* (2013) 'Characterization of Cells Expressing Lymphatic Marker LYVE-1 in Macaque Large Intestine during Simian Immunodeficiency Virus Infection Identifies A Large Population of Nonvascular LYVE-1 + /DC-SIGN + Cells', *Lymphatic Research and Biology*, 11(1), pp. 26–34. doi: 10.1089/lrb.2012.0019.
64. Christofk, H. R. *et al.* (2008) 'The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth', *Nature*, 452(7184), pp. 230–233. doi: 10.1038/nature06734.
65. Chu, C. S. *et al.* (2014) 'O-GlcNAcylation regulates EZH2 protein stability and function', *Proceedings of the National Academy of Sciences of the United States*

- of America*, 111(4), pp. 1355–1360. doi: 10.1073/pnas.1323226111.
66. Cimmino, L. *et al.* (2017) ‘Restoration of TET2 Function Blocks Aberrant Self-Renewal and Leukemia Progression’, *Cell*, 170(6), pp. 1079–1095.e20. doi: 10.1016/j.cell.2017.07.032.
  67. Clark, P. M. *et al.* (2008) ‘Direct in-gel fluorescence detection and cellular imaging of O-GlcNAc-modified proteins’, *Journal of the American Chemical Society*, 130(35), pp. 11576–11577. doi: 10.1021/ja8030467.
  68. Cohen, J. C., Horton, J. D. and Hobbs, H. H. (2011) ‘Human Fatty Liver Disease: Old Questions and New Insights’, *Science*, 332(6037), pp. 1519–1523. doi: 10.1126/science.1204265.
  69. Colletti, L. M. *et al.* (2000) ‘The ratio of ELR+ to ELR- CXC chemokines affects the lung and liver injury following hepatic ischemia/reperfusion in the rat’, *Hepatology*, 31(2), pp. 435–445. doi: 10.1002/hep.510310225.
  70. Comtesse, N., Maldener, E. and Meese, E. (2001) ‘Identification of a nuclear variant of MGEA5, a cytoplasmic hyaluronidase and a  $\beta$ -A-acetylglucosaminidase’, *Biochemical and Biophysical Research Communications*, 283(3), pp. 634–640. doi: 10.1006/bbrc.2001.4815.
  71. Cork, G. K., Thompson, J. and Slawson, C. (2018) ‘Real talk: The inter-play between the mTOR, AMPK, and hexosamine biosynthetic pathways in cell signaling’, *Frontiers in Endocrinology*, 9(SEP), pp. 1–9. doi: 10.3389/fendo.2018.00522.
  72. Cosentino, C., Grieco, D. and Costanzo, V. (2011) ‘ATM activates the pentose phosphate pathway promoting anti-oxidant defence and DNA repair.’, *The EMBO journal*, 30(3), pp. 546–55. doi: 10.1038/emboj.2010.330.
  73. Cox, J. S., Shamu, C. E. and Walter, P. (1993) ‘Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase’, *Cell*, 73(6), pp. 1197–1206. doi: 10.1016/0092-8674(93)90648-A.
  74. Dassarma, B. *et al.* (2018) ‘Hepatoprotective effect of food preservatives (butylated hydroxyanisole, butylated hydroxytoluene) on carbon tetrachloride-induced hepatotoxicity in rat.’, *Toxicology reports*, 5, pp. 31–37. doi: 10.1016/j.toxrep.2017.12.009.
  75. Davies, L. C. *et al.* (2013) ‘Tissue-resident macrophages.’, *Nature immunology*, 14(10), pp. 986–95. doi: 10.1038/ni.2705.
  76. Davis, J. G. (1978) ‘The case for fluoridised milk for children.’, *The Journal of the Dental Association of South Africa = Die Tydskrif van die Tandheekkundige*

- Vereniging van Suid-Afrika*, 33(10), pp. 556–7. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/295310>.
77. Decourcelle, A. *et al.* (2020) ‘Evidence of a compensatory regulation of colonic O-GlcNAc transferase and O-GlcNAcase expression in response to disruption of O-GlcNAc homeostasis’, *Biochemical and Biophysical Research Communications*, 521(1), pp. 125–130. doi: 10.1016/j.bbrc.2019.10.090.
  78. Decourcelle, A., Leprince, D. and Dehennaut, V. (2019) ‘Regulation of Polycomb Repression by O-GlcNAcylation: Linking Nutrition to Epigenetic Reprogramming in Embryonic Development and Cancer.’, *Frontiers in endocrinology*, 10, p. 117. doi: 10.3389/fendo.2019.00117.
  79. Delatte, B. and Fuks, F. (2013) ‘TET proteins: on the frenetic hunt for new cytosine modifications’, *Briefings in Functional Genomics*, 12(3), pp. 191–204. doi: 10.1093/bfpg/elt010.
  80. Deng, X. *et al.* (2012) ‘FoxO1 Inhibits Sterol Regulatory Element-binding Protein-1c (SREBP-1c) Gene Expression via Transcription Factors Sp1 and SREBP-1c’, *Journal of Biological Chemistry*, 287(24), pp. 20132–20143. doi: 10.1074/jbc.M112.347211.
  81. DeNicola, G. M. *et al.* (2011) ‘Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis.’, *Nature*, 475(7354), pp. 106–9. doi: 10.1038/nature10189.
  82. Dentin, R. *et al.* (2006) ‘Liver-specific inhibition of ChREBP improves hepatic steatosis and insulin resistance in ob/ob mice’, *Diabetes*, 55(8), pp. 2159–2170. doi: 10.2337/db06-0200.
  83. Dentin, R. *et al.* (2008) ‘Hepatic glucose sensing via the CREB coactivator CRT2’, *Science*, 319(5868), pp. 1402–1405. doi: 10.1126/science.1151363.
  84. Dentin, R. *et al.* (2012) ‘Glucose 6-phosphate, rather than xylulose 5-phosphate, is required for the activation of ChREBP in response to glucose in the liver’, *Journal of Hepatology*, 56(1), pp. 199–209. doi: 10.1016/j.jhep.2011.07.019.
  85. Deplus, R. *et al.* (2013) ‘TET2 and TET3 regulate GlcNAcylation and H3K4 methylation through OGT and SET1/COMPASS’, *EMBO Journal*, 32(5), pp. 645–655. doi: 10.1038/emboj.2012.357.
  86. Dewidar, B. *et al.* (2019) ‘TGF- $\beta$  in Hepatic Stellate Cell Activation and Liver Fibrogenesis-Updated 2019.’, *Cells*, 8(11). doi: 10.3390/cells8111419.
  87. Dibner, C., Schibler, U. and Albrecht, U. (2010) ‘The Mammalian Circadian Timing System: Organization and Coordination of Central and Peripheral

- Clocks', *Annual Review of Physiology*, 72(1), pp. 517–549. doi: 10.1146/annurev-physiol-021909-135821.
88. Diehl, J. A. *et al.* (1998) 'Glycogen synthase kinase-3 $\beta$  regulates cyclin D1 proteolysis and subcellular localization.', *Genes & development*, 12(22), pp. 3499–511. doi: 10.1101/gad.12.22.3499.
  89. Ding, C. *et al.* (2016) 'A cell-type-resolved liver proteome', *Molecular and Cellular Proteomics*, 15(10), pp. 3190–3202. doi: 10.1074/mcp.M116.060145.
  90. Ding, X. *et al.* (2015) 'Mixed Lineage Leukemia 5 (MLL5) Protein Stability Is Cooperatively Regulated by O-GlcNac Transferase (OGT) and Ubiquitin Specific Protease 7 (USP7)', *PLOS ONE*. Edited by M. S.-Y. Huen, 10(12), p. e0145023. doi: 10.1371/journal.pone.0145023.
  91. Dixon, L. J. *et al.* (2013) 'Kupffer Cells in the Liver', in *Comprehensive Physiology*. Hoboken, NJ, USA: John Wiley & Sons, Inc. doi: 10.1002/cphy.c120026.
  92. Dong, X. C. (2017) 'FOXO transcription factors in non-alcoholic fatty liver disease.', *Liver research*, 1(3), pp. 168–173. doi: 10.1016/j.livres.2017.11.004.
  93. Dorotea, D., Koya, D. and Ha, H. (2020) 'Recent Insights Into SREBP as a Direct Mediator of Kidney Fibrosis via Lipid-Independent Pathways', *Frontiers in Pharmacology*, 11. doi: 10.3389/fphar.2020.00265.
  94. Elsen, N. L. *et al.* (2017) 'Insights into activity and inhibition from the crystal structure of human O-GlcNAcase', *Nature Chemical Biology*. Nature Publishing Group, 13(6), pp. 613–615. doi: 10.1038/nchembio.2357.
  95. Estall, J. L. *et al.* (2009) 'Sensitivity of lipid metabolism and insulin signaling to genetic alterations in hepatic peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$  expression.', *Diabetes*, 58(7), pp. 1499–508. doi: 10.2337/db08-1571.
  96. Fang, R. *et al.* (2010) 'Human LSD2/KDM1b/AOF1 Regulates Gene Transcription by Modulating Intragenic H3K4me2 Methylation', *Molecular Cell*, 39(2), pp. 222–233. doi: 10.1016/j.molcel.2010.07.008.
  97. Fang, S. *et al.* (2015) 'Intestinal FXR agonism promotes adipose tissue browning and reduces obesity and insulin resistance', *Nature Medicine*, 21(2), pp. 159–165. doi: 10.1038/nm.3760.
  98. Fardini, Y. *et al.* (2014) 'O-GlcNAcylation of FoxO1 in pancreatic  $\beta$  cells promotes Akt inhibition through an IGFBP1-mediated autocrine mechanism', *FASEB Journal*, 28(2), pp. 1010–1021. doi: 10.1096/fj.13-238378.

99. Fardini, Y. *et al.* (2015) 'Regulatory O-GlcNAcylation sites on FoxO1 are yet to be identified.', *Biochemical and biophysical research communications*, 462(2), pp. 151–8. doi: 10.1016/j.bbrc.2015.04.114.
100. Feng, J. *et al.* (2020) 'Emerging roles and the regulation of aerobic glycolysis in hepatocellular carcinoma', *Journal of Experimental & Clinical Cancer Research*, 39(1), p. 126. doi: 10.1186/s13046-020-01629-4.
101. Ferrer, C. M. *et al.* (2014) 'O-GlcNAcylation Regulates Cancer Metabolism and Survival Stress Signaling via Regulation of the HIF-1 Pathway', *Molecular Cell*, 54(5), pp. 820–831. doi: 10.1016/j.molcel.2014.04.026.
102. Filhoulaud, G. *et al.* (2019) 'O-GlcNAcylation links TXNIP to inflammasome activation in pancreatic  $\beta$  cells', *Frontiers in Endocrinology*, 10(MAY), pp. 1–11. doi: 10.3389/fendo.2019.00291.
103. *FirstGlance in Jmol: a simple tool for macromolecular visualization, available on-line at firstglance.jmol.org v. 2.5.1 (free software, Creative Commons License)* (2006). Available at: <http://firstglance.jmol.org>.
104. Fong, J. J. *et al.* (2012) ' $\beta$ -N-acetylglucosamine (O-GlcNAc) is a novel regulator of mitosis-specific phosphorylations on histone H3', *Journal of Biological Chemistry*, 287(15), pp. 12195–12203. doi: 10.1074/jbc.M111.315804.
105. Foretz, M. *et al.* (1999) 'Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes', *Proceedings of the National Academy of Sciences*, 96(22), pp. 12737–12742. doi: 10.1073/pnas.96.22.12737.
106. Foretz, M., Even, P. C. and Viollet, B. (2018) 'AMPK Activation Reduces Hepatic Lipid Content by Increasing Fat Oxidation In Vivo.', *International journal of molecular sciences*, 19(9). doi: 10.3390/ijms19092826.
107. Franchitto, A. *et al.* (2013) 'Recent advances on the mechanisms regulating cholangiocyte proliferation and the significance of the neuroendocrine regulation of cholangiocyte pathophysiology.', *Annals of translational medicine*, 1(3), p. 27. doi: 10.3978/j.issn.2305-5839.2012.10.03.
108. Friedman, S. L. (2008a) 'Hepatic Stellate Cells: Protean, Multifunctional, and Enigmatic Cells of the Liver', *Physiological Reviews*, 88(1), pp. 125–172. doi: 10.1152/physrev.00013.2007.
109. Friedman, S. L. (2008b) 'Mechanisms of Hepatic Fibrogenesis', *Gastroenterology*, 134(6), pp. 1655–1669. doi: 10.1053/j.gastro.2008.03.003.
110. Fujiki, R. *et al.* (2011) 'GlcNAcylation of histone H2B facilitates its

- monoubiquitination', *Nature*, 480(7378), pp. 557–560. doi: 10.1038/nature10656.
111. Gao, J. *et al.* (2018) 'Proteomic analysis of the OGT interactome: Novel links to epithelial-mesenchymal transition and metastasis of cervical cancer', *Carcinogenesis*, 39(10), pp. 1222–1234. doi: 10.1093/carcin/bgy097.
  112. Gao, Y. *et al.* (2001) 'Dynamic O-glycosylation of nuclear and cytosolic proteins: Cloning and characterization of a neutral, cytosolic  $\beta$ -N-acetylglucosaminidase from human brain', *Journal of Biological Chemistry*, 276(13), pp. 9838–9845. doi: 10.1074/jbc.M010420200.
  113. Gaudio, E. *et al.* (2006) 'Vascular Endothelial Growth Factor Stimulates Rat Cholangiocyte Proliferation Via an Autocrine Mechanism', *Gastroenterology*, 130(4), pp. 1270–1282. doi: 10.1053/j.gastro.2005.12.034.
  114. Geerts, A. M. *et al.* (2008) 'Comparison of three research models of portal hypertension in mice: macroscopic, histological and portal pressure evaluation', *International Journal of Experimental Pathology*, 89(4), pp. 251–263. doi: 10.1111/j.1365-2613.2008.00597.x.
  115. Gekakis, N. (1998) 'Role of the CLOCK Protein in the Mammalian Circadian Mechanism', *Science*, 280(5369), pp. 1564–1569. doi: 10.1126/science.280.5369.1564.
  116. Géraud, C. *et al.* (2012) 'Unique Cell Type-Specific Junctional Complexes in Vascular Endothelium of Human and Rat Liver Sinusoids', *PLoS ONE*. Edited by A. Luttun, 7(4), p. e34206. doi: 10.1371/journal.pone.0034206.
  117. Gilbert, S. L., Pehrson, J. R. and Sharp, P. A. (2000) 'XIST RNA associates with specific regions of the inactive X chromatin.', *The Journal of biological chemistry*, 275(47), pp. 36491–4. doi: 10.1074/jbc.C000409200.
  118. Glaser, S. *et al.* (2010) 'Knockout of secretin receptor reduces large cholangiocyte hyperplasia in mice with extrahepatic cholestasis induced by bile duct ligation', *Hepatology*, 52(1), pp. 204–214. doi: 10.1002/hep.23657.
  119. Gonzalez, G. A. and Montminy, M. R. (1989) 'Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133', *Cell*, 59(4), pp. 675–680. doi: 10.1016/0092-8674(89)90013-5.
  120. Gorelik, A. *et al.* (2019) 'Genetic recoding to dissect the roles of site-specific protein O-GlcNAcylation', *Nature Structural and Molecular Biology*, 26(11), pp. 1071–1077. doi: 10.1038/s41594-019-0325-8.
  121. Grindheim, J. M. *et al.* (2019) 'Polycomb Repressive Complex 2 Proteins EZH1 and EZH2 Regulate Timing of Postnatal Hepatocyte Maturation and Fibrosis by

- Repressing Genes With Euchromatic Promoters in Mice.’, *Gastroenterology*, 156(6), pp. 1834–1848. doi: 10.1053/j.gastro.2019.01.041.
122. Groves, J. A. *et al.* (2017) ‘Fatty acid synthase inhibits the O-GlcNAcase during oxidative stress.’, *The Journal of biological chemistry*, 292(16), pp. 6493–6511. doi: 10.1074/jbc.M116.760785.
  123. Guibert, S. and Weber, M. (2013) ‘Functions of DNA Methylation and Hydroxymethylation in Mammalian Development’, in, pp. 47–83. doi: 10.1016/B978-0-12-416027-9.00002-4.
  124. Guinez, C. *et al.* (2011) ‘O-GlcNAcylation increases ChREBP protein content and transcriptional activity in the liver’, *Diabetes*, 60(5), pp. 1399–1413. doi: 10.2337/db10-0452.
  125. Guo, J. U. *et al.* (2011) ‘Hydroxylation of 5-Methylcytosine by TET1 Promotes Active DNA Demethylation in the Adult Brain’, *Cell*, 145(3), pp. 423–434. doi: 10.1016/j.cell.2011.03.022.
  126. Guo, Y. *et al.* (2020) ‘ERK/MAPK signalling pathway and tumorigenesis (Review)’, *Experimental and Therapeutic Medicine*. doi: 10.3892/etm.2020.8454.
  127. Van Haele, M., Snoeck, J. and Roskams, T. (2019) ‘Human Liver Regeneration: An Etiology Dependent Process’, *International Journal of Molecular Sciences*, 20(9), p. 2332. doi: 10.3390/ijms20092332.
  128. Han, C. Y. (2018) ‘Update on FXR Biology: Promising Therapeutic Target?’, *International journal of molecular sciences*, 19(7). doi: 10.3390/ijms19072069.
  129. Han, H.-S. *et al.* (2016) ‘Regulation of glucose metabolism from a liver-centric perspective’, *Experimental & Molecular Medicine*, 48(3), pp. e218–e218. doi: 10.1038/emm.2015.122.
  130. Han, Y. *et al.* (2013) ‘Recent advances in the morphological and functional heterogeneity of the biliary epithelium’, *Experimental Biology and Medicine*, 238(5), pp. 549–565. doi: 10.1177/1535370213489926.
  131. Hanover, J. A. *et al.* (2003) ‘Mitochondrial and nucleocytoplasmic isoforms of O-linked GlcNAc transferase encoded by a single mammalian gene’, *Archives of Biochemistry and Biophysics*, 409(2), pp. 287–297. doi: 10.1016/S0003-9861(02)00578-7.
  132. Harding, H. P., Zhang, Y. and Ron, D. (1999) ‘Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase’, *Nature*, 397(6716), pp. 271–274. doi: 10.1038/16729.
  133. Hart, G. W. (2015) ‘Nutrient regulation of transcription and signalling by O-

- GlcNAcylation', *Perspectives in Science*, 6, pp. 49–57. doi: 10.1016/j.pisc.2015.06.002.
134. Hart, G. W. (2019) 'Nutrient regulation of signaling and transcription', *Journal of Biological Chemistry*, 294(7), pp. 2211–2231. doi: 10.1074/jbc.AW119.003226.
135. Haze, K. *et al.* (1999) 'Mammalian Transcription Factor ATF6 Is Synthesized as a Transmembrane Protein and Activated by Proteolysis in Response to Endoplasmic Reticulum Stress', *Molecular Biology of the Cell*. Edited by P. Silver, 10(11), pp. 3787–3799. doi: 10.1091/mbc.10.11.3787.
136. Heckel, D. *et al.* (1998) 'Novel immunogenic antigen homologous to hyaluronidase in meningioma', *Human Molecular Genetics*, 7(12), pp. 1859–1872. doi: 10.1093/hmg/7.12.1859.
137. Hellerbrand, C. *et al.* (1999) 'The role of TGFbeta1 in initiating hepatic stellate cell activation in vivo.', *Journal of hepatology*, 30(1), pp. 77–87. doi: 10.1016/s0168-8278(99)80010-5.
138. Herman, M. A. *et al.* (2012) 'A novel ChREBP isoform in adipose tissue regulates systemic glucose metabolism', *Nature*, 484(7394), pp. 333–338. doi: 10.1038/nature10986.
139. Herman, M. a *et al.* (2012) 'A novel ChREBP isoform in adipose tissue regulates systemic glucose metabolism', *Nature*, 484(7394), pp. 333–338. doi: 10.1038/nature10986.
140. Herzig, S. *et al.* (2001) 'CREB regulates hepatic gluconeogenesis through the coactivator PGC-1', *Nature*, 413(6852), pp. 179–183. doi: 10.1038/35093131.
141. Hilf, R. *et al.* (1975) 'Multiple molecular forms of glucose-6-phosphate dehydrogenase in normal, preneoplastic, and neoplastic mammary tissues of mice.', *Cancer research*, 35(8), pp. 2109–16. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/238737>.
142. Hirosawa, M. *et al.* (2016) 'Novel O-GlcNAcylation on ser 40 of canonical H2A isoforms specific to viviparity', *Scientific Reports*, 6, pp. 1–11. doi: 10.1038/srep31785.
143. Hofmann, A. F. (2009) 'The enterohepatic circulation of bile acids in mammals: form and functions.', *Frontiers in bioscience (Landmark edition)*, 14, pp. 2584–98. doi: 10.2741/3399.
144. Holt, G. D. and Hart, G. W. (1986) 'The subcellular distribution of terminal N-acetylglucosamine moieties. Localization of a novel protein-saccharidic linkage, O-linked GlcNAc', *Journal of Biological Chemistry*, 261(17), pp. 8049–8057.

145. Hornbeck, P. *et al.* (2015) 'PhosphoSitePlus, 2014: mutations, PTMs and recalibrations.', *Nucleic Acids Res.*, pp. 43:D512–20.
146. Horton, J. D., Goldstein, J. L. and Brown, M. S. (2002) 'SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver', *Journal of Clinical Investigation*, 109(9), pp. 1125–1131. doi: 10.1172/JCI15593.
147. Hou, W. and Syn, W.-K. (2018) 'Role of Metabolism in Hepatic Stellate Cell Activation and Fibrogenesis', *Frontiers in Cell and Developmental Biology*, 6. doi: 10.3389/fcell.2018.00150.
148. Housley, M. P. *et al.* (2008) 'O-GlcNAc regulates FoxO activation in response to glucose.', *The Journal of biological chemistry*, 283(24), pp. 16283–92. doi: 10.1074/jbc.M802240200.
149. Housley, M. P. *et al.* (2009) 'A PGC-1 $\alpha$ - O -GlcNAc Transferase Complex Regulates FoxO Transcription Factor Activity in Response to Glucose', *Journal of Biological Chemistry*, 284(8), pp. 5148–5157. doi: 10.1074/jbc.M808890200.
150. Van Houten, B., Santa-Gonzalez, G. A. and Camargo, M. (2018) 'DNA repair after oxidative stress: current challenges.', *Current opinion in toxicology*, 7, pp. 9–16. doi: 10.1016/j.cotox.2017.10.009.
151. Hrit, J. *et al.* (2018) 'OGT binds a conserved C-terminal domain of TET1 to regulate TET1 activity and function in development', *eLife*, 7. doi: 10.7554/eLife.34870.
152. Hu, G. *et al.* (2019) 'FOXM1 promotes hepatocellular carcinoma progression by regulating KIF4A expression', *Journal of Experimental & Clinical Cancer Research*, 38(1), p. 188. doi: 10.1186/s13046-019-1202-3.
153. Huang, H. *et al.* (2005) 'Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation.', *Proceedings of the National Academy of Sciences of the United States of America*, 102(5), pp. 1649–54. doi: 10.1073/pnas.0406789102.
154. Ida, S. *et al.* (2017) 'Diverse metabolic effects of O-GlcNAcylation in the pancreas but limited effects in insulin-sensitive organs in mice', *Diabetologia*, 60(9), pp. 1761–1769. doi: 10.1007/s00125-017-4327-y.
155. Ido-Kitamura, Y. *et al.* (2012) 'Hepatic FoxO1 Integrates Glucose Utilization and Lipid Synthesis through Regulation of Chrebp O-Glycosylation', *PLoS ONE*, 7(10). doi: 10.1371/journal.pone.0047231.
156. Inoue, D. *et al.* (2018) 'A novel ASXL1–OGT axis plays roles in H3K4 methylation and tumor suppression in myeloid malignancies', *Leukemia*, 32(6),

- pp. 1327–1337. doi: 10.1038/s41375-018-0083-3.
157. Inoue, Y. *et al.* (2018) ‘Elevated O-GlcNAcylation stabilizes FOXM1 by its reduced degradation through GSK-3 $\beta$  inactivation in a human gastric carcinoma cell line, MKN45 cells’, *Biochemical and Biophysical Research Communications*, 495(2), pp. 1681–1687. doi: 10.1016/j.bbrc.2017.11.179.
  158. Iroz, A. *et al.* (2017) ‘A Specific ChREBP and PPAR $\alpha$  Cross-Talk Is Required for the Glucose-Mediated FGF21 Response’, *Cell Reports*, 21(2), pp. 403–416. doi: 10.1016/j.celrep.2017.09.065.
  159. Iyer, S. P. N. and Hart, G. W. (2003) ‘Roles of the Tetratricopeptide Repeat Domain in O-GlcNAc Transferase Targeting and Protein Substrate Specificity’, *Journal of Biological Chemistry*, 278(27), pp. 24608–24616. doi: 10.1074/jbc.M300036200.
  160. Jang, I. *et al.* (2015) ‘O-GlcNAcylation of eIF2 $\alpha$  regulates the phospho-eIF2 $\alpha$ -mediated ER stress response’, *Biochimica et Biophysica Acta - Molecular Cell Research*, 1853(8), pp. 1860–1869. doi: 10.1016/j.bbamcr.2015.04.017.
  161. Jeon, J. H. *et al.* (2014) ‘Glucosamine-induced OGT activation mediates glucose production through cleaved Notch1 and FoxO1, which coordinately contributed to the regulation of maintenance of self-renewal in mouse embryonic stem cells.’, *Stem cells and development*, 23(17), pp. 2067–79. doi: 10.1089/scd.2013.0583.
  162. Ji, S. *et al.* (2012) ‘O-GlcNAc modification of PPAR $\gamma$  reduces its transcriptional activity’, *Biochemical and Biophysical Research Communications*, 417(4), pp. 1158–1163. doi: 10.1016/j.bbrc.2011.12.086.
  163. Jia, C.-Z., Liu, T. and Wang, Z.-P. (2013) ‘O-GlcNAcPRED: a sensitive predictor to capture protein O-GlcNAcylation sites’, *Molecular BioSystems*, 9(11), p. 2909. doi: 10.1039/c3mb70326f.
  164. Jia, C., Zuo, Y. and Zou, Q. (2018) ‘O-GlcNAcPRED-II: An integrated classification algorithm for identifying O-GlcNAcylation sites based on fuzzy undersampling and a K -means PCA oversampling technique’, *Bioinformatics*, 34(12), pp. 2029–2036. doi: 10.1093/bioinformatics/bty039.
  165. Jiang, C. *et al.* (2015) ‘Intestine-selective farnesoid X receptor inhibition improves obesity-related metabolic dysfunction.’, *Nature communications*, 6, p. 10166. doi: 10.1038/ncomms10166.
  166. Jiang, M. *et al.* (2019) ‘O-GlcNAcylation promotes colorectal cancer metastasis via the miR-101-O-GlcNAc/EZH2 regulatory feedback circuit’, *Oncogene*, 38(3), pp. 301–316. doi: 10.1038/s41388-018-0435-5.

167. Jiang, P. *et al.* (2011) 'p53 regulates biosynthesis through direct inactivation of glucose-6-phosphate dehydrogenase.', *Nature cell biology*, 13(3), pp. 310–6. doi: 10.1038/ncb2172.
168. Jiang, Y. *et al.* (2015) 'Local generation of fumarate promotes DNA repair through inhibition of histone H3 demethylation', *Nature Cell Biology*, 17(9), pp. 1158–1168. doi: 10.1038/ncb3209.
169. Jin, C. and Felsenfeld, G. (2007) 'Nucleosome stability mediated by histone variants H3.3 and H2A.Z', *Genes & Development*, 21(12), pp. 1519–1529. doi: 10.1101/gad.1547707.
170. Jin, L. and Zhou, Y. (2019) 'Crucial role of the pentose phosphate pathway in malignant tumors (Review)', *Oncology Letters*. doi: 10.3892/ol.2019.10112.
171. Jinek, M. *et al.* (2004) 'The superhelical TPR-repeat domain of O-linked GlcNAc transferase exhibits structural similarities to importin  $\alpha$ ', *Nature Structural and Molecular Biology*, 11(10), pp. 1001–1007. doi: 10.1038/nsmb833.
172. Joiner, C. M. *et al.* (2019) 'Aspartate Residues Far from the Active Site Drive O-GlcNAc Transferase Substrate Selection', *Journal of the American Chemical Society*, 141(33), pp. 12974–12978. doi: 10.1021/jacs.9b06061.
173. Kaasik, K. *et al.* (2013) 'Glucose sensor O-GlcNAcylation coordinates with phosphorylation to regulate circadian clock', *Cell Metabolism*, 17(2), pp. 291–302. doi: 10.1016/j.cmet.2012.12.017.
174. Kamemura, K. *et al.* (2002) 'Dynamic interplay between O-glycosylation and O-phosphorylation of nucleocytoplasmic proteins: Alternative glycosylation/phosphorylation of Thr-58, a known mutational hot spot of c-Myc in lymphomas, is regulated by mitogens', *Journal of Biological Chemistry*, 277(21), pp. 19229–19235. doi: 10.1074/jbc.M201729200.
175. Kaneko, S. and Li, X. (2018) 'X chromosome protects against bladder cancer in females via a KDM6A -dependent epigenetic mechanism', *Science Advances*, 4(6), p. eaar5598. doi: 10.1126/sciadv.aar5598.
176. Kang, E.-S. *et al.* (2008) 'O-GlcNAc modulation at Akt1 Ser473 correlates with apoptosis of murine pancreatic beta cells.', *Experimental cell research*, 314(11–12), pp. 2238–48. doi: 10.1016/j.yexcr.2008.04.014.
177. Kantari-Mimoun, C. *et al.* (2015) 'Resolution of liver fibrosis requires myeloid cell-driven sinusoidal angiogenesis.', *Hepatology (Baltimore, Md.)*, 61(6), pp. 2042–55. doi: 10.1002/hep.27635.
178. Kao, H.-J. *et al.* (2015) 'A two-layered machine learning method to identify

- protein O-GlcNAcylation sites with O-GlcNAc transferase substrate motifs', *BMC Bioinformatics*, 16(Suppl 18), p. S10. doi: 10.1186/1471-2105-16-S18-S10.
179. Kawaguchi, T. *et al.* (2001) 'Glucose and cAMP regulate the L-type pyruvate kinase gene by phosphorylation/dephosphorylation of the carbohydrate response element binding protein', *Proceedings of the National Academy of Sciences*, 98(24), pp. 13710–13715. doi: 10.1073/pnas.231370798.
180. Keembiyehetty, C. N. *et al.* (2011) 'A lipid-droplet-targeted O-GlcNAcase isoform is a key regulator of the proteasome', *Journal of Cell Science*, 124(16), pp. 2851–2860. doi: 10.1242/jcs.083287.
181. Keum, Y.-S. *et al.* (2006) 'Induction of Heme Oxygenase-1 (HO-1) and NAD[P]H: Quinone Oxidoreductase 1 (NQO1) by a Phenolic Antioxidant, Butylated Hydroxyanisole (BHA) and Its Metabolite, tert-Butylhydroquinone (tBHQ) in Primary-Cultured Human and Rat Hepatocytes', *Pharmaceutical Research*, 23(11), pp. 2586–2594. doi: 10.1007/s11095-006-9094-2.
182. Khambu, B. *et al.* (2019) 'Hepatic Autophagy Deficiency Compromises Farnesoid X Receptor Functionality and Causes Cholestatic Injury.', *Hepatology (Baltimore, Md.)*, 69(5), pp. 2196–2213. doi: 10.1002/hep.30407.
183. Kim, E. *et al.* (2020) 'O -GlcNAcylation on LATS2 disrupts the Hippo pathway by inhibiting its activity', *Proceedings of the National Academy of Sciences*, p. 201913469. doi: 10.1073/pnas.1913469117.
184. Kim, E. J. *et al.* (2006) 'Enzymatic characterization of O-GlcNAcase isoforms using a fluorogenic GlcNAc substrate', *Carbohydrate Research*, 341(8), pp. 971–982. doi: 10.1016/j.carres.2006.03.004.
185. Kim, J. *et al.* (2006) 'HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia', *Cell Metabolism*, 3(3), pp. 177–185. doi: 10.1016/j.cmet.2006.02.002.
186. Kim, J. *et al.* (2010) 'A Myc network accounts for similarities between embryonic stem and cancer cell transcription programs.', *Cell*, 143(2), pp. 313–24. doi: 10.1016/j.cell.2010.09.010.
187. Kim, M. S. *et al.* (2016) 'ChREBP regulates fructose-induced glucose production independently of insulin signaling', *Journal of Clinical Investigation*, 126(11), pp. 4372–4386. doi: 10.1172/JCI81993.
188. Kisseleva, T. *et al.* (2012) 'Myofibroblasts revert to an inactive phenotype during regression of liver fibrosis', *Proceedings of the National Academy of Sciences*, 109(24), pp. 9448–9453. doi: 10.1073/pnas.1201840109.

189. Ko, S. *et al.* (2019) 'Hdac1 Regulates Differentiation of Bipotent Liver Progenitor Cells During Regeneration via Sox9b and Cdk8.', *Gastroenterology*, 156(1), pp. 187-202.e14. doi: 10.1053/j.gastro.2018.09.039.
190. Kocabayoglu, P. *et al.* (2015) ' $\beta$ -PDGF receptor expressed by hepatic stellate cells regulates fibrosis in murine liver injury, but not carcinogenesis.', *Journal of hepatology*, 63(1), pp. 141–7. doi: 10.1016/j.jhep.2015.01.036.
191. Krause, M. W. *et al.* (2018) 'Nutrient-Driven O-GlcNAcylation at Promoters Impacts Genome-Wide RNA Pol II Distribution', *Frontiers in Endocrinology*, 9. doi: 10.3389/fendo.2018.00521.
192. Kreppel, L. K., Blomberg, M. A. and Hart, G. W. (1997) 'Dynamic Glycosylation of Nuclear and Cytosolic Proteins', *Journal of Biological Chemistry*, 272(14), pp. 9308–9315. doi: 10.1074/jbc.272.14.9308.
193. Kreppel, L. K. and Hart, G. W. (1999) 'Regulation of a cytosolic and nuclear O-GlcNAc transferase. Role of the tetratricopeptide repeats', *Journal of Biological Chemistry*, 274(45), pp. 32015–32022. doi: 10.1074/jbc.274.45.32015.
194. Kuo, M. *et al.* (2008) 'O -glycosylation of FoxO1 increases its transcriptional activity towards the glucose 6-phosphatase gene', *FEBS Letters*, 582(5), pp. 829–834. doi: 10.1016/j.febslet.2008.02.010.
195. Kwei, K. A., Baker, J. B. and Pelham, R. J. (2012) 'Modulators of sensitivity and resistance to inhibition of PI3K identified in a pharmacogenomic screen of the NCI-60 human tumor cell line collection.', *PloS one*, 7(9), p. e46518. doi: 10.1371/journal.pone.0046518.
196. Laarse, S. A. M., Leney, A. C. and Heck, A. J. R. (2018) 'Crosstalk between phosphorylation and O-GlcNAcylation: friend or foe', *The FEBS Journal*, 285(17), pp. 3152–3167. doi: 10.1111/febs.14491.
197. Lagerlöf, O. *et al.* (2016) 'The nutrient sensor OGT in PVN neurons regulates feeding.', *Science (New York, N.Y.)*, 351(6279), pp. 1293–6. doi: 10.1126/science.aad5494.
198. Lalor, P. F. *et al.* (2002) 'Recruitment of lymphocytes to the human liver', *Immunology and Cell Biology*, 80(1), pp. 52–64. doi: 10.1046/j.1440-1711.2002.01062.x.
199. Lalor, P. F. *et al.* (2006) 'Human hepatic sinusoidal endothelial cells can be distinguished by expression of phenotypic markers related to their specialised functions in vivo.', *World journal of gastroenterology*, 12(34), pp. 5429–39. doi: 10.3748/wjg.v12.i34.5429.

200. Lambert, M., Bastide, B. and Cieniewski-Bernard, C. (2018) 'Involvement of O-GlcNAcylation in the skeletal muscle physiology and pathophysiology: Focus on muscle metabolism', *Frontiers in Endocrinology*, 9(OCT), pp. 1–12. doi: 10.3389/fendo.2018.00578.
201. Lane, E. A. *et al.* (2019) 'HCF-1 Regulates De Novo Lipogenesis through a Nutrient-Sensitive Complex with ChREBP', *Molecular Cell*. Elsevier Inc., 75(2), pp. 357-371.e7. doi: 10.1016/j.molcel.2019.05.019.
202. Lazarus, M. B. *et al.* (2011) 'Structure of human O-GlcNAc transferase and its complex with a peptide substrate', *Nature*. Nature Publishing Group, 469(7331), pp. 564–569. doi: 10.1038/nature09638.
203. Lee, E.-W. *et al.* (2017) 'Phosphorylation of p53 at threonine 155 is required for Jab1-mediated nuclear export of p53', *BMB Reports*, 50(7), pp. 373–378. doi: 10.5483/BMBRep.2017.50.7.077.
204. Lee, J.-S. and Zhang, Z. (2016) 'O-linked N -acetylglucosamine transferase (OGT) interacts with the histone chaperone HIRA complex and regulates nucleosome assembly and cellular senescence', *Proceedings of the National Academy of Sciences*, 113(23), pp. E3213–E3220. doi: 10.1073/pnas.1600509113.
205. Lefebvre, P. *et al.* (2006) 'Sorting out the roles of PPAR alpha in energy metabolism and vascular homeostasis.', *The Journal of clinical investigation*, 116(3), pp. 571–80. doi: 10.1172/JCI27989.
206. Lei, Y. *et al.* (2020) 'O-GlcNAcylation of PFKFB3 is required for tumor cell proliferation under hypoxia', *Oncogenesis*, 9(2), p. 21. doi: 10.1038/s41389-020-0208-1.
207. Leney, A. C. *et al.* (2017) 'Elucidating crosstalk mechanisms between phosphorylation and O-GlcNAcylation', *Proceedings of the National Academy of Sciences of the United States of America*, 114(35), pp. E7255–E7261. doi: 10.1073/pnas.1620529114.
208. Lercher, L. *et al.* (2015) 'Generation of a synthetic GlcNAcylated nucleosome reveals regulation of stability by H2A-Thr101 GlcNAcylation', *Nature Communications*. Nature Publishing Group, 6. doi: 10.1038/ncomms8978.
209. Leturcq, M., Lefebvre, T. and Vercoutter-Edouart, A. S. (2017) 'O-GlcNAcylation and chromatin remodeling in mammals: An up-to-date overview', *Biochemical Society Transactions*, 45(2), pp. 323–338. doi: 10.1042/BST20160388.
210. Lewis, B. A., Burlingame, A. L. and Myers, S. A. (2016) 'Human RNA

- polymerase II promoter recruitment in vitro is regulated by O-linked N-acetylglucosaminyltransferase (OGT)', *Journal of Biological Chemistry*, 291(27), pp. 14056–14061. doi: 10.1074/jbc.M115.684365.
211. Li, B., Li, H., Hu, C. W., *et al.* (2017) 'Structural insights into the substrate binding adaptability and specificity of human O-GlcNAcase', *Nature Communications*. Springer US, 8(1), pp. 1–5. doi: 10.1038/s41467-017-00865-1.
212. Li, B., Li, H., Lu, L., *et al.* (2017) 'Structures of human O-GlcNAcase and its complexes reveal a new substrate recognition mode', *Nature Structural & Molecular Biology*, 24(4), pp. 362–369. doi: 10.1038/nsmb.3390.
213. Li, F. *et al.* (2013) 'Microbiome remodelling leads to inhibition of intestinal farnesoid X receptor signalling and decreased obesity.', *Nature communications*, 4, p. 2384. doi: 10.1038/ncomms3384.
214. Li, L., Duan, M., Chen, W., *et al.* (2017) 'The spleen in liver cirrhosis: revisiting an old enemy with novel targets', *Journal of Translational Medicine*, 15(1), p. 111. doi: 10.1186/s12967-017-1214-8.
215. Li, L. *et al.* (2019) 'New guidelines for DNA methylome studies regarding 5-hydroxymethylcytosine for understanding transcriptional regulation', *Genome Research*, 29(4), pp. 543–553. doi: 10.1101/gr.240036.118.
216. Li, M.-D. *et al.* (2013) 'O-GlcNAc Signaling Entrain the Circadian Clock by Inhibiting BMAL1/CLOCK Ubiquitination', *Cell Metabolism*, 17(2), pp. 303–310. doi: 10.1016/j.cmet.2012.12.015.
217. Li, M. D. *et al.* (2018) 'Adipocyte OGT governs diet-induced hyperphagia and obesity', *Nature Communications*, 9(1). doi: 10.1038/s41467-018-07461-x.
218. Li, M. V. *et al.* (2006) 'Glucose-Dependent Transcriptional Regulation by an Evolutionarily Conserved Glucose-Sensing Module', *Diabetes*, 55(5), pp. 1179–1189. doi: 10.2337/db05-0822.
219. Li, P. *et al.* (2014) 'An efficient method to isolate and culture mouse Kupffer cells', *Immunology Letters*, 158(1–2), pp. 52–56. doi: 10.1016/j.imlet.2013.12.002.
220. Li, T. *et al.* (2018) 'O-GlcNAc Transferase Links Glucose Metabolism to MAVS-Mediated Antiviral Innate Immunity.', *Cell host & microbe*, 24(6), pp. 791–803.e6. doi: 10.1016/j.chom.2018.11.001.
221. Li, X.-F., Chen, C., Xiang, D.-M., *et al.* (2017) 'Chronic inflammation-elicited liver progenitor cell conversion to liver cancer stem cell with clinical significance', *Hepatology*, 66(6), pp. 1934–1951. doi: 10.1002/hep.29372.

222. Li, X. *et al.* (2019) 'O-GlcNAc Transferase Suppresses Inflammation and Necroptosis by Targeting Receptor-Interacting Serine/Threonine-Protein Kinase 3', *Immunity*, 50(3), pp. 576-590.e6. doi: 10.1016/j.immuni.2019.01.007.
223. Li, Y., Wang, L., Liu, J., *et al.* (2017) 'O-GlcNAcylation modulates Bmi-1 protein stability and potential oncogenic function in prostate cancer', *Oncogene*, 36(45), pp. 6293–6305. doi: 10.1038/onc.2017.223.
224. Li, Z., Li, X., Nai, S., *et al.* (2017) 'Checkpoint kinase 1–induced phosphorylation of O- linked  $\beta$ - N -acetylglucosamine transferase regulates the intermediate filament network during cytokinesis', *Journal of Biological Chemistry*, 292(48), pp. 19548–19555. doi: 10.1074/jbc.M117.811646.
225. Liu, Q. *et al.* (2016) 'Hyper-O-GlcNAcylation of YB-1 affects Ser102 phosphorylation and promotes cell proliferation in hepatocellular carcinoma', *Experimental Cell Research*, 349(2), pp. 230–238. doi: 10.1016/j.yexcr.2016.10.011.
226. Lo, P.-W. *et al.* (2018) 'O -GlcNAcylation regulates the stability and enzymatic activity of the histone methyltransferase EZH2', *Proceedings of the National Academy of Sciences*, 115(28), pp. 7302–7307. doi: 10.1073/pnas.1801850115.
227. Loppin, B. *et al.* (2005) 'The histone H3.3 chaperone HIRA is essential for chromatin assembly in the male pronucleus', *Nature*, 437(7063), pp. 1386–1390. doi: 10.1038/nature04059.
228. Lothrop, A. P., Torres, M. P. and Fuchs, S. M. (2013) 'Deciphering post-translational modification codes', *FEBS Letters*, 587(8), pp. 1247–1257. doi: 10.1016/j.febslet.2013.01.047.
229. Love, D. C. *et al.* (2003) 'Mitochondrial and nucleocytoplasmic targeting of O-linked GlcNAc transferase', *Journal of Cell Science*, 116(4), pp. 647–654. doi: 10.1242/jcs.00246.
230. Lubas, W. A. *et al.* (1997) 'O-linked GlcNAc transferase is a conserved nucleocytoplasmic protein containing tetratricopeptide repeats', *Journal of Biological Chemistry*, 272(14), pp. 9316–9324. doi: 10.1074/jbc.272.14.9316.
231. Lubas, W. A. and Hanover, J. A. (2000) 'Functional expression of O-linked GlcNAc transferase. Domain structure and substrate specificity.', *The Journal of biological chemistry*, 275(15), pp. 10983–8. doi: 10.1074/jbc.275.15.10983.
232. Lumpkin, R. J. *et al.* (2017) 'Site-specific identification and quantitation of endogenous SUMO modifications under native conditions.', *Nature communications*, 8(1), p. 1171. doi: 10.1038/s41467-017-01271-3.

233. Lundby, A. *et al.* (2012) ‘Proteomic analysis of lysine acetylation sites in rat tissues reveals organ specificity and subcellular patterns.’, *Cell reports*, 2(2), pp. 419–31. doi: 10.1016/j.celrep.2012.07.006.
234. Luo, X. *et al.* (2016) ‘Hyperglycemic Stress and Carbon Stress in Diabetic Glucotoxicity.’, *Aging and disease*, 7(1), pp. 90–110. doi: 10.14336/AD.2015.0702.
235. Lv, W.-W. *et al.* (2018) ‘Effects of PKM2 on global metabolic changes and prognosis in hepatocellular carcinoma: from gene expression to drug discovery.’, *BMC cancer*, 18(1), p. 1150. doi: 10.1186/s12885-018-5023-0.
236. Lyon, M. F. (1961) ‘Gene action in the X-chromosome of the mouse (*Mus musculus* L.)’, *Nature*, 190, pp. 372–3. doi: 10.1038/190372a0.
237. Ma, J. *et al.* (2016) ‘Comparative Proteomics Reveals Dysregulated Mitochondrial O-GlcNAcylation in Diabetic Hearts’, *Journal of Proteome Research*, 15(7), pp. 2254–2264. doi: 10.1021/acs.jproteome.6b00250.
238. Macauley, M. S. and Vocadlo, D. J. (2009) ‘Enzymatic characterization and inhibition of the nuclear variant of human O-GlcNAcase’, *Carbohydrate Research*. Elsevier Ltd, 344(9), pp. 1079–1084. doi: 10.1016/j.carres.2009.04.017.
239. Macsween, R. M. N. *et al.* (2003) ‘Pathology of the liver, 4th edition’, *Diagnostic Cytopathology*. Cham: Springer International Publishing, 29(1). doi: 10.1002/dc.10338.
240. Maik-Rachline, G., Hacoheh-Lev-Ran, A. and Seger, R. (2019) ‘Nuclear ERK: Mechanism of Translocation, Substrates, and Role in Cancer’, *International Journal of Molecular Sciences*, 20(5), p. 1194. doi: 10.3390/ijms20051194.
241. Majumdar, G. *et al.* (2006) ‘Insulin dynamically regulates calmodulin gene expression by sequential o-glycosylation and phosphorylation of sp1 and its subcellular compartmentalization in liver cells.’, *The Journal of biological chemistry*, 281(6), pp. 3642–50. doi: 10.1074/jbc.M511223200.
242. Makishima, M. *et al.* (1999) ‘Identification of a nuclear receptor for bile acids.’, *Science (New York, N.Y.)*, 284(5418), pp. 1362–5. doi: 10.1126/science.284.5418.1362.
243. Makwana, V. *et al.* (2019) ‘Essential role of O-GlcNAcylation in stabilization of oncogenic factors’, *Biochimica et Biophysica Acta - General Subjects*. Elsevier, 1863(8), pp. 1302–1317. doi: 10.1016/j.bbagen.2019.04.002.
244. Mancinelli, R. *et al.* (2009) ‘Follicle-stimulating hormone increases cholangiocyte

- proliferation by an autocrine mechanism via cAMP-dependent phosphorylation of ERK1/2 and Elk-1', *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 297(1), pp. G11–G26. doi: 10.1152/ajpgi.00025.2009.
245. Margueron, R. *et al.* (2008) 'Ezh1 and Ezh2 Maintain Repressive Chromatin through Different Mechanisms', *Molecular Cell*, 32(4), pp. 503–518. doi: 10.1016/j.molcel.2008.11.004.
246. Martínez-Limón, A. *et al.* (2020) 'The p38 Pathway: From Biology to Cancer Therapy', *International Journal of Molecular Sciences*, 21(6), p. 1913. doi: 10.3390/ijms21061913.
247. Matschinsky, F. M. and Wilson, D. F. (2019) 'The Central Role of Glucokinase in Glucose Homeostasis: A Perspective 50 Years After Demonstrating the Presence of the Enzyme in Islets of Langerhans', *Frontiers in Physiology*, 10. doi: 10.3389/fphys.2019.00148.
248. Matter, M. S. *et al.* (2014) 'Targeting the mTOR pathway in hepatocellular carcinoma: Current state and future trends', *Journal of Hepatology*, 60(4), pp. 855–865. doi: 10.1016/j.jhep.2013.11.031.
249. Maury, J. J. P. *et al.* (2015) 'RING1B O-GlcNAcylation regulates gene targeting of polycomb repressive complex 1 in human embryonic stem cells', *Stem Cell Research*, 15(1), pp. 182–189. doi: 10.1016/j.scr.2015.06.007.
250. McFerrin, L. G. and Atchley, W. R. (2012) 'A Novel N-Terminal Domain May Dictate the Glucose Response of Mondo Proteins', *PLoS ONE*. Edited by V. N. Uversky, 7(4), p. e34803. doi: 10.1371/journal.pone.0034803.
251. McGreal, S. R. *et al.* (2018) 'Modulation of O-GlcNAc Levels in the Liver Impacts Acetaminophen-Induced Liver Injury by Affecting Protein Adduct Formation and Glutathione Synthesis.', *Toxicological sciences: an official journal of the Society of Toxicology*, 162(2), pp. 599–610. doi: 10.1093/toxsci/kfy002.
252. Mele, L. *et al.* (2018) 'A new inhibitor of glucose-6-phosphate dehydrogenase blocks pentose phosphate pathway and suppresses malignant proliferation and metastasis in vivo', *Cell Death & Disease*, 9(5), p. 572. doi: 10.1038/s41419-018-0635-5.
253. Meng, F. *et al.* (2014) 'Regulation of the Histamine/VEGF Axis by miR-125b during Cholestatic Liver Injury in Mice', *The American Journal of Pathology*, 184(3), pp. 662–673. doi: 10.1016/j.ajpath.2013.11.008.
254. Meng, J. *et al.* (2016) 'Identification of HNF-4 $\alpha$  as a key transcription factor to

- promote ChREBP expression in response to glucose’, *Scientific Reports*. Nature Publishing Group, 6(March), pp. 1–14. doi: 10.1038/srep23944.
255. Mereiter, S. *et al.* (2019) ‘Glycosylation in the Era of Cancer-Targeted Therapy: Where Are We Heading?’, *Cancer Cell*, 36(1), pp. 6–16. doi: 10.1016/j.ccell.2019.06.006.
  256. Mermoud, J. E. *et al.* (1999) ‘Histone macroH2A1.2 relocates to the inactive X chromosome after initiation and propagation of X-inactivation.’, *The Journal of cell biology*, 147(7), pp. 1399–408. doi: 10.1083/jcb.147.7.1399.
  257. Mills, C. D. *et al.* (2000) ‘M-1/M-2 macrophages and the Th1/Th2 paradigm.’, *Journal of immunology (Baltimore, Md. : 1950)*, 164(12), pp. 6166–73. doi: 10.4049/jimmunol.164.12.6166.
  258. Mitsuishi, Y. *et al.* (2012) ‘Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming.’, *Cancer cell*, 22(1), pp. 66–79. doi: 10.1016/j.ccr.2012.05.016.
  259. Monte, M. J. *et al.* (2009) ‘Bile acids: chemistry, physiology, and pathophysiology.’, *World journal of gastroenterology*, 15(7), pp. 804–16. doi: 10.3748/wjg.15.804.
  260. Moon, Y.-A. *et al.* (2012) ‘The Scap/SREBP Pathway Is Essential for Developing Diabetic Fatty Liver and Carbohydrate-Induced Hypertriglyceridemia in Animals’, *Cell Metabolism*, 15(2), pp. 240–246. doi: 10.1016/j.cmet.2011.12.017.
  261. Morey, L. *et al.* (2013) ‘RYBP and Cbx7 Define Specific Biological Functions of Polycomb Complexes in Mouse Embryonic Stem Cells’, *Cell Reports*, 3(1), pp. 60–69. doi: 10.1016/j.celrep.2012.11.026.
  262. Mori, K. *et al.* (1993) ‘A transmembrane protein with a cdc2+CDC28-related kinase activity is required for signaling from the ER to the nucleus’, *Cell*, 74(4), pp. 743–756. doi: 10.1016/0092-8674(93)90521-Q.
  263. Moriwaki, K. and Asahi, M. (2017) ‘Augmented TME O<sup>-</sup>GlcNAcylation Promotes Tumor Proliferation through the Inhibition of p38 MAPK’, *Molecular Cancer Research*, 15(9), pp. 1287–1298. doi: 10.1158/1541-7786.MCR-16-0499.
  264. Morzyglod, L. *et al.* (2017) ‘Growth factor receptor binding protein 14 inhibition triggers insulin-induced mouse hepatocyte proliferation and is associated with hepatocellular carcinoma’, *Hepatology*, 65(4), pp. 1352–1368. doi: 10.1002/hep.28972.
  265. Mouly, E. *et al.* (2018) ‘B-cell tumor development in Tet2-deficient mice’, *Blood Advances*, 2(6), pp. 703–714. doi: 10.1182/bloodadvances.2017014118.

266. Mu, Y. *et al.* (2020) ‘O-linked  $\beta$ -N-acetylglucosamine transferase plays an essential role in heart development through regulating angiopoietin-1.’, *PLoS genetics*, 16(4), p. e1008730. doi: 10.1371/journal.pgen.1008730.
267. Murray, P. J. *et al.* (2014) ‘Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines’, *Immunity*, 41(1), pp. 14–20. doi: 10.1016/j.immuni.2014.06.008.
268. Myers, S. A., Panning, B. and Burlingame, A. L. (2011) ‘Polycomb repressive complex 2 is necessary for the normal site-specific O-GlcNAc distribution in mouse embryonic stem cells’, *Proceedings of the National Academy of Sciences*, 108(23), pp. 9490–9495. doi: 10.1073/pnas.1019289108.
269. Nagaraj, R. *et al.* (2017) ‘Nuclear Localization of Mitochondrial TCA Cycle Enzymes as a Critical Step in Mammalian Zygotic Genome Activation’, *Cell*, 168(1–2), pp. 210–223.e11. doi: 10.1016/j.cell.2016.12.026.
270. Nahrendorf, M. and Swirski, F. K. (2016) ‘Abandoning M1/M2 for a Network Model of Macrophage Function.’, *Circulation research*, 119(3), pp. 414–7. doi: 10.1161/CIRCRESAHA.116.309194.
271. de Napoles, M. *et al.* (2004) ‘Polycomb Group Proteins Ring1A/B Link Ubiquitylation of Histone H2A to Heritable Gene Silencing and X Inactivation’, *Developmental Cell*, 7(5), pp. 663–676. doi: 10.1016/j.devcel.2004.10.005.
272. Nelson, D. L. and Cox, M. (2017) *Lehninger principles of biochemistry*. 7th Editio, *Biochemistry and Molecular Biology Education*. 7th Editio. W.H. Freeman, New York, 1328. doi: 10.1002/bmb.2005.494033010419.
273. Ngoh, G. A. *et al.* (2009) ‘O -GlcNAc signaling attenuates ER stress-induced cardiomyocyte death’, *American Journal of Physiology-Heart and Circulatory Physiology*, 297(5), pp. H1711–H1719. doi: 10.1152/ajpheart.00553.2009.
274. Ni, M. *et al.* (2019) ‘Biological mechanisms and related natural modulators of liver X receptor in nonalcoholic fatty liver disease’, *Biomedicine & Pharmacotherapy*, 113, p. 108778. doi: 10.1016/j.biopha.2019.108778.
275. Nie, H. *et al.* (2020) ‘O-GlcNAcylation of PGK1 coordinates glycolysis and TCA cycle to promote tumor growth’, *Nature Communications*, 11(1), p. 36. doi: 10.1038/s41467-019-13601-8.
276. Nie, H. and Yi, W. (2019) ‘O-GlcNAcylation, a sweet link to the pathology of diseases’, *Journal of Zhejiang University: Science B*. doi: 10.1631/jzus.B1900150.
277. Nio, K., Yamashita, T. and Kaneko, S. (2017) ‘The evolving concept of liver

- cancer stem cells.’, *Molecular cancer*, 16(1), p. 4. doi: 10.1186/s12943-016-0572-9.
278. Novoa, I. *et al.* (2001) ‘Feedback Inhibition of the Unfolded Protein Response by GADD34-Mediated Dephosphorylation of eIF2 $\alpha$ ’, *The Journal of Cell Biology*, 153(5), pp. 1011–1022. doi: 10.1083/jcb.153.5.1011.
279. O’Donnell, N. *et al.* (2004) ‘Ogt-dependent X-chromosome-linked protein glycosylation is a requisite modification in somatic cell function and embryo viability.’, *Molecular and cellular biology*, 24(4), pp. 1680–90. doi: 10.1128/mcb.24.4.1680-1690.2004.
280. Ohashi, N. *et al.* (2017) ‘Pivotal Role of O -GlcNAc Modification in Cold-Induced Thermogenesis by Brown Adipose Tissue Through Mitochondrial Biogenesis’, *Diabetes*, 66(9), pp. 2351–2362. doi: 10.2337/db16-1427.
281. Ohn, T. *et al.* (2008) ‘A functional RNAi screen links O-GlcNAc modification of ribosomal proteins to stress granule and processing body assembly’, *Nature Cell Biology*, 10(10), pp. 1224–1231. doi: 10.1038/ncb1783.
282. Okano, K. *et al.* (1965) ‘Histochemical comparison of oxidative enzymes in adrenal glands of mammals.’, *Histochemie. Histochemistry. Histochimie*, 4(6), pp. 494–501. doi: 10.1007/BF00281902.
283. Olivier-Van Stichelen, S. *et al.* (2014) ‘O-GlcNAcylation stabilizes  $\beta$ -catenin through direct competition with phosphorylation at threonine 41.’, *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*, 28(8), pp. 3325–38. doi: 10.1096/fj.13-243535.
284. Olivier-Van Stichelen, S., Abramowitz, L. K. and Hanover, J. A. (2014) ‘X marks the spot: Does it matter that O-GlcNAc Transferase is an X-linked gene?’, *Biochemical and Biophysical Research Communications*, 453(2), pp. 201–207. doi: 10.1016/j.bbrc.2014.06.068.
285. Ortega-Prieto, P. and Postic, C. (2019) ‘Carbohydrate Sensing Through the Transcription Factor ChREBP’, *Frontiers in Genetics*, 10. doi: 10.3389/fgene.2019.00472.
286. Owen, J. L. *et al.* (2012) ‘Insulin stimulation of SREBP-1c processing in transgenic rat hepatocytes requires p70 S6-kinase’, *Proceedings of the National Academy of Sciences*, 109(40), pp. 16184–16189. doi: 10.1073/pnas.1213343109.
287. Paranjpe, S. *et al.* (2016) ‘Combined systemic elimination of MET and epidermal growth factor receptor signaling completely abolishes liver regeneration and leads to liver decompensation’, *Hepatology*, 64(5), pp. 1711–1724. doi:

- 10.1002/hep.28721.
288. Park, J. *et al.* (2005) ‘Overexpression of glucose-6-phosphate dehydrogenase is associated with lipid dysregulation and insulin resistance in obesity.’, *Molecular and cellular biology*, 25(12), pp. 5146–57. doi: 10.1128/MCB.25.12.5146-5157.2005.
289. Park, S. *et al.* (2014) ‘Inhibition of mTOR affects protein stability of OGT’, *Biochemical and Biophysical Research Communications*, 453(2), pp. 208–212. doi: 10.1016/j.bbrc.2014.05.047.
290. Park, S. K. *et al.* (2017) ‘A Conserved Splicing Silencer Dynamically Regulates O-GlcNAc Transferase Intron Retention and O-GlcNAc Homeostasis’, *Cell Reports*. ElsevierCompany., 20(5), pp. 1088–1099. doi: 10.1016/j.celrep.2017.07.017.
291. Pastor, W. A., Aravind, L. and Rao, A. (2013) ‘TETonic shift: biological roles of TET proteins in DNA demethylation and transcription’, *Nature Reviews Molecular Cell Biology*, 14(6), pp. 341–356. doi: 10.1038/nrm3589.
292. Patel, S. H., Camargo, F. D. and Yimlamai, D. (2017) ‘Hippo Signaling in the Liver Regulates Organ Size, Cell Fate, and Carcinogenesis.’, *Gastroenterology*, 152(3), pp. 533–545. doi: 10.1053/j.gastro.2016.10.047.
293. Pathak, S. *et al.* (2015) ‘The active site of O-GlcNAc transferase imposes constraints on substrate sequence’, *Nature Structural and Molecular Biology*, 22(9), pp. 744–749. doi: 10.1038/nsmb.3063.
294. Patra, K. C. and Hay, N. (2014) ‘The pentose phosphate pathway and cancer’, *Trends in Biochemical Sciences*, 39(8), pp. 347–354. doi: 10.1016/j.tibs.2014.06.005.
295. Pchelintsev, N. A. *et al.* (2013) ‘Placing the HIRA Histone Chaperone Complex in the Chromatin Landscape’, *Cell Reports*, 3(4), pp. 1012–1019. doi: 10.1016/j.celrep.2013.03.026.
296. Peixoto, A. *et al.* (2019) ‘Protein glycosylation and tumor microenvironment alterations driving cancer hallmarks’, *Frontiers in Oncology*, 9(MAY), pp. 1–24. doi: 10.3389/fonc.2019.00380.
297. Peng, C. *et al.* (2017) ‘Regulation of the Hippo-YAP Pathway by Glucose Sensor O-GlcNAcylation’, *Molecular Cell*, 68(3), pp. 591-604.e5. doi: 10.1016/j.molcel.2017.10.010.
298. Perez-Cervera, Y. *et al.* (2013) ‘Insulin signaling controls the expression of O-GlcNAc transferase and its interaction with lipid microdomains.’, *FASEB*

- journal: official publication of the Federation of American Societies for Experimental Biology*, 27(9), pp. 3478–86. doi: 10.1096/fj.12-217984.
299. Perreault, M. *et al.* (2013) ‘Role of Glucuronidation for Hepatic Detoxification and Urinary Elimination of Toxic Bile Acids during Biliary Obstruction’, *PLoS ONE*. Edited by M. A. Avila, 8(11), p. e80994. doi: 10.1371/journal.pone.0080994.
300. Perugorria, M. J. *et al.* (2012) ‘Histone methyltransferase ASH1 orchestrates fibrogenic gene transcription during myofibroblast transdifferentiation.’, *Hepatology (Baltimore, Md.)*, 56(3), pp. 1129–39. doi: 10.1002/hep.25754.
301. Phoomak, C. *et al.* (2012) ‘Overexpression of O-GlcNAc-Transferase Associates with Aggressiveness of Mass-Forming Cholangiocarcinoma’, *Asian Pacific Journal of Cancer Prevention*, 13(SUPPL.1), pp. 101–105. doi: 10.7314/APJCP.2012.13.KKSuppl.101.
302. Phoomak, C. *et al.* (2017) ‘High glucose levels boost the aggressiveness of highly metastatic cholangiocarcinoma cells via O-GlcNAcylation.’, *Scientific reports*, 7, p. 43842. doi: 10.1038/srep43842.
303. Phoomak, C., Silsirivanit, A., Park, D., Sawanyawisuth, K., Vaeteewoottacharn, K., Wongkham, C., Lam, E. W.-F., *et al.* (2018) ‘O-GlcNAcylation mediates metastasis of cholangiocarcinoma through FOXO3 and MAN1A1.’, *Oncogene*, 37(42), pp. 5648–5665. doi: 10.1038/s41388-018-0366-1.
304. Phoomak, C., Silsirivanit, A., Park, D., Sawanyawisuth, K., Vaeteewoottacharn, K., Wongkham, C., Lam, E. W. F., *et al.* (2018) ‘O-GlcNAcylation mediates metastasis of cholangiocarcinoma through FOXO3 and MAN1A1’, *Oncogene*. Springer US, 37(42), pp. 5648–5665. doi: 10.1038/s41388-018-0366-1.
305. Pinho, S. S. and Reis, C. A. (2015) ‘Glycosylation in cancer: Mechanisms and clinical implications’, *Nature Reviews Cancer*. Nature Publishing Group, 15(9), pp. 540–555. doi: 10.1038/nrc3982.
306. Plath, K. *et al.* (2003) ‘Role of histone H3 lysine 27 methylation in X inactivation.’, *Science (New York, N.Y.)*, 300(5616), pp. 131–5. doi: 10.1126/science.1084274.
307. Poisson, J. *et al.* (2017) ‘Liver sinusoidal endothelial cells: Physiology and role in liver diseases’, *Journal of Hepatology*, 66(1), pp. 212–227. doi: 10.1016/j.jhep.2016.07.009.
308. Pore, N. *et al.* (2004) ‘Sp1 is involved in Akt-mediated induction of VEGF expression through an HIF-1-independent mechanism.’, *Molecular biology of the*

- cell*, 15(11), pp. 4841–53. doi: 10.1091/mbc.e04-05-0374.
309. Potthoff, M. J. *et al.* (2011) ‘FGF15/19 regulates hepatic glucose metabolism by inhibiting the CREB-PGC-1 $\alpha$  pathway’, *Cell Metabolism*, 13(6), pp. 729–738. doi: 10.1016/j.cmet.2011.03.019.
310. Preitner, N. *et al.* (2002) ‘The Orphan Nuclear Receptor REV-ERB $\alpha$  Controls Circadian Transcription within the Positive Limb of the Mammalian Circadian Oscillator’, *Cell*, 110(2), pp. 251–260. doi: 10.1016/S0092-8674(02)00825-5.
311. Qu, Q. *et al.* (2016) ‘Fatty acid oxidation and carnitine palmitoyltransferase I: emerging therapeutic targets in cancer.’, *Cell death & disease*, 7, p. e2226. doi: 10.1038/cddis.2016.132.
312. Qu, S. *et al.* (2006) ‘Aberrant Forkhead box O1 function is associated with impaired hepatic metabolism.’, *Endocrinology*, 147(12), pp. 5641–52. doi: 10.1210/en.2006-0541.
313. De Queiroz, R. M. *et al.* (2016) ‘Changes in O-linked N-acetylglucosamine (O-GlcNAc) homeostasis activate the p53 pathway in ovarian cancer cells’, *Journal of Biological Chemistry*, 291(36), pp. 18897–18914. doi: 10.1074/jbc.M116.734533.
314. Rao, X. *et al.* (2015) ‘O-GlcNAcylation of G6PD promotes the pentose phosphate pathway and tumor growth.’, *Nature communications*, 6, p. 8468. doi: 10.1038/ncomms9468.
315. Reily, C. *et al.* (2019) ‘Glycosylation in health and disease’, *Nature Reviews Nephrology*. Springer US, 15(6), pp. 346–366. doi: 10.1038/s41581-019-0129-4.
316. Rena, G. *et al.* (1999) ‘Phosphorylation of the Transcription Factor Forkhead Family Member FKHR by Protein Kinase B’, *Journal of Biological Chemistry*, 274(24), pp. 17179–17183. doi: 10.1074/jbc.274.24.17179.
317. Rena, G. (2002) ‘Two novel phosphorylation sites on FKHR that are critical for its nuclear exclusion’, *The EMBO Journal*, 21(9), pp. 2263–2271. doi: 10.1093/emboj/21.9.2263.
318. Repa, J. J. *et al.* (2000) ‘Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXR $\alpha$  and LXR $\beta$ ’, *Genes and Development*, 14(22), pp. 2819–2830. doi: 10.1101/gad.844900.
319. Reppert, S. M. and Weaver, D. R. (2002) ‘Coordination of circadian timing in mammals’, *Nature*, 418(6901), pp. 935–941. doi: 10.1038/nature00965.
320. Resto, M. *et al.* (2016) ‘O-GlcNAcase is an RNA polymerase II elongation factor coupled to pausing factors SPT5 and TIF 1 $\beta$ ’, *Journal of Biological Chemistry*,

- 291(43), pp. 22703–22713. doi: 10.1074/jbc.M116.751420.
321. Rikimaru, T. *et al.* (2007) ‘Clinical Significance of Histone Deacetylase 1 Expression in Patients with Hepatocellular Carcinoma’, *Oncology*, 72(1–2), pp. 69–74. doi: 10.1159/000111106.
322. Rodríguez-Carballo, E., Gámez, B. and Ventura, F. (2016) ‘p38 MAPK Signaling in Osteoblast Differentiation’, *Frontiers in Cell and Developmental Biology*, 4. doi: 10.3389/fcell.2016.00040.
323. Roskams, T. A. *et al.* (2004) ‘Nomenclature of the finer branches of the biliary tree: Canals, ductules, and ductular reactions in human livers’, *Hepatology*, 39(6), pp. 1739–1745. doi: 10.1002/hep.20130.
324. Roth, C. *et al.* (2017) ‘Structural and functional insight into human O-GlcNAcase’, *Nature Chemical Biology*, 13(6), pp. 610–612. doi: 10.1038/nchembio.2358.
325. Ruan, H.-B. *et al.* (2012) ‘O-GlcNAc Transferase/Host Cell Factor C1 Complex Regulates Gluconeogenesis by Modulating PGC-1 $\alpha$  Stability’, *Cell Metabolism*, 16(2), pp. 226–237. doi: 10.1016/j.cmet.2012.07.006.
326. Ruan, H.-B. *et al.* (2014) ‘O-GlcNAc transferase enables AgRP neurons to suppress browning of white fat.’, *Cell*, 159(2), pp. 306–17. doi: 10.1016/j.cell.2014.09.010.
327. Ruan, H.-B. *et al.* (2017) ‘Calcium-dependent O-GlcNAc signaling drives liver autophagy in adaptation to starvation’, *Genes & Development*, 31(16), pp. 1655–1665. doi: 10.1101/gad.305441.117.
328. Ruan, H. Bin, Nie, Y. and Yang, X. (2013) ‘Regulation of protein degradation by O-GlcNAcylation: Crosstalk with ubiquitination’, *Molecular and Cellular Proteomics*, 12(12), pp. 3489–3497. doi: 10.1074/mcp.R113.029751.
329. Rudack, D., Chisholm, E. M. and Holten, D. (1971) ‘Rat liver glucose 6-phosphate dehydrogenase. Regulation by carbohydrate diet and insulin.’, *The Journal of biological chemistry*, 246(5), pp. 1249–54. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/5545067>.
330. Russell, J. O. and Monga, S. P. (2018) ‘Wnt/ $\beta$ -Catenin Signaling in Liver Development, Homeostasis, and Pathobiology.’, *Annual review of pathology*, 13, pp. 351–378. doi: 10.1146/annurev-pathol-020117-044010.
331. Saengboonmee, C. *et al.* (2016) ‘High glucose enhances progression of cholangiocarcinoma cells via STAT3 activation.’, *Scientific reports*, 6, p. 18995. doi: 10.1038/srep18995.

332. Sakabe, K., Wang, Z. and Hart, G. W. (2010) 'β-N-acetylglucosamine (O-GlcNAc) is part of the histone code', *Proceedings of the National Academy of Sciences of the United States of America*, 107(46), pp. 19915–19920. doi: 10.1073/pnas.1009023107.
333. Sakiyama, H. *et al.* (2010) 'The role of O-linked GlcNAc modification on the glucose response of ChREBP.', *Biochemical and biophysical research communications*, 402(4), pp. 784–9. doi: 10.1016/j.bbrc.2010.10.113.
334. Sato, T. K. *et al.* (2004) 'A Functional Genomics Strategy Reveals Rora as a Component of the Mammalian Circadian Clock', *Neuron*, 43(4), pp. 527–537. doi: 10.1016/j.neuron.2004.07.018.
335. Saunders, A. *et al.* (2017) 'The SIN3A/HDAC Corepressor Complex Functionally Cooperates with NANOG to Promote Pluripotency', *Cell Reports*, 18(7), pp. 1713–1726. doi: 10.1016/j.celrep.2017.01.055.
336. Van Schaftingen, E., Detheux, M. and Da Cunha, M. V. (1994) 'Short-term control of glucokinase activity: role of a regulatory protein', *The FASEB Journal*, 8(6), pp. 414–419. doi: 10.1096/fasebj.8.6.8168691.
337. Schuettengruber, B. *et al.* (2017) 'Genome Regulation by Polycomb and Trithorax: 70 Years and Counting', *Cell*, 171(1), pp. 34–57. doi: 10.1016/j.cell.2017.08.002.
338. Schultz, J. R. (2000) 'Role of LXRs in control of lipogenesis', *Genes & Development*, 14(22), pp. 2831–2838. doi: 10.1101/gad.850400.
339. Schulz, C. *et al.* (2012) 'A Lineage of Myeloid Cells Independent of Myb and Hematopoietic Stem Cells', *Science*, 336(6077), pp. 86–90. doi: 10.1126/science.1219179.
340. Seki, E., Brenner, D. A. and Karin, M. (2012) 'A liver full of JNK: signaling in regulation of cell function and disease pathogenesis, and clinical approaches.', *Gastroenterology*, 143(2), pp. 307–20. doi: 10.1053/j.gastro.2012.06.004.
341. Shafi, R. *et al.* (2000) 'The O-GlcNAc transferase gene resides on the X chromosome and is essential for embryonic stem cell viability and mouse ontogeny', *Proceedings of the National Academy of Sciences of the United States of America*, 97(11), pp. 5735–5739. doi: 10.1073/pnas.100471497.
342. Shamu, C. E. and Walter, P. (1996) 'Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus.', *The EMBO Journal*, 15(12), pp. 3028–3039. doi: 10.1002/j.1460-2075.1996.tb00666.x.

343. Shao, S. *et al.* (2020) ‘CHD4/NuRD complex regulates complement gene expression and correlates with CD8 T cell infiltration in human hepatocellular carcinoma’, *Clinical Epigenetics*, 12(1), p. 31. doi: 10.1186/s13148-020-00827-3.
344. Sharma, N. S. *et al.* (2019) ‘O-GlcNAc modification of Sox2 regulates self-renewal in pancreatic cancer by promoting its stability’, *Theranostics*, 9(12), pp. 3410–3424. doi: 10.7150/thno.32615.
345. Sheen-Chen, S.-M. *et al.* (2014) ‘Epigenetic histone methylation regulates transforming growth factor  $\beta$ -1 expression following bile duct ligation in rats’, *Journal of Gastroenterology*, 49(8), pp. 1285–1297. doi: 10.1007/s00535-013-0892-0.
346. Shen, J. *et al.* (2002) ‘ER Stress Regulation of ATF6 Localization by Dissociation of BiP/GRP78 Binding and Unmasking of Golgi Localization Signals’, *Developmental Cell*, 3(1), pp. 99–111. doi: 10.1016/S1534-5807(02)00203-4.
347. Shen, L. L. *et al.* (2011) ‘Effects of farnesoid X receptor on the expression of the fatty acid synthetase and hepatic lipase’, *Molecular Biology Reports*, 38(1), pp. 553–559. doi: 10.1007/s11033-010-0140-0.
348. Shetty, S., Lalor, P. F. and Adams, D. H. (2018) ‘Liver sinusoidal endothelial cells — gatekeepers of hepatic immunity’, *Nature Reviews Gastroenterology and Hepatology*. Springer US, 15(9), pp. 555–567. doi: 10.1038/s41575-018-0020-y.
349. Shi, C. *et al.* (2018) ‘Yap promotes hepatocellular carcinoma metastasis and mobilization via governing cofilin/F-actin/lamellipodium axis by regulation of JNK/Bnip3/SERCA/CaMKII pathways’, *Redox Biology*, 14, pp. 59–71. doi: 10.1016/j.redox.2017.08.013.
350. Shi, F.-T. *et al.* (2013) ‘Ten-Eleven Translocation 1 (Tet1) Is Regulated by O - Linked N -Acetylglucosamine Transferase (Ogt) for Target Gene Repression in Mouse Embryonic Stem Cells’, *Journal of Biological Chemistry*, 288(29), pp. 20776–20784. doi: 10.1074/jbc.M113.460386.
351. Shi, H. *et al.* (2018) ‘Skeletal muscle O-GlcNAc transferase is important for muscle energy homeostasis and whole-body insulin sensitivity’, *Molecular Metabolism*, 11, pp. 160–177. doi: 10.1016/j.molmet.2018.02.010.
352. Shi, J. *et al.* (2015) ‘O-GlcNAcylation regulates ischemia-induced neuronal apoptosis through AKT signaling.’, *Scientific reports*, 5, p. 14500. doi: 10.1038/srep14500.
353. Singh, J. P. *et al.* (2020) ‘O-GlcNAcase targets pyruvate kinase M2 to regulate tumor growth.’, *Oncogene*, 39(3), pp. 560–573. doi: 10.1038/s41388-019-0975-3.

354. Smalley, I. and Smalley, K. S. M. (2018) 'ERK Inhibition: A New Front in the War against MAPK Pathway-Driven Cancers?', *Cancer Discovery*, 8(2), pp. 140–142. doi: 10.1158/2159-8290.CD-17-1355.
355. Smoly, I. *et al.* (2017) 'An Asymmetrically Balanced Organization of Kinases versus Phosphatases across Eukaryotes Determines Their Distinct Impacts', *PLoS Computational Biology*, 13(1), pp. 1–21. doi: 10.1371/journal.pcbi.1005221.
356. Soesanto, Y. A. *et al.* (2008) 'Regulation of Akt signaling by O-GlcNAc in euglycemia.', *American journal of physiology. Endocrinology and metabolism*, 295(4), pp. E974-80. doi: 10.1152/ajpendo.90366.2008.
357. Solt, L. A., Kojetin, D. J. and Burris, T. P. (2011) 'The REV-ERBs and RORs: molecular links between circadian rhythms and lipid homeostasis.', *Future medicinal chemistry*, 3(5), pp. 623–38. doi: 10.4155/fmc.11.9.
358. Song, S. *et al.* (2010) 'Peroxisome proliferator activated receptor alpha (PPARalpha) and PPAR gamma coactivator (PGC-1alpha) induce carnitine palmitoyltransferase IA (CPT-1A) via independent gene elements.', *Molecular and cellular endocrinology*, 325(1–2), pp. 54–63. doi: 10.1016/j.mce.2010.05.019.
359. Stefan, N., Häring, H.-U. and Cusi, K. (2019) 'Non-alcoholic fatty liver disease: causes, diagnosis, cardiometabolic consequences, and treatment strategies', *The Lancet Diabetes & Endocrinology*, 7(4), pp. 313–324. doi: 10.1016/S2213-8587(18)30154-2.
360. Su, C. and Schwarz, T. L. (2017) 'O -GlcNAc Transferase Is Essential for Sensory Neuron Survival and Maintenance', *The Journal of Neuroscience*, 37(8), pp. 2125–2136. doi: 10.1523/JNEUROSCI.3384-16.2017.
361. Suh, H. N. *et al.* (2014) 'Glucosamine-Induced Sp1 O-GlcNAcylation Ameliorates Hypoxia-Induced SGLT Dysfunction in Primary Cultured Renal Proximal Tubule Cells', *Journal of Cellular Physiology*, 229(10), pp. 1557–1568. doi: 10.1002/jcp.24599.
362. Sun, Z. *et al.* (2013) 'Deacetylase-Independent function of HDAC3 in transcription and metabolism requires nuclear receptor corepressor', *Molecular Cell*, 52(6), pp. 769–782. doi: 10.1016/j.molcel.2013.10.022.
363. Svegliati-Baroni, G. *et al.* (2006) 'Estrogens maintain bile duct mass and reduce apoptosis after biliodigestive anastomosis in bile duct ligated rats', *Journal of Hepatology*, 44(6), pp. 1158–1166. doi: 10.1016/j.jhep.2005.10.032.
364. Tacke, F. (2017) 'Targeting hepatic macrophages to treat liver diseases.', *Journal*

- of hepatology*, 66(6), pp. 1300–1312. doi: 10.1016/j.jhep.2017.02.026.
365. El Taghdouini, A. and van Grunsven, L. A. (2016) ‘Epigenetic regulation of hepatic stellate cell activation and liver fibrosis.’, *Expert review of gastroenterology & hepatology*, 10(12), pp. 1397–1408. doi: 10.1080/17474124.2016.1251309.
  366. Takagi, N. and Sasaki, M. (1975) ‘Preferential inactivation of the paternally derived X chromosome in the extraembryonic membranes of the mouse.’, *Nature*, 256(5519), pp. 640–2. doi: 10.1038/256640a0.
  367. Tan, E. P. *et al.* (2014) ‘Altering O -Linked  $\beta$ - N -Acetylglucosamine Cycling Disrupts Mitochondrial Function’, *Journal of Biological Chemistry*, 289(21), pp. 14719–14730. doi: 10.1074/jbc.M113.525790.
  368. Tan, Z. W. *et al.* (2020) ‘O-GlcNAc regulates gene expression by controlling detained intron splicing’, *bioRxiv*, 48(10), pp. 5656–5669. doi: 10.1093/nar/gkaa263.
  369. Tanaka, M. and Iwakiri, Y. (2016) ‘The Hepatic Lymphatic Vascular System: Structure, Function, Markers, and Lymphangiogenesis’, *Cellular and Molecular Gastroenterology and Hepatology*, 2(6), pp. 733–749. doi: 10.1016/j.jcmgh.2016.09.002.
  370. Tannour-Louet, M. *et al.* (2002) ‘A tamoxifen-inducible chimeric Cre recombinase specifically effective in the fetal and adult mouse liver.’, *Hepatology (Baltimore, Md.)*, 35(5), pp. 1072–81. doi: 10.1053/jhep.2002.33164.
  371. Tessarz, P. and Kouzarides, T. (2014) ‘Histone core modifications regulating nucleosome structure and dynamics’, *Nature Reviews Molecular Cell Biology*, 15(11), pp. 703–708. doi: 10.1038/nrm3890.
  372. Thompson, J. W., Griffin, M. E. and Hsieh-Wilson, L. C. (2018) *Methods for the Detection, Study, and Dynamic Profiling of O-GlcNAc Glycosylation*. 1st edn, *Methods in Enzymology*. 1st edn. Elsevier Inc. doi: 10.1016/bs.mie.2017.06.009.
  373. Tirasophon, W., Welihinda, A. A. and Kaufman, R. J. (1998) ‘A stress response pathway from the endoplasmic reticulum to the nucleus requires a novel bifunctional protein kinase/endoribonuclease (Ire1p) in mammalian cells’, *Genes & Development*, 12(12), pp. 1812–1824. doi: 10.1101/gad.12.12.1812.
  374. Titchenell, P. M., Lazar, M. A. and Birnbaum, M. J. (2017) ‘Unraveling the Regulation of Hepatic Metabolism by Insulin’, *Trends in Endocrinology & Metabolism*, 28(7), pp. 497–505. doi: 10.1016/j.tem.2017.03.003.
  375. Toda, G. *et al.* (1980) ‘Mechanism of elevation of serum alkaline phosphatase

- activity in biliary obstruction: an experimental study', *Clinica Chimica Acta*, 107(1–2), pp. 85–96. doi: 10.1016/0009-8981(80)90417-9.
376. Toleman, C. *et al.* (2004) 'Characterization of the histone acetyltransferase (HAT) domain of a bifunctional protein with activable O-GlcNAcase and HAT activities', *Journal of Biological Chemistry*, 279(51), pp. 53665–53673. doi: 10.1074/jbc.M410406200.
377. Torres, C. R. and Hart, G. W. (1984) 'Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surfaces of intact lymphocytes. Evidence for O-linked GlcNAc', *Journal of Biological Chemistry*, 259(5), pp. 3308–3317.
378. Trapannone, R. *et al.* (2016) 'Nucleocytoplasmic human O-GlcNAc transferase is sufficient for O-GlcNAcylation of mitochondrial proteins', *Biochemical Journal*, 473(12), pp. 1693–1702. doi: 10.1042/BCJ20160092.
379. Trauner, M., Meier, P. J. and Boyer, J. L. (1998) 'Molecular Pathogenesis of Cholestasis', *New England Journal of Medicine*. Edited by F. H. Epstein, 339(17), pp. 1217–1227. doi: 10.1056/NEJM199810223391707.
380. Tsuchida, T. *et al.* (2018) 'A simple diet- and chemical-induced murine NASH model with rapid progression of steatohepatitis, fibrosis and liver cancer', *Journal of Hepatology*, 69(2), pp. 385–395. doi: 10.1016/j.jhep.2018.03.011.
381. Tsuchida, T. and Friedman, S. L. (2017) 'Mechanisms of hepatic stellate cell activation', *Nature Reviews Gastroenterology & Hepatology*, 14(7), pp. 397–411. doi: 10.1038/nrgastro.2017.38.
382. Ueda, Y. *et al.* (2020) 'O-GlcNAcylation-mediated degradation of FBXL2 stabilizes FOXM1 to induce cancer progression', *Biochemical and Biophysical Research Communications*. Elsevier Ltd, 521(3), pp. 632–638. doi: 10.1016/j.bbrc.2019.10.164.
383. Vattam, K. M. and Wek, R. C. (2004) 'Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells', *Proceedings of the National Academy of Sciences*, 101(31), pp. 11269–11274. doi: 10.1073/pnas.0400541101.
384. Védie, A.-L. *et al.* (2018) 'Molecular classification of hepatocellular adenomas: impact on clinical practice', *Hepatic Oncology*, 5(1), p. HEP04. doi: 10.2217/hep-2017-0023.
385. Vella, P. *et al.* (2013) 'Tet Proteins Connect the O-Linked N-acetylglucosamine Transferase Ogt to Chromatin in Embryonic Stem Cells', *Molecular Cell*. Elsevier

- Inc., 49(4), pp. 645–656. doi: 10.1016/j.molcel.2012.12.019.
386. Vidal, M. (2009) ‘Role of polycomb proteins Ring1A and Ring1B in the epigenetic regulation of gene expression’, *The International Journal of Developmental Biology*, 53(2–3), pp. 355–370. doi: 10.1387/ijdb.082690mv.
387. Wagner, S. A. *et al.* (2011) ‘A proteome-wide, quantitative survey of in vivo ubiquitylation sites reveals widespread regulatory roles.’, *Molecular & cellular proteomics : MCP*, 10(10), p. M111.013284. doi: 10.1074/mcp.M111.013284.
388. Walker, E. H. *et al.* (1999) ‘Structural insights into phosphoinositide 3-kinase catalysis and signalling’, *Nature*, 402(6759), pp. 313–320. doi: 10.1038/46319.
389. Walter, P. and Ron, D. (2011) ‘The Unfolded Protein Response: From Stress Pathway to Homeostatic Regulation’, *Science*, 334(6059), pp. 1081–1086. doi: 10.1126/science.1209038.
390. Wang, A. C. *et al.* (2016) ‘Loss of O-GlcNAc glycosylation in forebrain excitatory neurons induces neurodegeneration.’, *Proceedings of the National Academy of Sciences of the United States of America*, 113(52), pp. 15120–15125. doi: 10.1073/pnas.1606899113.
391. Wang, J. *et al.* (2011) ‘dbOGAP - An Integrated Bioinformatics Resource for Protein O-GlcNAcylation’, *BMC Bioinformatics*, 12(1), p. 91. doi: 10.1186/1471-2105-12-91.
392. Wang, J. *et al.* (2018) ‘Key signaling pathways, genes and transcription factors associated with hepatocellular carcinoma.’, *Molecular medicine reports*, 17(6), pp. 8153–8160. doi: 10.3892/mmr.2018.8871.
393. Wang, L. *et al.* (2018) ‘Suppressed OGT expression inhibits cell proliferation while inducing cell apoptosis in bladder cancer’, *BMC Cancer*, 18(1), p. 1141. doi: 10.1186/s12885-018-5033-y.
394. Wang, L. *et al.* (2019) ‘Suppressed OGT expression inhibits cell proliferation and modulates EGFR expression in renal cell carcinoma’, *Cancer Management and Research*, 11, pp. 2215–2223. doi: 10.2147/CMAR.S190642.
395. Wang, R. *et al.* (2010) ‘Polycomb Group Targeting through Different Binding Partners of RING1B C-Terminal Domain’, *Structure*, 18(8), pp. 966–975. doi: 10.1016/j.str.2010.04.013.
396. Wang, S. *et al.* (2012) ‘Extensive crosstalk between O-GlcNAcylation and phosphorylation regulates Akt signaling’, *PLoS ONE*, 7(5). doi: 10.1371/journal.pone.0037427.
397. Wang, T. *et al.* (2017) ‘O-GlcNAcylation of fumarase maintains tumour growth

- under glucose deficiency’, *Nature Cell Biology*, 19(7), pp. 833–843. doi: 10.1038/ncb3562.
398. Wang, Z. V *et al.* (2014) ‘Spliced X-box binding protein 1 couples the unfolded protein response to hexosamine biosynthetic pathway.’, *Cell*, 156(6), pp. 1179–1192. doi: 10.1016/j.cell.2014.01.014.
399. Wells, L. *et al.* (2002) ‘ Dynamic O- Glycosylation of Nuclear and Cytosolic Proteins ’, *Journal of Biological Chemistry*, 277(3), pp. 1755–1761. doi: 10.1074/jbc.M109656200.
400. Wells, L., Vosseller, K. and Hart, G. W. (2001) ‘Glycosylation of nucleocytoplasmic proteins: signal transduction and O-GlcNAc.’, *Science (New York, N.Y.)*, 291(5512), pp. 2376–8. doi: 10.1126/science.1058714.
401. Wheatley, E. G. *et al.* (2019) ‘Neuronal O-GlcNAcylation Improves Cognitive Function in the Aged Mouse Brain.’, *Current biology: CB*, 29(20), pp. 3359–3369.e4. doi: 10.1016/j.cub.2019.08.003.
402. Whelan, S. A. *et al.* (2010) ‘Regulation of Insulin Receptor Substrate 1 (IRS-1)/AKT Kinase-mediated Insulin Signaling by O -Linked  $\beta$ - N -Acetylglucosamine in 3T3-L1 Adipocytes’, *Journal of Biological Chemistry*, 285(8), pp. 5204–5211. doi: 10.1074/jbc.M109.077818.
403. Whelan, S. A., Lane, M. D. and Hart, G. W. (2008) ‘Regulation of the O -Linked  $\beta$ - N -Acetylglucosamine Transferase by Insulin Signaling’, *Journal of Biological Chemistry*, 283(31), pp. 21411–21417. doi: 10.1074/jbc.M800677200.
404. Whisenant, T. R. *et al.* (2006) ‘Disrupting the enzyme complex regulating O-GlcNAcylation blocks signaling and development’, *Glycobiology*, 16(6), pp. 551–563. doi: 10.1093/glycob/cwj096.
405. Williams, K. *et al.* (2011) ‘TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity’, *Nature*, 473(7347), pp. 343–348. doi: 10.1038/nature10066.
406. Wise, D. R. *et al.* (2008) ‘Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction’, *Proceedings of the National Academy of Sciences*, 105(48), pp. 18782–18787. doi: 10.1073/pnas.0810199105.
407. Wu, D. *et al.* (2017) ‘O -Linked N -acetylglucosamine transferase 1 regulates global histone H4 acetylation via stabilization of the nonspecific lethal protein NSL3’, *Journal of Biological Chemistry*, 292(24), pp. 10014–10025. doi: 10.1074/jbc.M117.781401.

408. Wu, D., Cai, Y. and Jin, J. (2017) 'Potential coordination role between O-GlcNAcylation and epigenetics', *Protein & Cell*, 8(10), pp. 713–723. doi: 10.1007/s13238-017-0416-4.
409. Wu, H. *et al.* (2011) 'Dual functions of Tet1 in transcriptional regulation in mouse embryonic stem cells', *Nature*, 473(7347), pp. 389–393. doi: 10.1038/nature09934.
410. Wu, H. and Zhang, Y. (2014) 'Reversing DNA Methylation: Mechanisms, Genomics, and Biological Functions', *Cell*, 156(1–2), pp. 45–68. doi: 10.1016/j.cell.2013.12.019.
411. Wu, Y. *et al.* (2020) 'Cancer Stem Cells: A Potential Breakthrough in HCC-Targeted Therapy.', *Frontiers in pharmacology*, 11, p. 198. doi: 10.3389/fphar.2020.00198.
412. Xiao, G. *et al.* (2019) 'EZH2 negatively regulates PD-L1 expression in hepatocellular carcinoma', *Journal for ImmunoTherapy of Cancer*, 7(1), p. 300. doi: 10.1186/s40425-019-0784-9.
413. Xie, Y. *et al.* (2019) 'New Insights Into the Circadian Rhythm and Its Related Diseases', *Frontiers in Physiology*, 10. doi: 10.3389/fphys.2019.00682.
414. Xu, B. *et al.* (2003) 'Capillarization of Hepatic Sinusoid by Liver Endothelial Cell-Reactive Autoantibodies in Patients with Cirrhosis and Chronic Hepatitis', *The American Journal of Pathology*, 163(4), pp. 1275–1289. doi: 10.1016/S0002-9440(10)63487-6.
415. Xu, H. *et al.* (2015) 'Cryptochrome 1 regulates the circadian clock through dynamic interactions with the BMAL1 C terminus', *Nature Structural & Molecular Biology*, 22(6), pp. 476–484. doi: 10.1038/nsmb.3018.
416. Xu, W. *et al.* (2017) 'O-GlcNAc transferase promotes fatty liver-associated liver cancer through inducing palmitic acid and activating endoplasmic reticulum stress', *Journal of Hepatology*, 67(2), pp. 310–320. doi: 10.1016/j.jhep.2017.03.017.
417. Xu, Y. *et al.* (2020) 'A Positive Feedback Loop of TET3 and TGF- $\beta$ 1 Promotes Liver Fibrosis', *Cell Reports*, 30(5), pp. 1310-1318.e5. doi: 10.1016/j.celrep.2019.12.092.
418. Yamaji, S. *et al.* (2010) 'Hepatocyte-specific deletion of DDB1 induces liver regeneration and tumorigenesis', *Proceedings of the National Academy of Sciences of the United States of America*, 107(51), pp. 22237–22242. doi: 10.1073/pnas.1015793108.

419. Yamashita, H. *et al.* (2001) 'A glucose-responsive transcription factor that regulates carbohydrate metabolism in the liver', *Proc Natl Acad Sci U S A*, 98(16), pp. 9116–9121. doi: 10.1073/pnas.161284298.
420. Yang, A.-Q. *et al.* (2017) 'Validation, Identification, and Biological Consequences of the Site-specific O -GlcNAcylation Dynamics of Carbohydrate-responsive Element-binding Protein (ChREBP)', *Molecular & Cellular Proteomics*, 16(7), pp. 1233–1243. doi: 10.1074/mcp.M116.061416.
421. Yang, C. *et al.* (2014) 'Glutamine oxidation maintains the TCA cycle and cell survival during impaired mitochondrial pyruvate transport.', *Molecular cell*, 56(3), pp. 414–24. doi: 10.1016/j.molcel.2014.09.025.
422. Yang, H.-C., Stern, A. and Chiu, D. T.-Y. (2020) 'G6PD: A hub for metabolic reprogramming and redox signaling in cancer', *Biomedical Journal*. doi: 10.1016/j.bj.2020.08.001.
423. Yang, L. *et al.* (2014) 'Vascular endothelial growth factor promotes fibrosis resolution and repair in mice.', *Gastroenterology*, 146(5), pp. 1339–50.e1. doi: 10.1053/j.gastro.2014.01.061.
424. Yang, W. H. *et al.* (2006) 'Modification of p53 with O-linked N-acetylglucosamine regulates p53 activity and stability', *Nature Cell Biology*, 8(10), pp. 1074–1083. doi: 10.1038/ncb1470.
425. Yang, W. H. *et al.* (2008) 'NF B activation is associated with its O-GlcNAcylation state under hyperglycemic conditions', *Proceedings of the National Academy of Sciences*, 105(45), pp. 17345–17350. doi: 10.1073/pnas.0806198105.
426. Yang, X. *et al.* (2008) 'Phosphoinositide signalling links O-GlcNAc transferase to insulin resistance.', *Nature*, 451(7181), pp. 964–9. doi: 10.1038/nature06668.
427. Yang, X. and Qian, K. (2017) 'Protein O-GlcNAcylation: Emerging mechanisms and functions', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 18(7), pp. 452–465. doi: 10.1038/nrm.2017.22.
428. Yang, X., Zhang, F. and Kudlow, J. E. (2002) 'Recruitment of O-GlcNAc transferase to promoters by corepressor mSin3A: Coupling protein O-GlcNAcylation to transcriptional repression', *Cell*, 110(1), pp. 69–80. doi: 10.1016/S0092-8674(02)00810-3.
429. Yang, Y. *et al.* (2015) 'Histone Demethylase LSD2 Acts as an E3 Ubiquitin Ligase and Inhibits Cancer Cell Growth through Promoting Proteasomal Degradation of OGT', *Molecular Cell*, 58(1), pp. 47–59. doi:

10.1016/j.molcel.2015.01.038.

430. Yang, Y. *et al.* (2020) 'O-GlcNAc transferase inhibits visceral fat lipolysis and promotes diet-induced obesity.', *Nature communications*, 11(1), p. 181. doi: 10.1038/s41467-019-13914-8.
431. Yi Chen, Q., Costa, M. and Sun, H. (2015) 'Structure and function of histone acetyltransferase MOF', *AIMS Biophysics*, 2(4), pp. 555–569. doi: 10.3934/biophy.2015.4.555.
432. Yi, Wen *et al.* (2012) 'Phosphofructokinase 1 glycosylation regulates cell growth and metabolism.', *Science (New York, N.Y.)*, 337(6097), pp. 975–80. doi: 10.1126/science.1222278.
433. Yi, W. *et al.* (2012) 'Phosphofructokinase 1 Glycosylation Regulates Cell Growth and Metabolism', *Science*, 337(6097), pp. 975–980. doi: 10.1126/science.1222278.
434. Yildirim, E. *et al.* (2013) 'Xist RNA is a potent suppressor of hematologic cancer in mice.', *Cell*, 152(4), pp. 727–42. doi: 10.1016/j.cell.2013.01.034.
435. Yoon, J. C. *et al.* (2001) 'Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1.', *Nature*, 413(6852), pp. 131–8. doi: 10.1038/35093050.
436. Zammit, V. A. (2008) 'Carnitine palmitoyltransferase 1: Central to cell function', *IUBMB Life*, 60(5), pp. 347–354. doi: 10.1002/iub.78.
437. Zeke, A. *et al.* (2016) 'JNK Signaling: Regulation and Functions Based on Complex Protein-Protein Partnerships', *Microbiology and Molecular Biology Reviews*, 80(3), pp. 793–835. doi: 10.1128/MMBR.00043-14.
438. Zeng, W. *et al.* (2013) 'A new method to isolate and culture rat kupffer cells.', *PloS one*, 8(8), p. e70832. doi: 10.1371/journal.pone.0070832.
439. Zhang, B., Li, M.-D., *et al.* (2019) 'O-GlcNAc transferase suppresses necroptosis and liver fibrosis.', *JCI insight*, 4(21). doi: 10.1172/jci.insight.127709.
440. Zhang, B., Li, M. D., *et al.* (2019) 'O-GlcNAc transferase suppresses necroptosis and liver fibrosis', *JCI Insight*, 4(21), pp. 1–16. doi: 10.1172/jci.insight.127709.
441. Zhang, F., Lu, Y. and Zheng, S. (2012) 'Peroxisome proliferator-activated receptor- $\gamma$  cross-regulation of signaling events implicated in liver fibrogenesis', *Cellular Signalling*, 24(3), pp. 596–605. doi: 10.1016/j.cellsig.2011.11.008.
442. Zhang, J. *et al.* (2014) 'The histone acetyltransferase hMOF suppresses hepatocellular carcinoma growth', *Biochemical and Biophysical Research Communications*, 452(3), pp. 575–580. doi: 10.1016/j.bbrc.2014.08.122.

443. Zhang, K., Yin, R. and Yang, X. (2014) 'O-GlcNAc: A bittersweet switch in liver', *Frontiers in Endocrinology*, 5(DEC), pp. 1–7. doi: 10.3389/fendo.2014.00221.
444. Zhang, N. *et al.* (2010) 'The Merlin/NF2 tumor suppressor functions through the YAP oncoprotein to regulate tissue homeostasis in mammals.', *Developmental cell*, 19(1), pp. 27–38. doi: 10.1016/j.devcel.2010.06.015.
445. Zhang, Q. *et al.* (2014) 'Differential Regulation of the Ten-Eleven Translocation (TET) Family of Dioxygenases by O -Linked  $\beta$ - N -Acetylglucosamine Transferase (OGT)', *Journal of Biological Chemistry*, 289(9), pp. 5986–5996. doi: 10.1074/jbc.M113.524140.
446. Zhang, S. *et al.* (2011) 'Modification of histones by sugar  $\beta$ -N-acetylglucosamine (GlcNAc) occurs on multiple residues, including histone H3 serine 10, and is cell cycle-regulated', *Journal of Biological Chemistry*, 286(43), pp. 37483–37495. doi: 10.1074/jbc.M111.284885.
447. Zhang, T. *et al.* (2020) 'Symphony of epigenetic and metabolic regulation—interaction between the histone methyltransferase EZH2 and metabolism of tumor', *Clinical Epigenetics*, 12(1), p. 72. doi: 10.1186/s13148-020-00862-0.
448. Zhang, W. *et al.* (2006) 'FoxO1 Regulates Multiple Metabolic Pathways in the Liver', *Journal of Biological Chemistry*, 281(15), pp. 10105–10117. doi: 10.1074/jbc.M600272200.
449. Zhang, W. *et al.* (2016) 'Integrated Regulation of Hepatic Lipid and Glucose Metabolism by Adipose Triacylglycerol Lipase and FoxO Proteins', *Cell Reports*, 15(2), pp. 349–359. doi: 10.1016/j.celrep.2016.03.021.
450. Zhang, X. *et al.* (2015) 'MAPK/ERK signaling pathway-induced hyper-O-GlcNAcylation enhances cancer malignancy', *Molecular and Cellular Biochemistry*, 410(1–2), pp. 101–110. doi: 10.1007/s11010-015-2542-8.
451. Zhang, X., Yang, S., *et al.* (2019) 'Unraveling the regulation of hepatic gluconeogenesis', *Frontiers in Endocrinology*, 10(JAN). doi: 10.3389/fendo.2018.00802.
452. Zhang, Y. *et al.* (2006) 'Activation of the nuclear receptor FXR improves hyperglycemia and hyperlipidemia in diabetic mice.', *Proceedings of the National Academy of Sciences of the United States of America*, 103(4), pp. 1006–11. doi: 10.1073/pnas.0506982103.
453. Zhang, Z., Deng, X., *et al.* (2019) 'PKM2, function and expression and regulation', *Cell & Bioscience*, 9(1), p. 52. doi: 10.1186/s13578-019-0317-8.

454. Zhao, M. *et al.* (2018) 'Deficiency in intestinal epithelial O-GlcNAcylation predisposes to gut inflammation', *EMBO Molecular Medicine*, 10(8). doi: 10.15252/emmm.201708736.
455. Zhao, M. *et al.* (2020) 'Protein O-GlcNAc Modification Links Dietary and Gut Microbial Cues to the Differentiation of Enteroendocrine L Cells', *Cell Reports*, 32(6), p. 108013. doi: 10.1016/j.celrep.2020.108013.
456. Zhao, P. *et al.* (2011) 'Combining High-Energy C-Trap Dissociation and Electron Transfer Dissociation for Protein O-GlcNAc Modification Site Assignment', *Journal of Proteome Research*, 10(9), pp. 4088–4104. doi: 10.1021/pr2002726.
457. Zheng, D. *et al.* (2020) 'Pyruvate Kinase M2 Tetramerization Protects against Hepatic Stellate Cell Activation and Liver Fibrosis', *The American Journal of Pathology*. doi: 10.1016/j.ajpath.2020.08.002.
458. Zhou, P. *et al.* (2013) 'Mixed Lineage Leukemia 5 (MLL5) Protein Regulates Cell Cycle Progression and E2F1-responsive Gene Expression via Association with Host Cell Factor-1 (HCF-1)', *Journal of Biological Chemistry*, 288(24), pp. 17532–17543. doi: 10.1074/jbc.M112.439729.
459. Zhu, G. *et al.* (2016) 'O-GlcNAcylation of histone deacetylases 1 in hepatocellular carcinoma promotes cancer progression', *Glycobiology*, 26(8), pp. 820–833. doi: 10.1093/glycob/cww025.
460. Zhu, Q. *et al.* (2012) 'O-GlcNAcylation plays a role in tumor recurrence of hepatocellular carcinoma following liver transplantation', *Medical Oncology*, 29(2), pp. 985–993. doi: 10.1007/s12032-011-9912-1.
461. Zografos, G. N. *et al.* (2011) 'Cholangiocarcinoma: principles and current trends.', *Hepatobiliary & pancreatic diseases international: HBPD INT*, 10(1), pp. 10–20. doi: 10.1016/s1499-3872(11)60001-5.

# **Annex**

**Review article: Carbohydrate sensing through  
the transcription factor ChREBP**



# Carbohydrate Sensing Through the Transcription Factor ChREBP

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Carbohydrate response element binding protein (ChREBP) is a carbohydrate-signaling transcription factor that in the past years has emerged as a central metabolic regulator. ChREBP expression is mostly abundant in active sites of *de novo* lipogenesis including liver and white and brown adipose tissues. ChREBP is also expressed in pancreatic islets, small intestine and to a lesser extent in the kidney and the brain. In response to glucose, ChREBP undergoes several post-translational modifications (PTMs) (phosphorylation, acetylation and/or O-GlcNAcylation) that will either modulate its cellular location, stability and/or its transcriptional activity. ChREBP $\beta$  is a shorter isoform of ChREBP that was first described in adipose tissue and later found to be expressed in other sites including liver and pancreatic  $\beta$  cells. ChREBP $\beta$  lacks an important regulatory inhibitory domain, known as LID (low glucose inhibitory domain), in its N-terminal domain and is therefore reported as a highly active isoform. In this review, we recapitulate a recent progress concerning the mechanisms governing the activity of the ChREBP isoforms, including PTMs, partners/cofactors as well as novel metabolic pathways regulated by ChREBP in key metabolic tissues, by discussing phenotypes associated with tissue-specific deletion of ChREBP in knockout mice.

**Keywords:** ChREBP, carbohydrate sensing, transcriptional regulation, metabolism, insulin sensitivity

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## INTRODUCTION

Increased consumption of simple sugars such as sucrose and high-fructose corn syrup in recent years has led to an increased risk of metabolic diseases such as obesity, dyslipidemia, type 2 diabetes and/or nonalcoholic fatty liver disease (NAFLD). The liver is the principal organ responsible for the conversion of excess dietary carbohydrates into fat. The resulting triglycerides (TG) can be packed into very low density lipoproteins (VLDL) and either secreted into the circulation, stored as lipid droplets, or metabolized through the beta-oxidation pathway. Insulin secreted in response to elevated blood glucose, stimulates the expression of genes of *de novo* fatty acid synthesis (lipogenesis) through the transcription factor sterol regulatory element binding protein-1c (SREBP-1c) (Foretz et al., 1999). SREBP-1c acts in synergy with another transcription factor called carbohydrate response element binding protein (ChREBP), which mediates the response to dietary carbohydrates. The ChREBP protein structure contains a low glucose inhibitory domain (LID) and a glucose-response activation conserved element (GRACE) located in its N-terminus (Li et al., 2006). Activation of the GRACE domain by glucose metabolites promotes ChREBP transcriptional activity and binding to a highly conserved sequence called carbohydrate response element (ChoRE). ChoRE is present on the promoters of ChREBP

target genes, which encode key enzymes of *de novo* lipogenesis including L-pyruvate kinase (*L-pk*), a rate-limiting enzyme in glycolysis, fatty acid synthase (*Fas*), acetyl-CoA carboxylase (*Acc*) and stearoyl-CoA desaturase (*Scd1*) (Kawaguchi et al., 2001). A recent study reported the interdependence between ChREBP (activated by glucose) and SREBP-1c (activated by insulin) for the full induction of glycolytic and lipogenic gene expression in liver (Linden et al., 2018). Viral re-establishment of the nuclear active form of SREBP-1c in the liver of ChREBP-deficient mice (ChREBP<sup>KO</sup>) normalized lipogenic gene expression, while having no effect on rescuing glycolytic gene expression. The mirror experiment, in which ChREBP expression was induced in the liver of SREBP-1c knockout mice, rescued glycolytic gene expression but surprisingly not lipogenic gene expression, despite the well-known role of ChREBP in the control of fatty acid synthesis genes. Nevertheless, this study suggests the importance of the dual action of ChREBP and SREBP-1c in the control genes involved in the regulation of fatty acid synthesis (Linden et al., 2018).

ChREBP is highly enriched in the liver and has been studied as a master regulator of lipid metabolism (Iizuka et al., 2004; Osorio et al., 2016). ChREBP is also significantly expressed in pancreatic islets, small intestine, skeletal muscle and to a lesser extent in the kidney and the brain (see Richards et al., 2017 for review). Interestingly, another isoform of ChREBP, ChREBP $\beta$ , originating from an alternative first exon promoter, was first identified in adipose tissue (Herman et al., 2012) and later described in other cell types (see Abdul-Wahed et al., 2017 for review). As we will discuss, ChREBP $\beta$  is described as a constitutively active isoform. It is hoped that future work will address the respective roles of ChREBP and ChREBP $\beta$  in the regulation of glucose and lipid metabolism as well as identify their specific and/or overlapping targets.

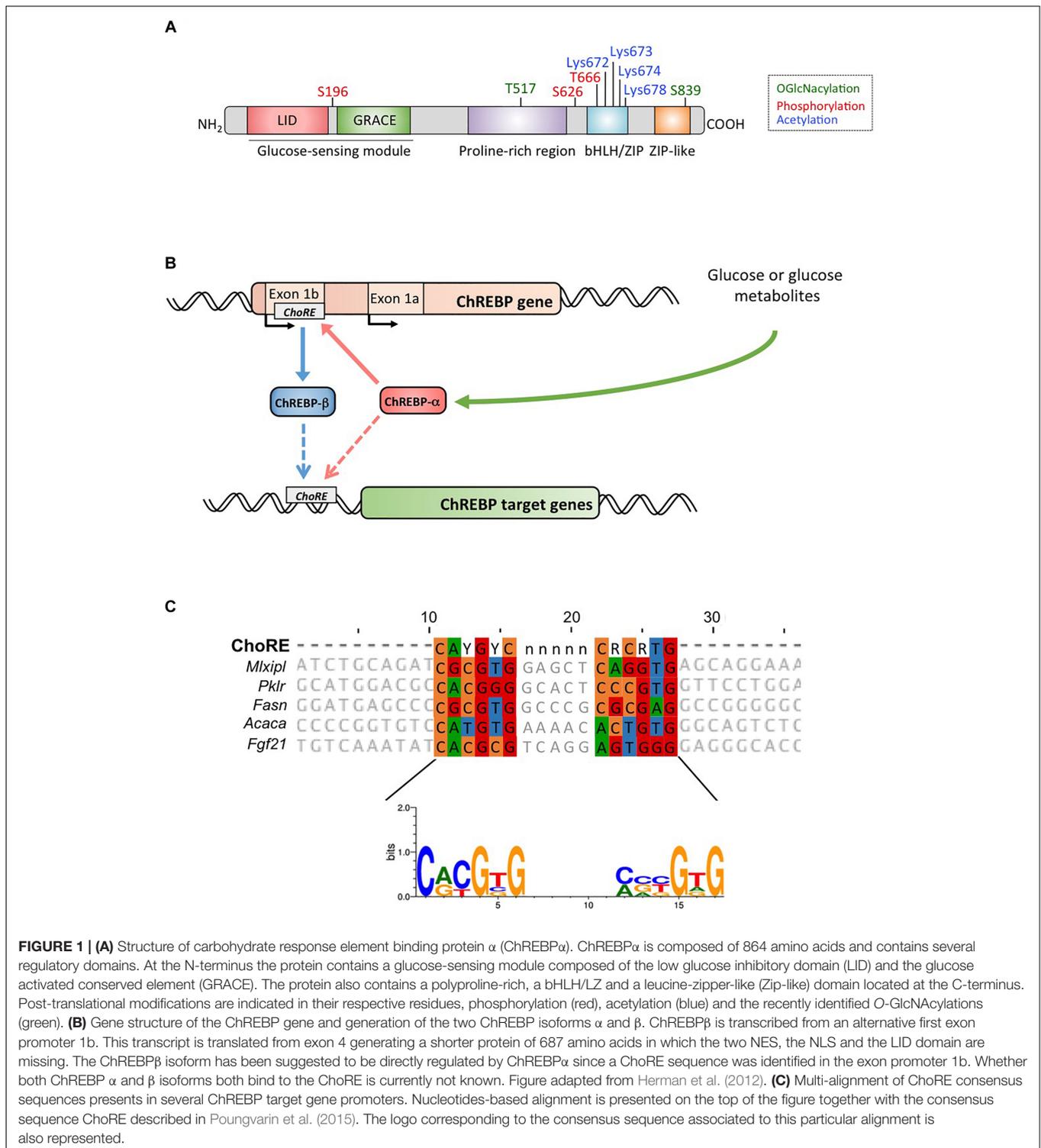
## ChREBP STRUCTURE AND REGULATION VIA THE LID/GRACE DOMAINS

ChREBP belongs to the Mondo family of bHLH/Zip transcription factors. The N-terminus domain (1-251 residues) contains two nuclear export signal (NES) and a nuclear localization signal (NLS) regulating subcellular localization by interacting with chromosome region maintenance 1 (CRM1) also referred to as exportin 1 and/or 14-3-3 proteins (Sakiyama et al., 2008). The C-terminus region contains a polyproline domain, a bHLH/LZ domain (660-737 residues) and a leucine zipper-like domain (Zip-like, 807-847 residues) that are associated with co-factors and DNA binding (Yamashita et al., 2001; Fukasawa et al., 2010; Ge et al., 2012). Localization and transcriptional activation of ChREBP are determined by nutrient availability. Glucose-mediated regulation of ChREBP occurs mostly at the level of the glucose-sensing module (GSM) or mondo conserved region (MCR), which is composed of the LID and the GRACE domains, as mentioned in the introduction (Figure 1A; Li et al., 2006; Singh and Irwin, 2016). In 2012, Herman et al. (2012) described

another ChREBP isoform, ChREBP $\beta$ , that is transcribed from an alternative first exon promoter 1b to exon 2 (Figure 1B). This transcript is translated from exon 4, generating a shorter protein of 687 amino acids (the full length ChREBP isoform, renamed  $\alpha$ , contains 864 amino acids, called ChREBP in the manuscript) in which the two NES, NLS and the LID domain are missing. ChREBP $\beta$  is highly active in white adipose tissue in a GLUT-4 dependent manner and is suggested to be directly regulated by ChREBP $\alpha$  since a ChoRE sequence was identified in the exon promoter 1 $\beta$  (Herman et al., 2012; Figure 1B). The regulation of ChREBP $\beta$  by ChREBP $\alpha$  suggests the existence of a feed-forward loop that potentially exacerbates the response to glucose under hyperglycemic conditions. However, the regulatory mechanism(s) of the ChREBP $\beta$  isoform, and more importantly its specific function, need to be elucidated.

## ACTIVATION OF ChREBP BY GLUCOSE METABOLITES

Under fasting conditions, the glucagon-dependent activation of protein kinase A (PKA) (Kawaguchi et al., 2002) phosphorylates ChREBP on residues Ser196 and Thr666, leading to ChREBP binding to the protein 14-3-3 and its retention in the cytosol (Kawaguchi et al., 2001, 2002; Davies et al., 2008). AMP activated protein kinase (AMPK), a central cellular energy sensor, also phosphorylates ChREBP on residue Ser568, which in turn decreases binding of ChREBP to promoters of its target genes (Kawaguchi et al., 2002; Sato et al., 2016). It was demonstrated that metabolites generated during fasting, such as AMP and ketone bodies produced from fatty acid oxidation, play an allosteric inhibitory role by altering ChREBP and 14-3-3 protein affinity, enhancing complex stabilization and favoring cytosolic retention (Sakiyama et al., 2008; Nakagawa et al., 2013; Sato et al., 2016). In response to carbohydrates, ChREBP is regulated at the transcriptional, translational and post-translational levels. Increased glucose concentrations after a meal promote the synthesis of intermediate metabolites such as xylulose-5-phosphate (X5P), initially proposed as an activator of protein phosphatase 2A (PP2A) (Kawaguchi et al., 2001; Kabashima et al., 2003). PP2A was previously described to dephosphorylate ChREBP at the Ser196 residue allowing its translocation to the nucleus where it is further dephosphorylated in a X5P- and PP2A-dependent manner (on Thr666 and Ser568). However, this model was challenged over the years and other metabolites such as glucose-6-phosphate (G6P) were proposed to be potential activators of ChREBP translocation/activity (Dentin et al., 2012). McFerrin et al. (2012) identified a putative motif for G6P binding (253-SDTLFT-258) on the GRACE domain, which is also conserved in MondoA, a ChREBP/MondoB ortholog (see Richards et al., 2017 for review). According to this hypothesis, G6P could promote an allosteric conformation change that induces an open conformation for ChREBP, facilitating the interaction with co-factors and subsequent translocation to the nucleus (McFerrin et al., 2012).



**FIGURE 1 | (A)** Structure of carbohydrate response element binding protein  $\alpha$  (ChREBP $\alpha$ ). ChREBP $\alpha$  is composed of 864 amino acids and contains several regulatory domains. At the N-terminus the protein contains a glucose-sensing module composed of the low glucose inhibitory domain (LID) and the glucose activated conserved element (GRACE). The protein also contains a polyproline-rich, a bHLH/LZ and a leucine-zipper-like (Zip-like) domain located at the C-terminus. Post-translational modifications are indicated in their respective residues, phosphorylation (red), acetylation (blue) and the recently identified O-GlcNAcylation (green). **(B)** Gene structure of the ChREBP gene and generation of the two ChREBP isoforms  $\alpha$  and  $\beta$ . ChREBP $\beta$  is transcribed from an alternative first exon promoter 1b. This transcript is translated from exon 4 generating a shorter protein of 687 amino acids in which the two NES, the NLS and the LID domain are missing. The ChREBP $\beta$  isoform has been suggested to be directly regulated by ChREBP $\alpha$  since a ChoRE sequence was identified in the exon promoter 1b. Whether both ChREBP  $\alpha$  and  $\beta$  isoforms both bind to the ChoRE is currently not known. Figure adapted from Herman et al. (2012). **(C)** Multi-alignment of ChoRE consensus sequences presents in several ChREBP target gene promoters. Nucleotides-based alignment is presented on the top of the figure together with the consensus sequence ChoRE described in Pongvarin et al. (2015). The logo corresponding to the consensus sequence associated to this particular alignment is also represented.

Within the nucleus, ChREBP can be modified through O-GlcNAcylation, a post-translational modification dependent on glucose metabolism, and identified to be important for ChREBP transcriptional activity (Guinez et al., 2010). O-GlcNAcylation occurs on serine and threonine residues through

the activity of O-GlcNAc transferase (OGT), an enzyme that adds N-acetylglucosamine (GlcNAc) residues to target proteins thereby modifying their activity, stability and/or subcellular location. Yang et al. (2017) recently revealed several ChREBP residues modified by O-GlcNAcylation. Mutations

of these residues at the bHLH/ZIP and dimerization and cytoplasmic location domain (DCD) domains have allowed for the identification of Thr517 and Ser839 as essential sites for the glucose-dependent activation of ChREBP (Figure 1A). ChREBP can also be modified by acetylation via the histone acetyltransferase activity of p300 (Bricambert et al., 2010). Glucose-activated p300 acetylates ChREBP on Lys672 and increases its transcriptional activity by enhancing its recruitment to the ChoRE sequence, which's optimal consensus binding sequence is CAYGYCnnnnnCRCRTG (Figure 1C). Pongvarin et al. (2015) analyzed ChREBP binding sites by ChIP-seq in liver and white adipose tissue of mice re-fed with a high-carbohydrate, fat-free diet. They reported that ChREBP binding is enriched in pathways involved in insulin signaling, adherent junctions and cancer, suggesting a novel involvement of ChREBP in tumorigenesis and cancer progression. Further, a recent study reported the importance of ChREBP in hepatocellular carcinoma (HCC) (Ribback et al., 2017). The authors found that genetic deletion of ChREBP (in ChREBP<sup>KO</sup> mice) impaired hepatocarcinogenesis driven by protein kinase B/Akt overexpression in mice. Furthermore, siRNA-mediated inhibition of ChREBP in mouse and/or human HCC cells resulted in decreased proliferation and apoptosis.

## ChREBP CO-FACTORS AND PARTNERS

Several co-factors and/or partners of ChREBP were identified over the past years (see Richards et al., 2017 for review). Max like protein x (Mlx), a bHLH/LZ transcription factor, was the first identified as a common binding partner of the Mondo family (Stoeckman et al., 2004). Dimerization of ChREBP with Mlx is required for both nuclear translocation in response to glucose and binding to ChoRE elements. Nuclear receptors such as hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ) and farnesoid X receptor (FXR) were also described as ChREBP partners. HNF4 $\alpha$  physically interacts with ChREBP by binding to the direct repeat-1 (DR-1) region on the promoter of ChREBP target genes (Adamson et al., 2006; Meng et al., 2016). Moreover, the p300/CBP transcriptional co-activator proteins were shown to stabilize the ChREBP/HNF4 $\alpha$  complex (Burke et al., 2009). The p300/CBP transcriptional co-activator proteins play a central role in coordinating and integrating multiple signal-dependent events with the transcription apparatus. Another key property of p300/CBP is the presence of histone acetyltransferase (HAT) activity, which endows p300/CBP with the capacity to influence chromatin activity by modulating nucleosomal histones. In human hepatocytes, FXR binding to the ChREBP-HNF4 $\alpha$  complex triggers the release of ChREBP from CBP/p300, leading to the recruitment of the histone deacetylase SMRT on the *Lpk* promoter, thereby acting as a co-repressor of ChREBP transcriptional activity (Caron et al., 2013). In addition, CBP/p300 HAT activity modifies ChREBP on Lys 672, leading to its transcriptional activation in response to glucose (Bricambert et al., 2010).

Bricambert et al. (2018) recently identified the histone demethylase plant homeodomain finger 2 (Phf2), which belongs

to the histone lysine demethylase (KDM7) family, as a novel co-factor of ChREBP. Interaction between Phf2 and ChREBP enhances ChREBP transcriptional activation by erasing H3K9 methyl-marks on the promoter of its target genes. Interestingly, specific co-recruitment of Phf2 and ChREBP to the promoter of nuclear factor erythroid 2 like 2 (Nrf2) contributes to the protective effect of Phf2 against increased reactive oxygen species (ROS) and NAFLD progression in the context of hyperglycemia (Bricambert et al., 2018).

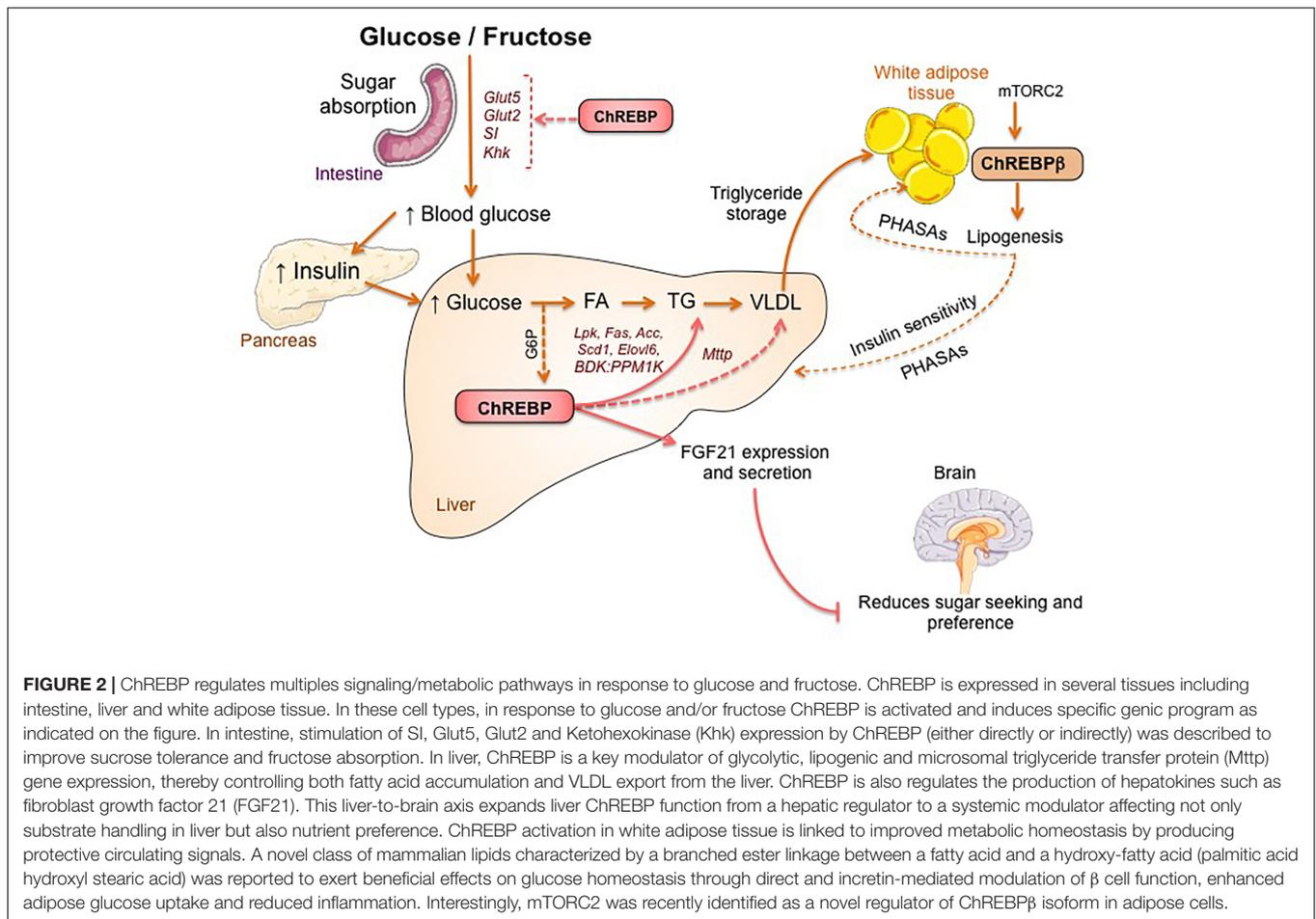
## ROLE OF ChREBP IN CARBOHYDRATE METABOLISM AND HEPATOKINE PRODUCTION

### ChREBP as Regulator of Hepatic Fatty Acid Synthesis and VLDL Secretion

Non-alcoholic fatty liver disease is a hallmark of metabolic syndrome, and studies in humans reveal that *de novo* lipogenesis contributes to about 25% of total liver lipids in patients with NAFLD (Donnelly et al., 2005). In insulin resistant states, hyperglycemia and hyperinsulinemia enhance lipogenesis partly through the activation of ChREBP and SREBP-1c. ChREBP inhibition in liver of obese and insulin resistant *ob/ob* mice, through RNAi or genetic ablation leads to reversal of hepatic steatosis (Dentin et al., 2006; Iizuka et al., 2006). Altered secretion of VLDL by the liver also contributes to the pathogenesis of NAFLD. Microsomal triglyceride transfer protein (MTTP) is the protein in charge of assembly and secreting apolipoprotein B-containing lipoproteins. Deficiency of MTTP in mice and humans causes hypolipidemia and fatty liver. Regulation of this protein has been associated with a few highly conserved cis-elements in its promoter including critical positive [HNF1, HNF4, DR-1 and forkhead box (FOX)] and negative [regulatory sterol and insulin response elements (SRE/IRE)] regulatory domains (Cuchel et al., 2013; Hussain et al., 2011). Recently, ChREBP was pointed out as a potential regulator of MTTP since lack of functional ChREBP in liver suppresses *Mttp* expression and VLDL assembly and secretion (Niwa et al., 2018). However, since no ChoRE could be clearly identified on the *Mttp* promoter, further analysis will be needed to identify the mechanism with which ChREBP regulates *Mttp*.

### Regulation of Fructose Metabolism by ChREBP in Liver and Intestine

The link between ChREBP and fructose metabolism was first evidenced by the phenotypic analysis of ChREBP knockout mice (ChREBP<sup>KO</sup> mice). ChREBP<sup>KO</sup> mice were reported to die within several days of high-fructose diet (HFrD) feeding (Iizuka et al., 2004). This major intolerance to fructose was attributed to the reduction in the expression of fructokinase and triose kinase, two enzymes required for fructose metabolism (Iizuka et al., 2004). Kim et al. (2016) later reported the importance of ChREBP for the efficient conversion of fructose to glucose in liver and whole-body fructose clearance but also, under



**FIGURE 2 |** ChREBP regulates multiples signaling/metabolic pathways in response to glucose and fructose. ChREBP is expressed in several tissues including intestine, liver and white adipose tissue. In these cell types, in response to glucose and/or fructose ChREBP is activated and induces specific genic program as indicated on the figure. In intestine, stimulation of SI, Glut5, Glut2 and Ketoheokinase (Khk) expression by ChREBP (either directly or indirectly) was described to improve sucrose tolerance and fructose absorption. In liver, ChREBP is a key modulator of glycolytic, lipogenic and microsomal triglyceride transfer protein (Mttp) gene expression, thereby controlling both fatty acid accumulation and VLDL export from the liver. ChREBP is also regulates the production of hepatokines such as fibroblast growth factor 21 (FGF21). This liver-to-brain axis expands liver ChREBP function from a hepatic regulator to a systemic modulator affecting not only substrate handling in liver but also nutrient preference. ChREBP activation in white adipose tissue is linked to improved metabolic homeostasis by producing protective circulating signals. A novel class of mammalian lipids characterized by a branched ester linkage between a fatty acid and a hydroxy-fatty acid (palmitic acid hydroxyl stearic acid) was reported to exert beneficial effects on glucose homeostasis through direct and incretin-mediated modulation of  $\beta$  cell function, enhanced adipose glucose uptake and reduced inflammation. Interestingly, mTORC2 was recently identified as a novel regulator of ChREBP $\beta$  isoform in adipose cells.

ingestion of fructose, ChREBP could contribute to hyperglycemia by directly trans-activating *G6pc* expression, a key gene of gluconeogenesis. This effect could lead to a vicious cycle in which fructose consumption exacerbates glucose production though ChREBP activity (Kim et al., 2016). The following year, the study by Zhang et al. (2017) reported that ChREBP<sup>KO</sup> mice fed with HFrD develop severe liver injury due to over-activation of endoplasmic reticulum stress and CCAAT-enhancer-binding protein homologous protein (CHOP)-mediated hepatocyte apoptosis. Apoptosis in hepatocytes in these mice was most likely linked to increased cholesterol biosynthesis since inhibition of this pathway via HMG-CoA reductase (HMGCR) or SREBP2 inhibition rescued ChREBP<sup>KO</sup> mice from HFrD-induced liver injury. A lack of ChREBP was also recently associated with a dysregulation of sucrose and fructose metabolism leading to sugar intolerance and malabsorption in mice (Kato et al., 2018). These effects were associated with decreased expression of intestinal sucrose-isomaltase (SI), which digests sucrose in glucose and fructose, the glucose transporters 5 (Glut5) and 2 (Glut2) and the ketoheokinase (Khk) enzyme, which regulates fructolysis (Figure 2). Dysregulation of these enzymes may lead to the accumulation of undigested sucrose and fructose with potential repercussions in gut microbiota composition. The comparison between ChREBP<sup>KO</sup> and liver-specific ChREBP

knockout (ChREBP<sup>LiverKO</sup>) mice fed with HFrD had previously revealed that hepatic ChREBP deficiency alone does not lead to fructose intolerance but that ChREBP deficiency in the small intestine is most likely responsible for the impairment in fructose tolerance observed in these mice (Kim et al., 2017). Altogether, these studies underline the importance of ChREBP in the regulation of fructose metabolism and underscore the need for a better understanding of its role and regulation in the small intestine.

## Regulation of the BDK:PPM1K Axis in Liver

The first committed step of branched-chain amino acid (BCAAs) catabolism is regulated by the branched-chain ketoacid dehydrogenase (BCKDH) complex which is controlled by two enzymes, the branched-chain alpha-keto acid dehydrogenase kinase (BDK) and the protein phosphatase,  $Mg^{2+}/Mn^{2+}$  dependent 1K (PPM1K). White et al. (2018) recently associated ChREBP with the upregulation of BDK and down-regulation of PPM1K in liver and identified a conserved ChoRE motif in the promoter of both of these genes. A positive correlation between the expression of BDK and other typical ChREBP target genes (*Fasn*, *Pklr*, ChREBP $\beta$ ) was observed in livers of rats fed

with a high glucose or fructose diet. At the physiological level, increase in the BDK:PPM1K ratio led to the phosphorylation and activation of ATP-citrate lyase (ACLY), thereby stimulating *de novo* lipogenesis. These findings reveal that BDK and PPM1K may be novel lipogenesis-activating genes regulated by ChREBP $\beta$ . Given their role in the regulation of lipid, glucose and amino acid metabolism, BDK and PPM1K could be considered as potential therapeutic targets in the liver in the near future (White et al., 2018).

## ChREBP Is Required for the Glucose-Mediated Regulation of FGF21

ChREBP was recently associated to the production and secretion of hepatokines such as Fibroblast growth factor 21 (FGF21) (Iizuka et al., 2009; Dushay et al., 2015; Iroz et al., 2017). FGF21 is a metabolic hormone synthesized by the liver with multiple beneficial effects in peripheral tissues (Kharitonov et al., 2005; Badman et al., 2007; Markan et al., 2014). Until recently, FGF21 was considered as a fasting hormone that enhances fatty acid oxidation, ketogenesis and lipolysis under the transcriptional control of peroxisome proliferator activated receptor  $\alpha$  (PPAR $\alpha$ ) (Inagaki et al., 2007). A ChoRE on the *Fgf21* promoter has been previously identified in both mice (−74 to −52 bp) and humans (−380 to −366 bp) (Iizuka et al., 2009) but functional studies have been lacking until recently. Consumption of glucose and fructose was reported to lead to a rapid elevation of FGF21 levels in healthy volunteers and metabolic syndrome patients (Dushay et al., 2015). Additional studies also reported a mechanistic link between ChREBP-derived FGF21 and macronutrient preference through a liver-brain axis (Talukdar et al., 2016; von Holstein-Rathlou et al., 2016). This liver-to-brain axis expands ChREBP function from a hepatic metabolic regulator to a systemic modulator, affecting not only liver substrate handling but also global feeding behavior (Abdul-Wahed et al., 2017).

## ROLE OF ChREBP IN THE INTER-ORGAN NETWORK THAT CONTROLS ENERGY HOMEOSTASIS

### Role of Hepatic ChREBP in the Control of Insulin Sensitivity Balance

Our laboratory previously reported that ChREBP acts as a key modulator of hepatic fatty acid composition and insulin sensitivity in the context of non-alcoholic and alcoholic liver diseases (see Abdul-Wahed et al., 2017 for review). Mice overexpressing ChREBP developed greater hepatic steatosis than controls, but interestingly stayed free of metabolic complications and did not develop insulin resistance. Lipidomic analysis has revealed that ChREBP-mediated steatosis is associated with a decrease in saturated fatty acids and an increase in monounsaturated fatty acids, the latter which have been shown to be associated with ChREBP-mediated beneficial effects on insulin sensitivity (Benhamed et al., 2012). These results demonstrate the role of ChREBP in lipid partitioning

and suggest that specific lipid species, when present in the proper location and time, may trigger signals that modulate adaptation to metabolic stress (Benhamed et al., 2012; Bricambert et al., 2018). Interestingly, Jois et al. (2017) also suggested a protective role for hepatic ChREBP regarding whole body glucose homeostasis and insulin sensitivity. ChREBP<sup>LiverKO</sup> mice exhibit worsened glucose tolerance, while protected from hepatic steatosis. Hepatic ChREBP deletion also resulted in gene expression changes in white and brown adipose tissues, suggesting inter-tissue communication. The contribution of ChREBP to whole body energy balance may therefore rely on its regulation of lipid species and/or hepatokine production contributing to inter tissue coordination of energy homeostasis (Jois et al., 2017).

### Adipose ChREBP Links Lipogenesis to Insulin Sensitivity

Impaired insulin signaling in adipose tissue is a critical feature of insulin resistance. Studies have reported that ChREBP activation in white adipose tissue can improve metabolic homeostasis by producing protective circulating signals (Yore et al., 2014; Tang et al., 2016). A class of mammalian lipids characterized by a branched ester linkage between a fatty acid and a hydroxy-fatty acid, palmitic acid hydroxyl stearic acid (PAHSA), was reported to exert beneficial effects on glucose homeostasis through direct and incretin-mediated modulation of  $\beta$  cell function, glucose uptake and reduction in inflammation (Yore et al., 2014). Similarly, adipose-specific ChREBP knockout (ChREBP<sup>adiposeKO</sup>), which exhibit low lipogenesis rates in adipose tissue, are insulin resistant with impaired insulin action in liver, muscle and white adipose tissue under both chow and high fat diet conditions. ChREBP<sup>adiposeKO</sup> mice have lower serum levels of PAHSAs, while PAHSA supplementation, in particular the 9-PAHSA isomer, rescues ChREBP<sup>adiposeKO</sup> global insulin resistance and adipose tissue inflammation, confirming that loss of adipose-ChREBP is sufficient to cause insulin resistance (Vijayakumar et al., 2017). A recent study identified the mechanistic target of rapamycin complex 2 (mTORC2) as a novel regulator of ChREBP (especially the  $\beta$  isoform) in adipose cells. Specific ablation of rapamycin-insensitive companion of mTOR (Rictor) in mature adipocytes impaired insulin-stimulated glucose uptake in adipose tissue leading to the down-regulation of ChREBP $\beta$  and target gene expression involved in lipogenesis control (Tang et al., 2016). In agreement with an important adipose–liver crosstalk mediated by ChREBP, these effects are associated with hepatic insulin resistance and enhanced gluconeogenesis. Altogether, these studies support an important role for adipose ChREBP in triggering insulin-sensitive signals (Tang et al., 2016).

### Novel Interaction Between Hormone-Sensitive Lipase and ChREBP in Adipose Tissue

ChREBP was recently identified as a partner of the lipolytic enzyme hormone-sensitive lipase (HSL) in adipose tissue (Morigny et al., 2019). Knockdown of HSL in human

adipocytes and mouse adipose tissue was shown to enhance insulin sensitivity and induce the elongation of very long chain fatty acid enzyme (Elovl6). Elovl6 is a microsomal enzyme that regulates the elongation of C12-16 saturated and monounsaturated fatty acids in a ChREBP-dependent manner (Morigny et al., 2019). At the mechanistic level, physical interaction between HSL and ChREBP impaired the nuclear translocation of ChREBP $\alpha$  and the subsequent induction of ChREBP $\beta$  and target genes, in particular Elovl6 (Morigny et al., 2019). This study reveals a novel regulation for ChREBP in adipose tissue. Inhibiting the interaction between HSL and ChREBP may lead to potential therapeutic strategies for improving insulin sensitivity in fat cells.

## CONCLUSION AND FUTURE DIRECTIONS

ChREBP is now a well-established carbohydrate sensor. Although most studies have been dedicated to its implication in the control of the glycolytic and lipogenic pathways, recent data have also unraveled novel contributions of ChREBP in hepatocytes and in fat cells where it could be instrumental in producing hepatokines and/or lipokines triggering inter-organ crosstalk. As discussed, newly identified co-factors

(epigenetic modifiers) and/or partners (adipose HSL) in these tissues may also represent potential therapeutic strategies for NAFLD and/or for improving systemic insulin sensitivity. Recent studies have also supported the importance of ChREBP in the regulation of fructose metabolism and have underscored the need for a better understanding of its role and regulation in the small intestine. Lastly, identifying specific and/or overlapping targets of ChREBP $\alpha$  and ChREBP $\beta$  in key cell types as well as determining their specific impact on insulin sensitivity will be of particular importance in the coming years.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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## REFERENCES

- Abdul-Wahed, A., Guilmeau, S., and Postic, C. (2017). Sweet sixteenth for ChREBP: established roles and future goals. *Cell Metab.* 26, 324–341. doi: 10.1016/j.cmet.2017.07.004
- Adamson, A. W., Suchankova, G., Rufo, C., Nakamura, M. T., Teran-Garcia, M., Clarke, S. D., et al. (2006). Hepatocyte nuclear factor-4 $\alpha$  contributes to carbohydrate-induced transcriptional activation of hepatic fatty acid synthase. *Biochem. J.* 399, 285–295. doi: 10.1042/BJ20060659
- Badman, M. K., Pissios, P., Kennedy, A. R., Koukos, G., Flier, J. S., and Maratos-Flier, E. (2007). Hepatic fibroblast growth factor 21 is regulated by PPAR $\alpha$  and is a key mediator of hepatic lipid metabolism in ketotic states. *Cell Metab.* 5, 426–437. doi: 10.1016/j.cmet.2007.05.002
- Benhamed, F., Denechaud, P. D., Lemoine, M., Robichon, C., Moldes, M., Bertrand-Michel, J., et al. (2012). The lipogenic transcription factor ChREBP dissociates hepatic steatosis from insulin resistance in mice and humans. *J. Clin. Invest.* 122, 2176–2194. doi: 10.1172/JCI41636
- Bricambert, J., Alves-Guerra, M. C., Esteves, P., Prip-Buus, C., Bertrand-Michel, J., Guillou, H., et al. (2018). The histone demethylase Phf2 acts as a molecular checkpoint to prevent NAFLD progression during obesity. *Nat. Commun.* 9:2092. doi: 10.1038/s41467-018-04361-y
- Bricambert, J., Miranda, J., Benhamed, F., Girard, J., Postic, C., and Dentin, R. (2010). Salt-inducible kinase 2 links transcriptional coactivator p300 phosphorylation to the prevention of ChREBP-dependent hepatic steatosis in mice. *J. Clin. Invest.* 120, 4316–4331. doi: 10.1172/JCI41624
- Burke, S. J., Collier, J. J., and Scott, D. K. (2009). cAMP opposes the glucose-mediated induction of the L-PK gene by preventing the recruitment of a complex containing ChREBP, HNF4 $\alpha$ , and CBP. *FASEB J.* 23, 2855–2865. doi: 10.1096/fj.08-126631
- Caron, S., Huaman Samanez, C., Dehondt, H., Ploton, M., Briand, O., Lien, F., et al. (2013). Receptor inhibits the transcriptional activity of carbohydrate response element binding protein in human hepatocytes. *Mol. Cell. Biol.* 33, 2202–2211. doi: 10.1128/MCB.01004-12
- Cuchel, M., Meagher, E., du Toit, T. H., Blom, D., Marais, A., Hegele, R., et al. (2013). Efficacy and safety of a microsomal triglyceride transfer protein inhibitor in homozygous familial hypercholesterolemia. *Lancet* 381, 40–46. doi: 10.1016/S0140-6736(12)61731-0.Efficacy
- Davies, M. N., O'Callaghan, B. L., and Towle, H. C. (2008). Glucose activates ChREBP by increasing its rate of nuclear entry and relieving repression of its transcriptional activity. *J. Biol. Chem.* 283, 24029–24038. doi: 10.1074/jbc.M801539200
- Dentin, R., Benhamed, F., Hainault, I., Fauveau, V., Foufelle, F., Dyck, J. R. B., et al. (2006). Liver-specific inhibition of ChREBP improves hepatic steatosis and insulin resistance in ob/ob mice. *Diabetes* 55, 2159–2170. doi: 10.2337/db06-0200
- Dentin, R., Tomas-Cobos, L., Fofelle, F., Leopold, J., Girard, J., Postic, C., et al. (2012). Glucose 6-phosphate, rather than xylulose 5-phosphate, is required for the activation of ChREBP in response to glucose in the liver. *J. Hepatol.* 56, 199–209. doi: 10.1016/j.jhep.2011.07.019
- Donnelly, K. L., Smith, C. I., Schwarzenberg, S. J., Jessurun, J., Boldt, M. D., and Parks, E. J. (2005). Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J. Clin. Invest.* 115, 1343–1351. doi: 10.1172/JCI23621
- Dushay, J. R., Toschi, E., Mitten, E. K., Fisher, F. M., Herman, M. A., and Maratos-Flier, E. (2015). Fructose ingestion acutely stimulates circulating FGF21 levels in humans. *Mol. Metab.* 4, 51–57. doi: 10.1016/j.molmet.2014.09.008
- Foretz, M., Pacot, C., Dugail, I., Lemarchand, P., Guichard, C., Le Liepvre, X., et al. (1999). ADD1/SREBP-1c is required in the activation of hepatic lipogenic gene expression by glucose. *Mol. Cell Biol.* 19, 3760–3768. doi: 10.1128/MCB.19.5.3760
- Fukasawa, M., Ge, Q., Wynn, R. M., Ishii, S., and Uyeda, K. (2010). Coordinate regulation/localization of the carbohydrate responsive binding protein (ChREBP) by two nuclear export signal sites: discovery of a new leucine-rich nuclear export signal site. *Biochem. Biophys. Res. Commun.* 391, 1166–1169. doi: 10.1016/j.bbrc.2009.11.115

- Ge, Q., Huang, N., Wynn, R. M., Li, Y., Du, X., Miller, B., et al. (2012). Structural characterization of a unique interface between carbohydrate response element-binding protein (ChREBP) and 14-3-3 $\beta$  Protein. *J. Biol. Chem.* 287, 41914–41921. doi: 10.1074/jbc.M112.418855
- Guinez, C., Filhoulaud, G., Rayah-Benhamed, F., Marmier, S., Dubuquoy, C., Dentin, R., et al. (2010). O-GlcNAcylation increases ChREBP protein content and transcriptional activity in the liver. *Diabetes* 60, 1399–1413. doi: 10.2337/db10-0452
- Herman, M. A., Peroni, O. D., Villoria, J., Schön, M. R., Abumrad, N. A., Blüher, M., et al. (2012). A novel ChREBP isoform in adipose tissue regulates systemic glucose metabolism. *Nature* 484, 333–338. doi: 10.1038/nature10986
- Hussain, M., Nijstad, N., and Franceschini, L. (2011). Regulation of microsomal triglyceride transfer protein. *Clin. Lipidol.* 6, 293–303. doi: 10.2217/clp.11.21. Regulation
- Iizuka, K., Bruick, R. K., Liang, G., Horton, J. D., and Uyeda, K. (2004). Deficiency of carbohydrate response element-binding protein (ChREBP) reduces lipogenesis as well as glycolysis. *Proc. Natl. Acad. Sci. U.S.A.* 101, 7281–7286. doi: 10.1073/pnas.0401516101
- Iizuka, K., Miller, B., and Uyeda, K. (2006). Deficiency of carbohydrate-activated transcription factor ChREBP prevents obesity and improves plasma glucose control in leptin-deficient (ob/ob) mice. *Am. J. Physiol. Metab.* 291, E358–E364. doi: 10.1152/ajpendo.00027.2006
- Iizuka, K., Takeda, J., and Horikawa, Y. (2009). Glucose induces FGF21 mRNA expression through ChREBP activation in rat hepatocytes. *FEBS Lett.* 583, 2882–2886. doi: 10.1016/j.febslet.2009.07.053
- Inagaki, T., Dutchak, P., Zhao, G., Ding, X., Gautron, L., Parameswara, V., et al. (2007). Endocrine regulation of the fasting response by PPAR $\alpha$ -mediated induction of fibroblast growth factor 21. *Cell Metab.* 5, 415–425. doi: 10.1016/j.cmet.2007.05.003
- Iroz, A., Montagner, A., Benhamed, F., Levavasseur, F., Polizzi, A., Anthony, E., et al. (2017). A specific ChREBP and PPAR $\alpha$  cross-talk is required for the glucose-mediated FGF21 response. *Cell Rep.* 21, 403–416. doi: 10.1016/j.celrep.2017.09.065
- Jois, T., Chen, W., Howard, V., Harvey, R., Youngs, K., Thalmann, C., et al. (2017). Deletion of hepatic carbohydrate response element binding protein (ChREBP) impairs glucose homeostasis and hepatic insulin sensitivity in mice. *Mol. Metab.* 6, 1381–1394. doi: 10.1016/j.molmet.2017.07.006
- Kabashima, T., Kawaguchi, T., Wadzinski, B. E., and Uyeda, K. (2003). Xylulose 5-phosphate mediates glucose-induced lipogenesis by xylulose 5-phosphate-activated protein phosphatase in rat liver. *Proc. Natl. Acad. Sci. U.S.A.* 100, 5107–5112. doi: 10.1073/pnas.0730817100
- Kato, T., Iizuka, K., Takao, K., Horikawa, Y., Kitamura, T., and Takeda, J. (2018). ChREBP-knockout mice show sucrose intolerance and fructose malabsorption. *Nutrients* 10:E340. doi: 10.3390/nu10030340
- Kawaguchi, T., Osatomi, K., Yamashita, H., Kabashima, T., and Uyeda, K. (2002). Mechanism for fatty acid “sparing” effect on glucose-induced transcription: regulation of carbohydrate-responsive element-binding protein by AMP-activated protein kinase. *J. Biol. Chem.* 277, 3829–3835. doi: 10.1074/jbc.M107895200
- Kawaguchi, T., Takenoshita, M., Kabashima, T., and Uyeda, K. (2001). Glucose and cAMP regulate the L-type pyruvate kinase gene by phosphorylation and dephosphorylation of the carbohydrate response element binding protein. *Proc. Natl. Acad. Sci. U.S.A.* 98, 13710–13715. doi: 10.1073/pnas.231370798
- Kharitonov, A., Shyanova, T. L., Koester, A., Ford, A. M., Micanovic, R., Galbreath, E. J., et al. (2005). FGF-21 as a novel metabolic regulator. *J. Clin. Invest.* 115, 1627–1635. doi: 10.1172/JCI23606
- Kim, M., Astapova, I. I., Flier, S. N., Hannou, S. A., Doridot, L., Sargsyan, A., et al. (2017). Intestinal, but not hepatic, ChREBP is required for fructose tolerance. *JCI Insight* 2:96703. doi: 10.1172/jci.insight.96703
- Kim, M. S., Krawczyk, S. A., Doridot, L., Fowler, A. J., Wang, J. X., Trauger, S. A., et al. (2016). ChREBP regulates fructose-induced glucose production independently of insulin signaling. *J. Clin. Invest.* 126, 4372–4386. doi: 10.1172/JCI81993
- Li, M. V., Chang, B., Imamura, M., Pongvarin, N., and Chan, L. (2006). Glucose-dependent transcriptional regulation by an evolutionarily conserved glucose-sensing module. *Diabetes* 55, 1179–1189. doi: 10.2337/db05-0822
- Linden, A. G., Li, S., Choi, H. Y., Fang, F., Fukasawa, M., Uyeda, K., et al. (2018). Interplay between ChREBP and SREBP-1c coordinates postprandial glycolysis and lipogenesis in livers of mice. *J. Lipid Res.* 59, 475–487. doi: 10.1194/jlr.M081836
- Markan, K. R., Naber, M. C., Ameka, M. K., Anderegg, M. D., Mangelsdorf, D. J., Kliewer, S. A., et al. (2014). Circulating FGF21 is liver derived and enhances glucose uptake during refeeding and overfeeding. *Diabetes* 63, 4057–4063. doi: 10.2337/db14-0595
- McFerrin, L. G., Atchley, W. R. A., and Novel, N. (2012). -Terminal domain may dictate the glucose response of mondo proteins. *PLoS One* 7:e34803. doi: 10.1371/journal.pone.0034803
- Meng, J., Feng, M., Dong, W., Zhu, Y., Li, Y., Zhang, P., et al. (2016). Identification of HNF-4 $\alpha$  as a key transcription factor to promote ChREBP expression in response to glucose. *Sci. Rep.* 6:2394.
- Morigny, P., Houssier, M., Mairal, A., Ghilain, C., Mouisel, E., Benhamed, F., et al. (2019). Interaction between hormone-sensitive lipase and ChREBP in fat cells controls insulin sensitivity. *Nat. Metab.* 1, 133–146. doi: 10.1038/s42255-018-0007-6
- Nakagawa, T., Ge, Q., Pawlosky, R., Wynn, R. M., Veech, R. L., and Uyeda, K. (2013). Metabolite regulation of nucleo-cytosolic trafficking of carbohydrate response element-binding protein (ChREBP). *J. Biol. Chem.* 288, 28358–28367. doi: 10.1074/jbc.M113.498550
- Niwa, H., Iizuka, K., Kato, T., Wu, W., Tsuchida, H., Takao, K., et al. (2018). ChREBP rather than SHP regulates hepatic VLDL secretion. *Nutrients* 10:E321. doi: 10.3390/nu10030321
- Osorio, J. S., Lohakare, J., and Bionaz, M. (2016). Biosynthesis of milk fat, protein, and lactose: roles of transcriptional and posttranscriptional regulation. *Physiol. Genomics* 48, 231–256. doi: 10.1152/physiolgenomics.00016.2015
- Pongvarin, N., Chang, B., Imamura, M., Chen, J., Moolsuwan, K., Sae-Lee, C., et al. (2015). Genome-wide analysis of ChREBP binding sites on male mouse liver and white adipose chromatin. *Endocrinology* 156, 1982–1994. doi: 10.1210/en.2014-1666
- Ribback, S., Sonke, J., Lohr, A., Frohme, J., Peters, K., Holm, J., et al. (2017). Hepatocellular glycogenotic foci after combined intraportal pancreatic islet transplantation and knockout of the carbohydrate responsive element binding protein in diabetic mice. *Oncotarget* 8, 104315–104329. doi: 10.18632/oncotarget.22234
- Richards, P., Ourabah, S., Montagne, J., Burnol, A.-F., Postic, C., and Guilmeau, S. (2017). MondoA/ChREBP: the usual suspects of transcriptional glucose sensing; implication in pathophysiology. *Metabolism* 70, 133–151. doi: 10.1016/j.metabol.2017.01.033
- Sakiyama, H., Wynn, R. M., Lee, W. R., Fukasawa, M., Mizuguchi, H., Gardner, K. H., et al. (2008). Regulation of nuclear import/export of carbohydrate response element-binding protein (ChREBP): interaction of  $\alpha$ -helix of ChREBP with the 14-3-3 proteins and regulation by phosphorylation. *J. Biol. Chem.* 283, 24899–24908. doi: 10.1074/jbc.M804308200
- Sato, S., Jung, H., Nakagawa, T., Pawlosky, R., Takeshima, T., Lee, W. R., et al. (2016). Metabolite regulation of nuclear localization of carbohydrate-response element-binding protein (ChREBP): role of amp as an allosteric inhibitor. *J. Biol. Chem.* 291, 10515–10527. doi: 10.1074/jbc.M115.708982
- Singh, P., and Irwin, D. M. (2016). Contrasting patterns in the evolution of vertebrate MLX interacting protein (MLXIP) and MLX interacting protein-like (MLXIPL) genes. *PLoS One* 11:e0149682. doi: 10.1371/journal.pone.0149682
- Stoekman, A. K., Ma, L., and Towle, H. C. (2004). Mlx is the functional heteromeric partner of the carbohydrate response element-binding protein in glucose regulation of lipogenic enzyme genes. *J. Biol. Chem.* 279, 15662–15669. doi: 10.1074/jbc.M311301200
- Talukdar, S., Owen, B. M., Song, P., Hernandez, G., Zhang, Y., Zhou, Y., et al. (2016). FGF21 regulates sweet and alcohol preference. *Cell Metab.* 23, 344–349. doi: 10.1016/j.cmet.2015.12.008
- Tang, Y., Wallace, M., Sanchez-Gurmaches, J., Hsiao, W.-Y., Li, H., Lee, P. L., et al. (2016). Adipose tissue mTORC2 regulates ChREBP-driven de novo lipogenesis and hepatic glucose metabolism. *Nat. Commun.* 7:11365. doi: 10.1038/ncomms11365
- Vijayakumar, A., Aryal, P., Wen, J., Syed, I., Vazirani, R. P., Moraes-Vieira, P. M., et al. (2017). Absence of carbohydrate response element

- binding protein in adipocytes causes systemic insulin resistance and impairs glucose transport. *Cell Rep.* 21, 1021–1035. doi: 10.1016/j.celrep.2017.09.091
- von Holstein-Rathlou, S., Bondurant, L. D., Peltekian, L., Naber, M. C., Yin, T. C., Clafin, K. E., et al. (2016). FGF21 mediates endocrine control of simple sugar intake and sweet taste preference by the liver. *Cell Metab.* 23, 335–343. doi: 10.1016/j.cmet.2015.12.003
- White, P. J., McGarrah, R. W., Grimsrud, P. A., Tso, S. C., Yang, W. H., Haldeman, J. M., et al. (2018). The BCKDH kinase and phosphatase integrate BCAA and lipid metabolism via regulation of ATP-Citrate Lyase. *Cell Metab.* 27, 1281.e7–1293.e7. doi: 10.1016/j.cmet.2018.04.015
- Yamashita, H., Takenoshita, M., Sakurai, M., Bruick, R. K., Henzel, W. J., Shillinglaw, W., et al. (2001). A glucose-responsive transcription factor that regulates carbohydrate metabolism in the liver. *Proc. Natl. Acad. Sci. U.S.A.* 98, 9116–9121. doi: 10.1073/pnas.161284298
- Yang, A.-Q., Li, D., Chi, L., and Ye, X.-S. (2017). Validation, identification, and biological consequences of the site-specific O -GlcNAcylation dynamics of carbohydrate-responsive element-binding protein (ChREBP). *Mol. Cell. Proteomics* 16, 1233–1243. doi: 10.1074/mcp.M116.061416
- Yore, M. M., Syed, I., Moraes-Vieira, P. M., Zhang, T., Herman, M. A., Homan, E. A., et al. (2014). Discovery of a class of endogenous mammalian lipids with anti-diabetic and anti-inflammatory effects. *Cell* 159, 318–332. doi: 10.1016/j.cell.2014.09.035
- Zhang, D., Tong, X., Van Dommelen, K., Gupta, N., Stamper, K., Brady, G. F., et al. (2017). Lipogenic transcription factor ChREBP mediates fructose-induced metabolic adaptations to prevent hepatotoxicity. *J. Clin. Invest.* 127, 2855–2867. doi: 10.1172/JCI89934

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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