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L'implication de Tip60 sur la marque H4K12ac et le rôle du ratio Tip60/P400 dans les cancers du sein triple négatifs

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Mouhamed Idrissou. L'implication de Tip60 sur la marque H4K12ac et le rôle du ratio Tip60/P400 dans les cancers du sein triple négatifs. Cancer. Université Clermont Auvergne [2017-2020], 2020. Français. NNT : 2020CLFAC089 . tel-04013287

HAL Id: tel-04013287

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*ECOLE DOCTORALE DES SCIENCES DE
LA VIE ET DE LA SANTE – AGRONOMIE - ENVIRONNEMENT*

Thèse

Présentée à l'Université d'Auvergne
pour l'obtention du grade de

DOCTEUR D'UNIVERSITE

Spécialité: Epigénétique et Cancer

Soutenue publiquement le **3 décembre 2020**,
Devant la commission d'examen par

Mouhamed IDRISOU

**L'implication de Tip60 sur la marque H4K12ac et Le rôle du
ratio Tip60/P400 dans les cancers du sein triple négatifs.**

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ABREVIATIONS

ADN: Acide désoxyribonucléique.
AJCC: American Joint Committee on Cancer.
AKT: Serine/threonine-specific protein kinase.
Antagomir: Micro-ARN antagoniste.
AP-1: Activator protein 1.
ARN: Acide ribonucléique.
ATAC: The Ada2a-containing.
ATF2: Activating Transcription Factor 2.
ATM: Ataxia Telangiectasia Mutated.
ATP: Adénosine-Triphosphate.
BALB/c: Bagg and Albino/ colony.
BRCA1: Breast cancer 1.
BRCA2: Breast cancer 2.
BRD2/4: Bromodomain-containing protein 2/4.
CBP: CREB-binding protein.
Cdc20: Cell division cycle protein 20 homolog.
ChIP: Chromatin immunoprecipitation.
CpG: Cytosine-phosphate-guanine.
DNMT: DNA Methyl Transferase.
DZNep: 3-Deazaneplanocine A.
EGF: Epidermal Growth Factor.
EP300: EA1 binding protein p300.
ERBB2: Erb-b2 receptor tyrosine kinase 2.
ESR1 et 2 : Estrogen receptor 1 et 2.
EZH2: Enhancer of zeste homolog 2.
GNAT: GCN5-related N-acetyltransferases.
GPR50: G Protein-Coupled Receptor 5.
HAT: Histone acetyltransferase.
HDAC: Histone déacetylase.
HDM: Histone demethylase.
HER2: Human epidermal growth factor receptor 2.
HIV-1: Human Immunodeficiency Virus-1.
HMT: Histone méthyltransférase.
HP1 : Heterochromatin Protein 1.
HTATIP : HIV-1 TAT Interactive Protein, 60kDa.
JAK: Janus kinases.
KAT: Lysine acetyl-transférase.
KISS1: Kisspeptine 1.
LAR: Luminal androgen receptor.
LET-7: Lethal-7.
Mdm2: Mouse Double Minute.
MET: Mesenchymal-epithelial transition.
miR-22: MicroRNA-22.
MYST: MOZ, Ybf2, Sas2, Sas3 et TIP60.
NK: Natural killer.
NPI: Nottingham Prognostic Index.
NR3C2: Nuclear Receptor Subfamily 3, Group C, Member 2.

NUA4: Nucleosome acetyltransferase of H4.
OMS: Organisation mondiale de la Santé.
Oncomir: Micro-ARN oncogène.
P/CAF: p300/CBP Associated Factor.
P53: Protéine 53.
PI3K: Phosphoinositide 3-kinases.
PPAR γ : peroxisome proliferator activated receptor gamma.
PRC2 : Polycomb Repressive Complexe 2.
qPCR : Quantitative polymerase chain reaction.
RA : Récepteur aux androgènes.
RE : Récepteur aux oestrogènes.
RP : Récepteur à la progestérone.
SAGA: Spt-Ada-Gcn5 acetyltransferase.
SBR: Scarff, Bloom et Richardson.
Sh-RNA: Short hairpin-RNA.
SiRNA: small interfering RNA.
SIRT1: Sirtuin 1.
SRC/RAS/ERK
SRC3: Steroid Receptor Coactivateur-3.
STAT3: signal transducer activator of transcription 3.
SUV39H1: Suppressor of variegation 3-9 homolog 1.
TH1/TH2: T helper 1/2.
TIP60: Tat interactive protein 60kDa.
TLDA: TaqMan low-density array.
TNBC: Triple negative breast cancer.
TNM : Taille adénopathie métastase.
USP7: ubiquitin specific peptidase 7.
XTT: XML Tunneling Technology .

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Dédicaces

Je dédie cette thèse :

*A mes très chers parents **IDDY Mama** et **SAINABU Saini***

Vous avez toujours été pour moi un exemple de parents respectueux, honnêtes, des personnes méticuleuses, je tiens à honorer les parents que vous êtes.

Grâce à vous j'ai appris le sens du travail et de la responsabilité. Je voudrais vous remercier pour votre amour, votre générosité, votre compréhension... votre soutien fut une lumière dans tout mon parcours. Aucune dédicace ne saurait exprimer l'amour, l'estime et le respect que j'ai toujours eu pour vous.

Ce modeste travail est le fruit de tous les sacrifices que vous aviez déployés pour mon éducation et ma formation. Je vous aime papa et maman qu'Allah vous accorde une bonne santé et une vie longue et heureuse.

*A mes chers frères et Sœur **Lawal TANKO**, **SANI Mama** et **Assana IDDY***

A tous les moments d'enfance passés avec vous mes frères, en gage de ma profonde estime pour l'aide que vous m'aviez apportée. Vous m'aviez soutenu, réconforté et encouragé. Puissent nos liens fraternels se consolider et se pérenniser encore plus.

A TOUTE MA FAMILLE

*Spécialement à mes cousins **Abdelkader Ousman** et **Ibrahim** « Masta », aucun langage ne saurait exprimer mon respect et ma considération pour votre soutien et encouragements. Je vous dédie ce travail en reconnaissance de l'amour que vous m'offrez quotidiennement et votre bonté exceptionnelle. Qu'Allah vous garde et vous procure santé et bonheur.*

*A mes amies **Fatiha**, **Ji sung**, **Helene**, **Rodrigue**, **Majid** « T-Fly », **Djibril** « Toums », **Ospice**, **Patrick** « Epso », **Christian** « JC », **Mouhamad** « Bago », **Yvan** « Tapsiri », **Cédric** « Eméca », **Innocent** « Sac », **Herman** « Oporco », **Ibrahim** « coach », **Quentin** « Zadi », **Abdoul** « Momo », **William** « Balbo », **Aslane**, **Maxim**, **Ibtisem** et **Nicolas** Je ne peux trouver les mots justes et sincères pour vous exprimer mon affection et mes pensées, vous êtes pour moi des sœurs et des amies sur qui je peux compter. En témoignage de l'amitié qui nous unit et des souvenirs de tous les moments que nous avons passés ensemble, je vous dédie ce travail et je vous souhaite une vie pleine de santé et de bonheur.*

Remerciements

*Je souhaite avant tout remercier ma directrice de thèse, **Dominique BERNARD-GALLON**, pour le temps qu'elle a consacré à m'apporter les outils méthodologiques indispensables à la conduite de cette recherche. Pour sa patience, sa disponibilité et surtout ses judicieux conseils, qui ont contribué à alimenter ma réflexion, son exigence m'a grandement stimulé.*

*Je tiens à témoigner toute ma reconnaissance aux personnes suivantes, pour leur aide dans la réalisation de cette thèse : **Anna SANCHEZ, Gaëlle JUDES, Khaldoun RIFAÏ, Marine DAURES, Driss EL OUARDI, Tiphonie BOISNIER et Fatma Zohra Houfaf Khoufaf.***

« Arrivez comme le vent et partez comme l'éclair... »

Sun tzu.

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I. INTRODUCTION

Le cancer du sein est le cancer le plus diagnostiqué avec une incidence de 24,2% chez la femme et il est aussi la principale cause de décès avec 15% de mortalité chez la femme (Bray et al., 2018). Le cancer est une pathologie provoquée par des dérèglements dans la cellule entraînant une prolifération anormale et l'immortalité de cette dernière. Ces dérèglements ont été longtemps associés à des aberrations au niveau du génome. L'avancée des recherches sur les mécanismes de cancérogénèse ont montré qu'il y a d'autres dérèglements tels que ceux qui se produisent au niveau épigénétique. Dans le cancer du sein 10 à 15% des cancers sont dus à une mutation génétique héréditaire le plus souvent associée à la mutation des gènes *BRCA1* et *BRCA2*, les 85 à 90% restants sont des cancers sporadiques ou non héréditaires (Sobol et al., 1994). Parmi ces cancers sporadiques on retrouve ceux dû à l'influence environnementale sur l'épigénome.

La transformation d'une cellule normale en cellule cancéreuse peut être provoquée par des altérations d'origines génétiques telles que les mutations des oncogènes (*erb-B2*, *c-myc*) et des gènes suppresseurs de tumeurs (*BRCA1*, *BRCA2*, *P53* etc.), Mais aussi des altérations épigénétiques (Calip et al., 2020; Easton et al., 1993; Liu, 1993; Mariani-Costantini et al., 1989; Wooster and Weber, 2003). Ces altérations se font au niveau des modifications dites épigénétiques qui permettent de moduler (activation ou répression) l'expression des gènes. Parmi ces mécanismes épigénétiques, on peut citer la méthylation de l'ADN qui a longtemps été étudiée dans les cancers, notamment le cancer du sein montrant ainsi son implication dans l'expression des suppresseurs de tumeur tels que *BRCA1* et *BRCA2* et des oncogènes tel que *erb-B2* (Bosviel et al., 2012a; Bosviel et al., 2012b; Dagdemir et al., 2016; Mariani-Costantini et al., 1989; Yan et al., 2003). Les micros-ARN qui sont de plus en plus étudiés et montrent leur implication dans la progression tumorale comme les oncomiRs tels que miR-21 et miR-221/222, ou au contraire leur rôle de suppresseur de tumeurs tels que la famille des LET-7 et des miR-200 (Finoux and Chartrand, 2008; Gambari et al., 2016; Huang et al., 2011; M et al., 2018). Enfin, les modifications post-traductionnelles des histones permettent le remodelage de la chromatine en activant ou en réprimant des gènes impliqués dans l'initiation, l'activation ou le développement tumoral. Parmi ces modifications post-traductionnelles des histones, la méthylation des histones connue pour être le plus souvent répressive, a longtemps été étudiée et utilisée comme biomarqueurs dans certains cancers comme le cancer de la prostate (Daures et al., 2018; Ngollo et al., 2014; Ngollo et al., 2017).

Ou encore l'acétylation de l'histone qui quant à elle est considérée comme activatrice, en particulier l'acétylation des histones H3 et H4 qui font l'objet de nombreuses recherches sur leurs implications dans le développement tumoral (Grezy et al., 2016; Judes et al., 2016; Judes et al., 2015; Rifai et al., 2018a; Rifai et al., 2018b).

En effet, notre équipe s'est focalisée sur le rôle de l'acétylation des histones H3 et H4 dans le cancer du sein. Cette acétylation régulée par des protéines antagonistes à savoir les histones acétyl-transferases (HAT) et les histones déacétylases (HDAC) qui respectivement peuvent placer ou retirer des groupements acétyles sur les queues N-terminales des histones. Deux de ces protéines dont TIP60 qui est une acétyl-transférase et SIRT1 une déacétylase sont les principaux acteurs de notre investigation, pour mieux comprendre l'implication de l'acétylation des histones dans le développement du cancer du sein. La compréhension de ce mécanisme pourrait être un atout majeur dans le diagnostic, la prise en charge et le traitement de cette maladie.

Cette thèse a pour but de permettre une meilleure compréhension du mécanisme d'acétylation des histones à travers le rôle de TIP60 et de SIRT1 dans l'initiation, l'activation et le développement du cancer du sein.

II. ETUDE BIBLIOGRAPHIQUE

1. Cancer du sein

1.1 Épidémiologie du cancer du sein

Le cancer du sein est le deuxième cancer le plus diagnostiqué dans le monde derrière le cancer du poumon tous sexes confondus avec une incidence de 11,6% et le cinquième cancer le plus meurtrier dans le monde avec une mortalité de 6,6% par cancer dans le monde (Figure 1) (Bray et al., 2018). Chez la femme le cancer du sein est de loin le cancer avec une forte incidence de 24,2% de nouveaux cas par an, suivi du cancer colorectal et du poumon qui ont respectivement des incidences de 9,5% et 8,4% de nouveaux cas par an (Figure 1). Avec un taux de mortalité de 15%, le cancer du sein est le cancer le plus meurtrier chez la femme suivie du cancer du poumon avec un taux de mortalité de 13,8% (Figure 1). Le taux d'incidence du cancer du sein est plus élevé dans les pays développés (54,4%) par rapport au pays moins développés (31,3%), alors que le taux de mortalité est plus faible dans les pays développés (11,6%) pour (14,9%) dans les pays moins développés (Figure 2).

En France, le cancer du sein est le cancer le plus fréquent chez la femme, il est estimé à environ 59 000 nouveaux cas par an et environ 12 000 décès en 2017, ce qui fait de lui la première cause de décès par cancer chez la femme. Une femme sur 8 sera touchée par le cancer du sein au cours de sa vie. Huit femmes sur 10 atteintes de cancer du sein sont âgées de plus de 50 ans, l'âge moyen du cancer du sein est de 63 ans (©Les cancers en France, Les Données, INCa, janvier 2019). Lorsque le cancer est rapidement dépisté, il est guéri dans 85% des cas, à l'état métastatique le taux de guérison est beaucoup plus faible.

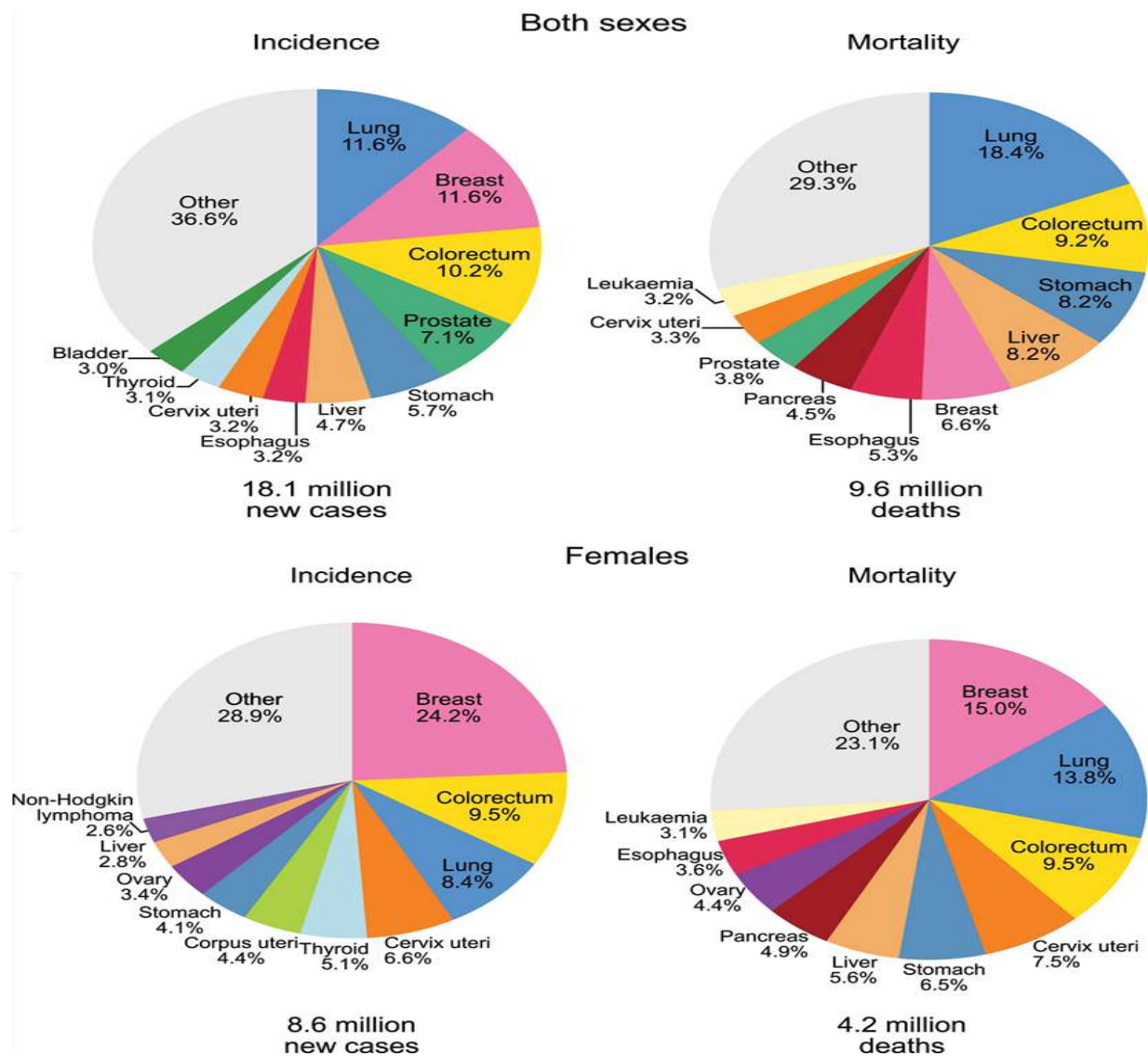


Figure 1 : Les diagrammes représentent la répartition des incidences et des décès pour les 10 cancers les plus courants en 2018 pour les deux sexes et les femmes : Pour chaque sexe, la surface du diagramme circulaire reflète la proportion du nombre total d'incidence ou de décès. Source : © GLOBOCAN 2018.

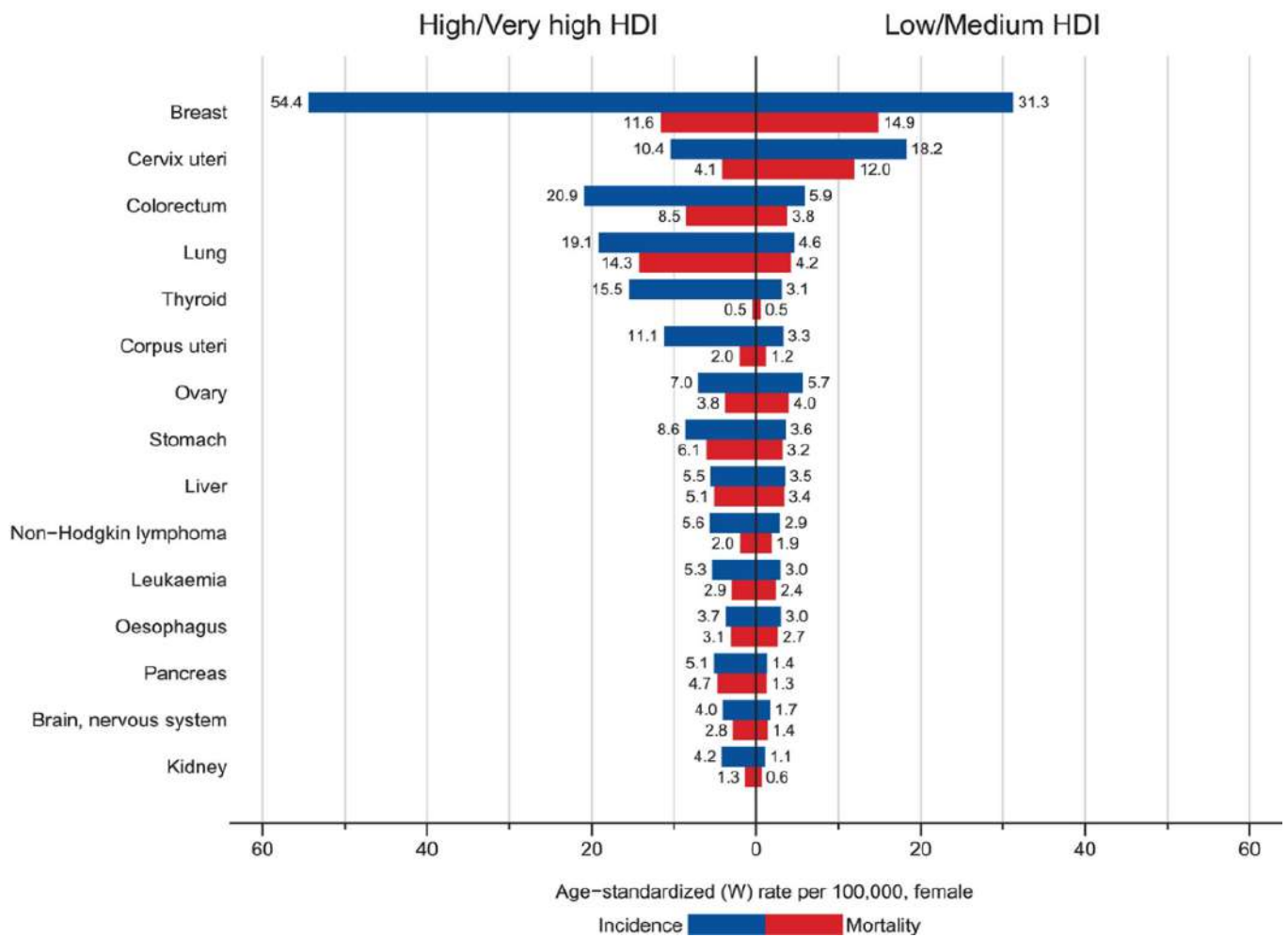


Figure 2 : Les barres représentent les taux d'incidence et de mortalité normalisés selon l'âge dans les régions à indice de développement humain (IDH) élevé/très élevé par rapport aux régions à IDH faible/moyen chez les femmes en 2018 : Les 15 cancers les plus répandus dans le monde (W) en 2018 sont présentés par ordre décroissant du taux global normalisé par âge. Source : © GLOBOCAN 2018.

1.2 Étiologie du cancer du sein

1.2.1 *Origine du cancer du sein*

Avant de parler de l'origine du cancer du sein, il est important de savoir qu'elle est l'anatomie du sein. Le sein est un organe féminin qui a pour fonction biologique de produire du lait afin de nourrir un nouveau-né. Il est constitué de tissus adipeux, de peau, du mamelon et des canaux lactifères qui débouchent dans les glandes mammaires formées de plusieurs lobules (Figure3).

L'origine des cellules qui donnent naissance à une tumeur dans le sein a fait naître plusieurs hypothèses. L'hypothèse la plus utilisée suggère que la tumeur prend naissance dans les cellules épithéliales de la glande mammaire (adénocarcinome) de l'unité terminale ducto-lobulaire (unité de base du sein). L'adénocarcinome représente 95% des cancers du sein, les 5% restant sont des cancers du sein rares tels que le lymphome primitif du sein, la tumeur phyllode ou encore le sarcome (Ghanem et al., 2013; J et al., 2008). Pour qu'une cellule normale se transforme en cellule tumorale elle traverse trois grandes étapes qui sont : L'initiation qui est due à un facteur mutagène ou oncogène (chimique, physique, biologique...) entraînant une modification au niveau du gène de la cellule, la promotion qui consiste à la formation d'un clone de cellules anormales et la progression qui consiste à une multiplication, la formation d'un amas et vascularisation des cellules anormales (Figure 4) (Burgio and Migliore, 2015).

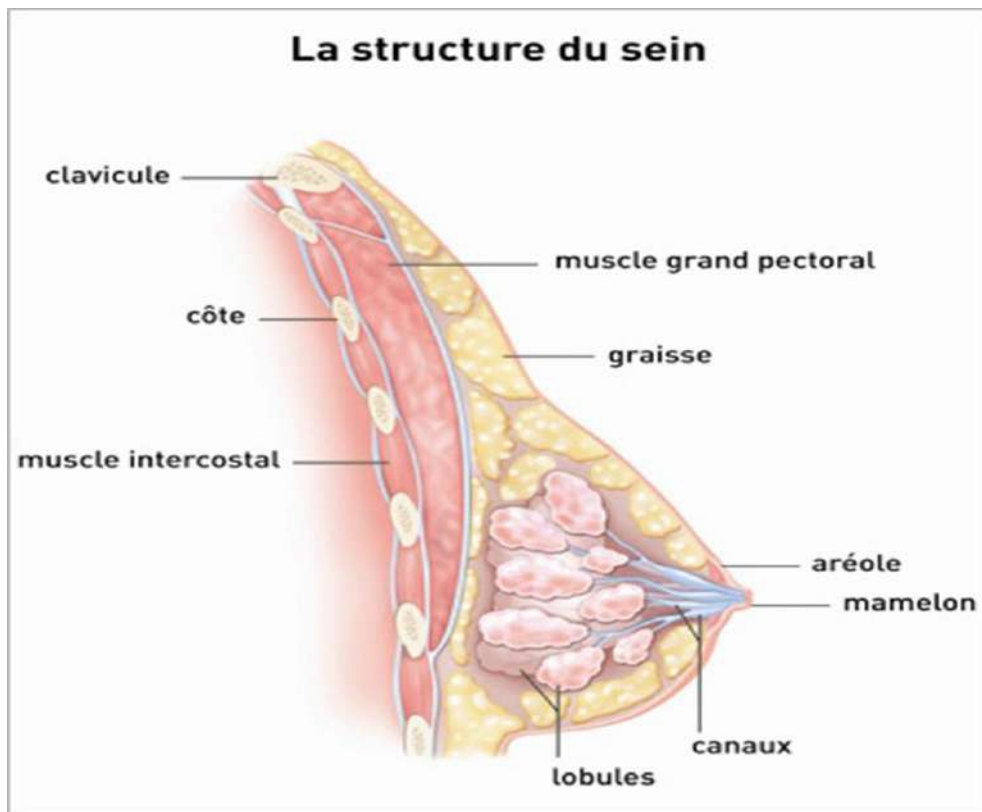


Figure 3 : Représentation de l'anatomie du sein : les différentes structures formant le sein sont représentées : Aréole, canaux, clavicule, côte, graisse, lobules, mamelon et muscles intercostal et du grand pectoral. © Institut National du Cancer (INCA).

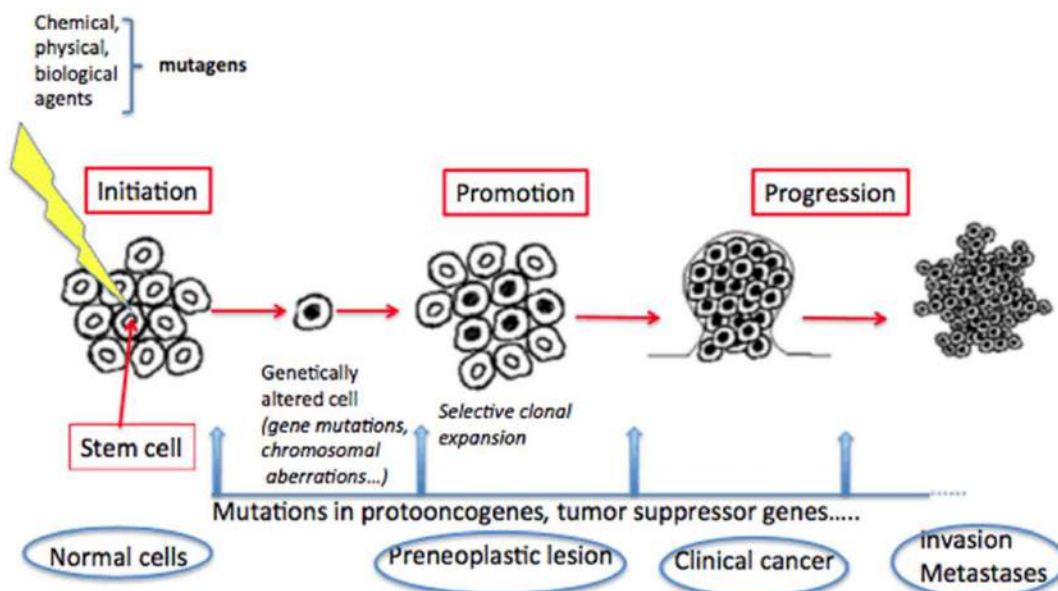


Figure 4 : Représentation des différentes étapes du processus de carcinogénèse : Figure récupéré de (Burgio and Migliore, 2015).

1.2.2 Classification des cancers du sein

Compte tenu de son hétérogénéité, pour mieux comprendre et mieux traiter le cancer du sein de nombreuses classifications ont été établies. Ces classifications se basent sur le lieu de formation de la tumeur ou encore l'expression moléculaire de certaines hormones.

a. Classification histologique du cancer du sein

Le cancer du sein a été subdivisé en différents types selon le lieu où prend naissance la tumeur, cette classification a été validée par l'OMS et comprend:

Les carcinomes non invasifs ou encore carcinomes *in situ*, qui prennent naissance aux niveaux des canaux sont appelés carcinomes canaux *in situ* et représentent 85 à 90% de ces types de ce cancer. Dix à 15% des carcinomes *in situ* prennent naissance aux niveaux des lobules et sont appelés carcinomes lobulaires *in situ* (Bloom, 1958).

Les carcinomes invasifs ou infiltrants qui présentent une capacité à métastaser prennent naissance à 75% dans les canaux (carcinomes invasifs canaux), de 5 à 15% dans les lobules (carcinomes invasifs lobulaires). Il existe d'autres types de carcinomes représentant 1 à 5% des carcinomes infiltrants qui sont les carcinomes mucineux (2%), les carcinomes médullaires (1 à 5%), les carcinomes tubulaires (1 à 5%) et enfin les carcinomes papillaires (1 à 5%) (Tableau 1).

Tableau 1 : Classification histologique des carcinomes mammaires selon l'Institut national du cancer. © Institut National du Cancer (INCA).

Carcinome Non Invasif (in situ)		Carcinome Invasif		
Canalaire (Ductal) 85-90%	Lobulaire 10-15%	Canalaire 75%	Lobulaire 4-11%	Autres : 5% Médullaire Mucineux Tubulaire Canalaire-Lobulaire

Cette classification histologique a permis d'élaborer des méthodes de détermination de l'agressivité de la tumeur en les classant en grade ou en stade.

- La première méthode établie par Patey et Scarff en 1928 est la classification en grade. En 1957, Bloom et Richardson ont rafraîchi et nommé la méthode le grade SBR (Scarff, Bloom and Richardson) et enfin en 2008 l'équipe de Ellis et Elston ont modifié la méthode et adapté à une meilleure reproductibilité et pronostic du grade qu'ils ont nommé NPI (Nottingham Prognostic Index) (Bloom and Richardson, 1957; Rakha et al., 2010).

Ces grades sont basés sur 3 critères et notés de 1 à 3.

- Le premier critère est l'architecture de la tumeur donc « 1 » correspond à une tumeur uniquement composée de tubes, « 2 » à une tumeur partiellement tubulaire et « 3 » à une tumeur sans tube.

- Le second critère est l'observation des anomalies des noyaux cellulaires (atypies cytonucléaires), « 1 » correspond à des noyaux réguliers et monomorphes, « 2 » à une anomalie modérée ou atypie modérée et « 3 » aux noyaux pléomorphes ou atypie marquée.

- Le troisième critère mesure l'activité mitotique (l'index mitotique) des cellules tumorales sur 10 champs cellulaires, « 1 » correspond à 0-9 mitoses, « 2 » à 10-19 mitoses et « 3 » supérieur à 19 mitoses.

Enfin le grade d'un cancer du sein correspond à la somme des notes obtenues pour chaque critère. Un score de 3 à 5 correspond au grade 1 (les tumeurs les moins agressives), de 6 à 7 au grade 2 (tumeurs intermédiaires) et enfin le grade 3 correspond à un score de 8 à 9 (tumeurs les plus agressives).

- La deuxième méthode a été établie par The American Joint Committee on Cancer (AJCC) en 2010 et classe les cancers du sein en stade de 0 à IV en prenant en compte la taille de la tumeur primaire, le statut ganglionnaire de la

tumeur et enfin l'existence de métastases d'où son nom TNM (Tumor Node Metastasis) (Figure 5) (Chavez-MacGregor et al., 2017).

-Le stade 0 correspond à un carcinome *in situ* (Tis) sans envahissement ganglionnaire régional (N0) et sans métastase (M0) (Figure 5. A).

-Le stade I correspond à une tumeur de 2 cm de diamètre ou moins (T1) sans envahissement ganglionnaire régional (N0) et sans métastase (M0) (Figure 5. B).

-Le stade II est subdivisé en 2 types, le stade IIA correspond à une tumeur primitive non palpable (T0) ou une tumeur d'un diamètre entre ≤ 2 à ≤ 5 cm avec ou sans envahissement des ganglions et sans métastase (Figure 5. C); le stade IIB correspond à une tumeur de diamètre > 5 cm avec ou sans envahissement des ganglions et sans métastase (Figure 5. D).

-Le stade III est lui aussi subdivisé en plusieurs types dont le stade IIIA allant des tumeurs non palpables à des tumeurs de diamètre > 5 cm (T0-T3) avec un envahissement ≤ 9 ganglions (N1-N2) et sans métastase (M0) (Figure 5. E); le stade IIIB ayant une tumeur de taille variable avec une extension directe à la paroi thoracique ou la peau (T4), un envahissement ≤ 9 ganglions (N0-N2) et sans métastase (M0) (Figure 5. F); le stade IIIC ayant des tumeurs de toutes tailles avec un envahissement d'au moins 10 ganglions (N3) sans métastase (M0) (Figure 5. G).

-Le stade IV qui est caractérisé par des tumeurs de toutes tailles avec ou sans envahissement des ganglions et avec une métastase (M1) (Figure 5. H).

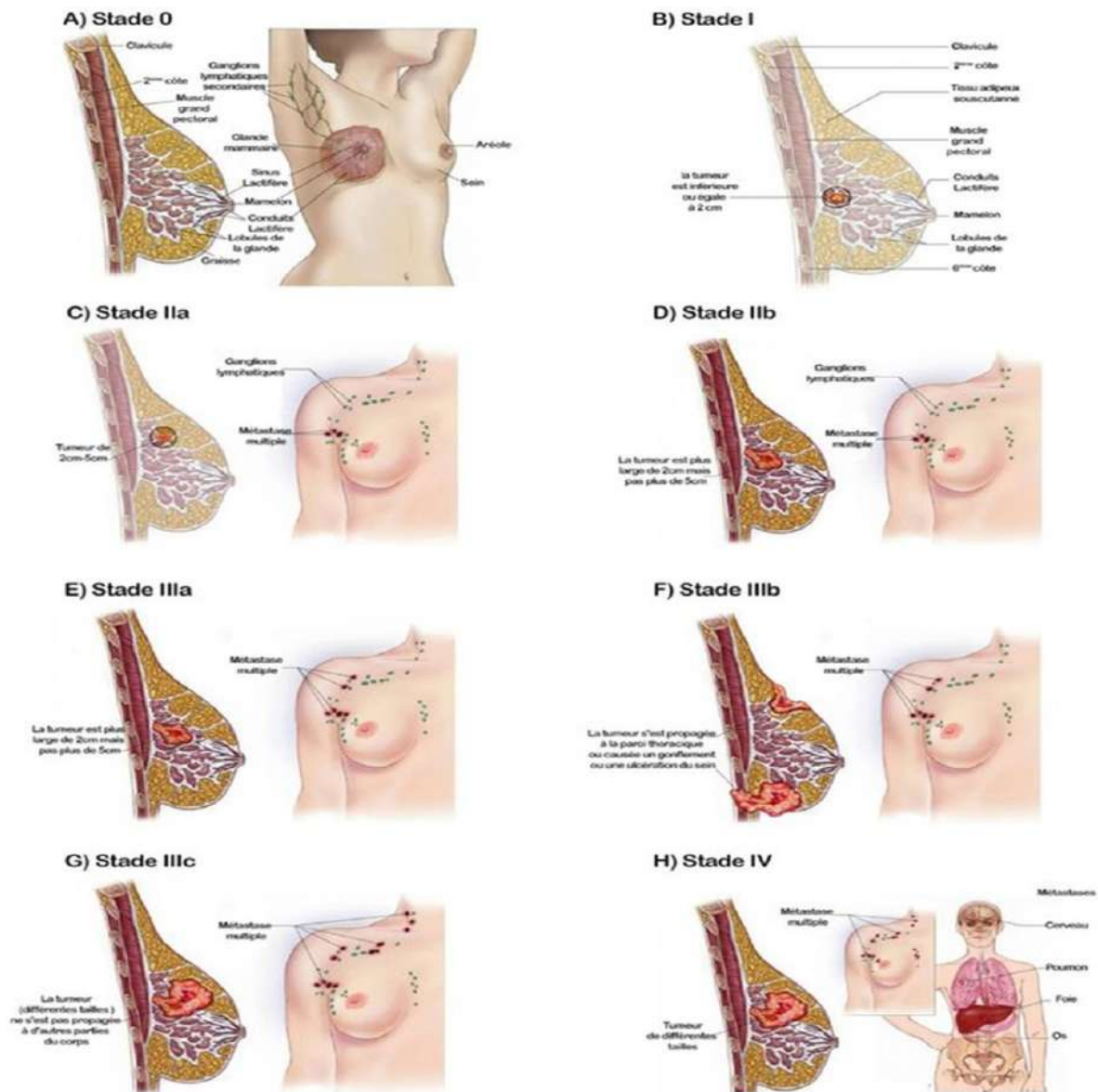


Figure 5 : Méthode de classification histologique du cancer du sein établi par The American Joint Committee on Cancer (AJCC). A) Stade 0: Le premier stade du carcinome canalaire in situ. B) Stade I : la tumeur est ≤ 2 cm sans atteinte ganglionnaire. C) Stade IIa : La tumeur est ≤ 2 cm avec une atteinte des ganglions lymphatiques axillaires, ou tumeur se situe entre 2 cm et 5 cm sans atteinte des ganglions axillaires. D) Tumeur entre 2 - 5 cm avec atteinte de 1 à 3 ganglions lymphatiques ou tumeur > 5 cm sans atteinte ganglionnaire. E) Stade IIIa : Cancer localement avancé. Tumeur < 5 cm avec métastases fixées ou emmêlées dans les ganglions lymphatiques axillaires, ou tumeur est > 5 cm avec atteinte des ganglions lymphatiques axillaires. F) Stade IIIb : La tumeur croit dans la paroi thoracique ou dans la peau du sein ; ou le cancer s'est propagé aux ganglions sous-claviculaires homolatéraux ; ou le cancer du sein inflammatoire. G) Stade IIIc : Métastases dans les ganglions lymphatiques axillaires et les ganglions lymphatiques claviculaires homolatéraux ; ou métastases dans les ganglions lymphatiques sus-claviculaires homolatéraux. H) La tumeur s'est propagée à des sites distants tels que le foie, les poumons, les os ou le cerveau. Figure tirée de (<https://www.cancerresearchuk.org/>).

b. Classifications moléculaires du cancer du sein

L'utilisation de la classification histologique pour le traitement des cancers n'était pas optimale d'où la mise en place d'une nouvelle classification qui prend en compte l'hétérogénéité des cancers du sein dans l'expression de certains gènes. Peaufiné par le consensus international de ST Gallen et permettant une meilleure prise en charge et traitement de la tumeur, la classification moléculaire se base sur l'expression de certains récepteurs hormonaux considérés comme biomarqueurs du cancer du sein tels que le récepteur aux œstrogènes (ER), le récepteur à la progestérone (PR) et le récepteur épidermique humain 2 (HER2) (Ignatiadis et al., 2015). Cette classification est composée de 4 sous types qui sont le Luminal A, Luminal B, HER2 positif et les triples négatifs (Tableau 2).

- Les tumeurs lumineales sont des tumeurs ayant le même profil d'expression génique que les cellules épithéliales lumineales d'où leurs noms. Elles expriment les récepteurs aux œstrogènes et à la progestérone. Elles sont de faible grade donc moins agressives. Ces tumeurs se présentent en deux sous types qui sont :

-La tumeur Luminaire A caractérisée par l'expression des récepteurs ER (+) et PR (+) et l'absence d'expression de HER2 (-), elle représente 50 à 60 % des cancers du sein ce qui fait d'elle le cancer le plus commun et le moins agressif (bon pronostic). Son étude histochimique montre qu'elle a une expression du Ki 67 $< 14\%$.

-La tumeur Luminaire B caractérisée par une expression des récepteurs ER (+) moins importante que celle de la tumeur lumineale A, exprime faiblement ou pas le récepteur PR (+) avec un faible taux d'expression du Ki 67 $\geq 14\%$. Elle est subdivisée en deux sous-groupes en fonction de l'expression de HER2 en Luminaire B HER2 (+) et Luminaire B HER2 (-). Ce type de tumeur représente 10 à 30 % des cancers du sein et est considéré comme plus agressif que la lumineale A.

- La tumeur enrichie en HER2 ou HER2 (+) est caractérisée par l'absence des récepteurs ER (-), PR (-) et une expression du récepteur HER2 (+) d'où son nom. Elle représente 15 à 25 % des cancers du sein et a un mauvais pronostic dû à l'absence des récepteurs ER et PR.

Tableau 2 : Classification du cancer du sein selon le consensus de St. Gallen 2013.

Tableau tirée de (Cho, 2016).

Surrogate definitions of intrinsic subtypes of breast cancer classification from the St. Gallen Consensus 2013

Intrinsic subtype		Clinicopathologic surrogate definition					Type of therapy
		ER	PR	HER2	Ki-67	Recurrence risk ^{a)}	
Luminal A	Luminal A-like	+	+ ^{b)}	-	Low <14%	Low (if available)	Endocrine therapy is often used alone Cytotoxic therapy may be added
Luminal B	Luminal B-like ^{c)} (HER2-negative)	+	- or low	-	High	High (if available)	Endocrine therapy for all patients, cytotoxic therapy for most
	Luminal B-like (HER2-positive)	+	Any	Over-expressed or amplified	Any	NA	Cytotoxics+anti-HER2+endocrine therapy
ErbB-2 overexpression	HER2-positive (non-luminal)	Absent	Absent	Over-expressed or amplified	NA	NA	Cytotoxics+anti-HER2
Basal-like	Triple negative (ductal)	-	-	-	NA	NA	Cytotoxics

- Les tumeurs triples négatifs comme leurs noms l'indiquent, n'expriment ni le récepteur ER (-), PR (-) et HER2(-). Elles présentent le même profil génétique que les cellules myoépithéliales que l'on trouve dans la lame basale, elles portent aussi le nom de tumeurs basales. Les tumeurs triples négatives représentent 10 à 20 % des cancers du sein et sont le sous type le plus agressif avec un mauvais pronostic. Ce sous type est composé de différents groupes de tumeurs triples négatives classées en fonction de leurs expressions de gènes, on distingue :

-Les basal like 1 qui expriment un fort enrichissement en gènes de la prolifération, du cycle et de la division cellulaire.

-Basal like 2 sur-expriment uniquement les gènes impliqués dans la voie signalisation des facteurs de croissance telle que la voie d'EGF, MET et Wnt/ β -catenin.

-Immunomodulatory comme son nom l'indique exprime les gènes impliqués dans les processus de signalisation des cellules immunitaires tels que les voies de signalisation des cellules TH1/TH2 et NK, mais aussi les voies de transduction JAK/STAT.

-Mesenchymal like quant à eux sur-expriment les gènes impliqués dans les voies de la motilité et la différenciation cellulaire.

-Mesenchymal stem like sur-expriment les gènes impliqués dans les processus liés à la signalisation des facteurs de croissance tel que le mécanisme de l'inositol phosphate, mais les mésenchymes stem like partagent aussi certains profils d'expression avec le mésenchyme like tels que les gènes impliqués dans la différenciation cellulaire.

-Luminal androgen receptor exprime fortement le récepteur aux androgènes et certains gènes des voies de la synthèse stéroïdienne, du métabolisme des androgènes / œstrogènes et le métabolisme des porphyrines.

1.3 Facteurs de risque du cancer du sein.

Comme on l'a défini dans l'introduction, le cancer est dû à une dérégulation dans la cellule entraînant une prolifération anarchique et une immortalité de cette dernière. Les causes de ces dérégulations dans le cancer du sein sont difficiles à déterminer. Cependant, de plus en plus d'études scientifiques montrent une association entre certaines caractéristiques propres à la personne (facteurs internes) ou à son environnement (facteurs externes) et le risque de développer un cancer du sein. Les facteurs de risque les plus importants qui jouent un rôle essentiel en augmentant le risque de développer un cancer du sein chez les femmes peuvent être classés en deux groupes distinctes : l'âge, les antécédents familiaux, la densité mammaire, l'alimentation, les mutations dans certains gènes, l'inactivité physique et le mode de vie. (Figure 6).

a. Facteurs externes.

- **Le mode de vie** peut influencer le risque de cancer du sein. Une consommation élevée d'alcool ou de tabac peuvent augmenter le niveau de certaines hormones telles que l'œstrogène, et lorsque les niveaux de ces hormones sont élevés, surtout après un certain âge, les cellules du sein peuvent devenir cancéreuses. L'alcool risque de réduire la quantité de certains éléments nutritifs essentiels qui protègent contre les dommages cellulaires, comme l'acide folique (type de vitamine B) et les vitamines A et C. Des

études récentes démontrent que le tabagisme actif est lié au cancer du sein tant chez la femme pré-ménopausée que la femme post-ménopausée.

- **L'inactivité physique** accroît le risque de cancer du sein chez la femme pré-ménopausée ou post-ménopausée car elle favorise le surpoids et l'obésité qui sont identifiés comme facteurs de risque des cancers du sein. La pratique régulière d'une activité physique contribue aussi à réduire le risque de développer un cancer du sein de 10 à 27 % (©Anses, 2016).
- **L'alimentation** : Une alimentation favorisant l'obésité ou le surpoids est une alimentation qui augmente les risques d'avoir un cancer du sein. Par contre de nombreuses études ont montré que certaines alimentations diminuaient les risques de cancer du sein. Une alimentation riche en fruits et légumes est bonne pour prévenir le cancer du sein.

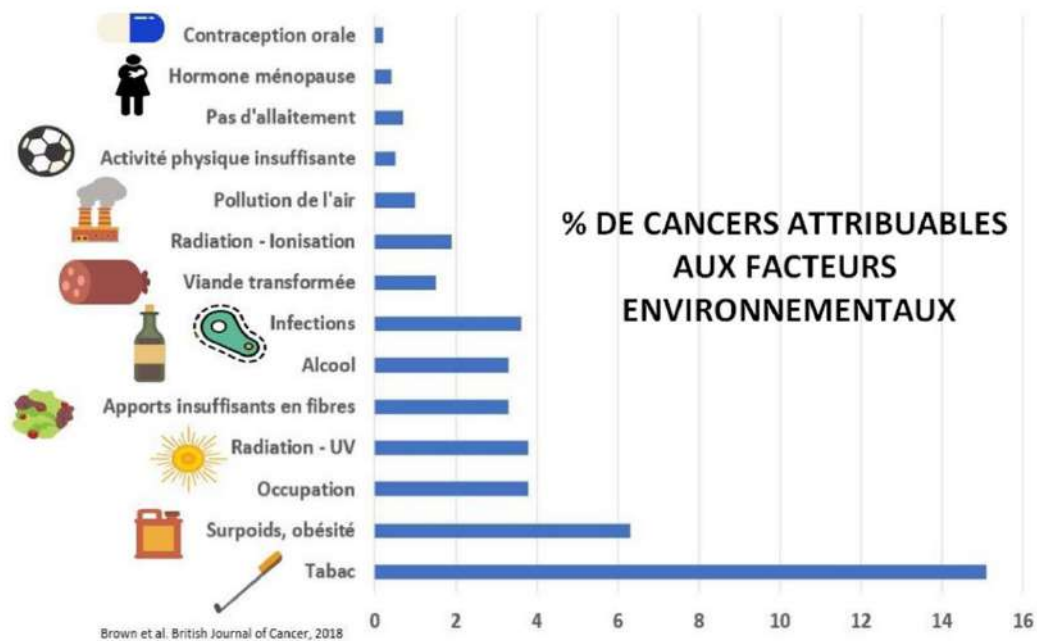


Figure 6 : Principaux facteurs entraînant la survenue de cancers. Figure tirée de <https://quoidansmonassiette.fr/prevention-cancer-40-cas-evitables-tabac-surpoids-obesite-facteurs-de-risque/>.

b. Facteurs internes.

- **L'âge** comme pratiquement tous les cancers, est le principal facteur du cancer du sein. Deux cancers sur trois se déclarent après la ménopause (après 40 ans) (©EUROPA DONNA). L'âge moyen au moment du diagnostic du cancer du sein est de 61 ans (Bolton, 2016).
- **Les antécédents familiaux**, Par exemple, avoir une mère et/ou une sœur atteinte d'un cancer du sein augmente le risque, particulièrement si elles ont été diagnostiquées à un âge précoce, ceci augmente le risque de cancer du sein.
- **Les prédispositions génétiques** à certaines mutations, qui rendront les femmes 10 fois plus à risque de développer un cancer du sein. Les gènes les plus mutés sont particulièrement les gènes de réparation de l'ADN, *BRCA1* (Breast Cancer 1), *BRCA2* (Breast Cancer 2) qui jouent un rôle dans la stabilité du génome et *P53* gène suppresseur de tumeur,
- **La densité mammaire** peut modifier aussi le risque de développer un cancer du sein. Une forte densité à l'âge adulte augmente le risque de développer un cancer du sein après la ménopause. Les seins denses contiennent plus de tissu conjonctif, de glandes et de canaux galactophores que le tissu graisseux. La densité du sein est un caractère héréditaire. Cette densité mammaire ne peut être observée qu'à la mammographie, mais des seins denses rendent plus difficile la lecture du cliché. Le tissu graisseux apparaît foncé alors que le tissu dense apparaît blanc, tout comme les tumeurs, pouvant ainsi masquer un cancer (figure 7).

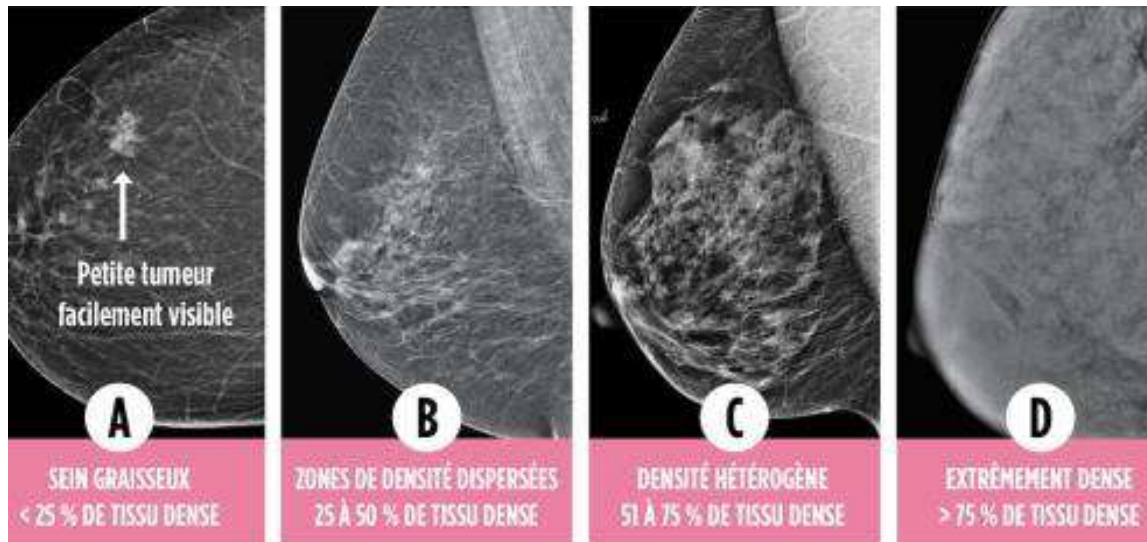


Figure 7 : Mammographie représentant différents pourcentages de densité mammaire.

A) sein à densité très faible, B) sein à densité faible, C) sein à densité élevée, D) sein à densité très élevée. Figure tirée de <https://www.canalvie.com/sante-beaute/sante/densite-seins-information-sauver-vies-1.10793256>.

2. Epigénétique

L'épigénétique est définie comme l'étude des modifications de l'expression des gènes qui sont transmissibles lors de la mitose et /ou méiose, mais ne découlent pas de modifications dans la séquence de l'ADN (Deltour et al., 2005). Contrairement aux mutations qui affectent la séquence d'ADN, les modifications épigénétiques sont réversibles. Il existe trois modifications ou régulations épigénétiques qui sont la méthylation d'ADN, modifications post-traductionnelles d'histone et la régulation des gènes par les micro-ARN (Figure 8). Ces trois modifications correspondent à la façon dont le gène sera lu (actif ou pas actif, allumé ou éteint, surexprimé ou sous exprimé, etc.) ce qui permet à toutes les cellules d'un organisme d'avoir la même information génétique mais de l'exprimer différemment en fonction de leurs rôles et de leurs emplacements dans l'organisme (Biswas and Rao, 2017; You and Jones, 2012). La cible des principaux mécanismes épigénétiques est le nucléosome, qui est l'unité de base du matériel génétique, constitué d'un complexe formé de protéines (histones) autour duquel s'enroule l'ADN (Figure 9). Ces nucléosomes s'enroulent ensuite sur eux même de manière compactée (hétérochromatine) ou décompactée (euchromatine) (Figure 10). L'état de la chromatine dépend plus de la régulation post-traductionnelle des histones. En effet, les connaissances sur l'expression des gènes, la structure et les mécanismes post-traductionnelles

de la chromatine ont montré l'importance de ces régulations épigénétiques. L'altération de ces modifications sont associées à de nombreuses pathologies (diabète, obésité...) et plus particulièrement dans les différents cancers.

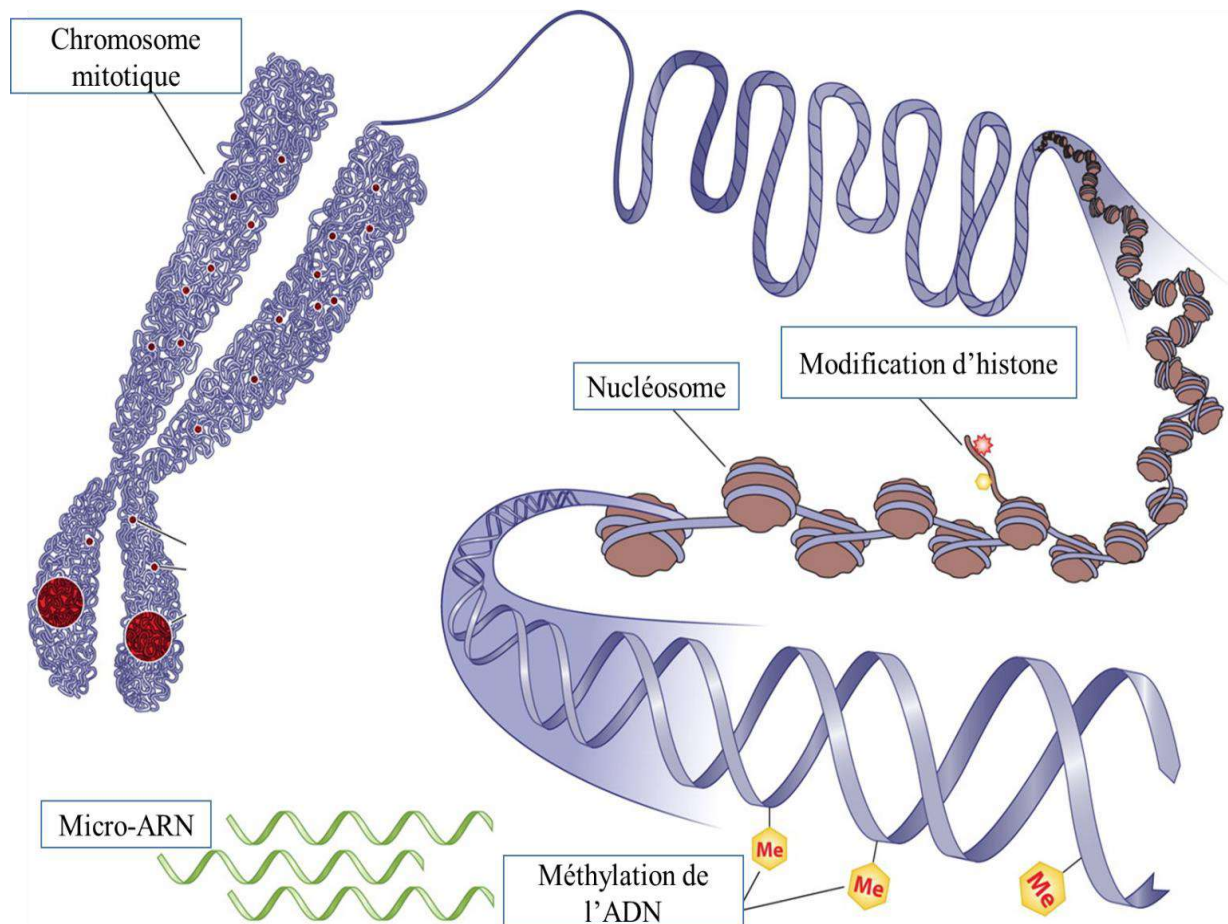


Figure 8 : Représentation des trois principaux mécanismes déterminant l'architecture épigénétique. La méthylation de l'ADN, la modification post-traductionnelle des histones et les micros-ARN sont des mécanismes régulant la transcription des gènes et déterminant le phénotype d'une cellule. Figure tirée et modifiée de (Zaidi et al., 2010)

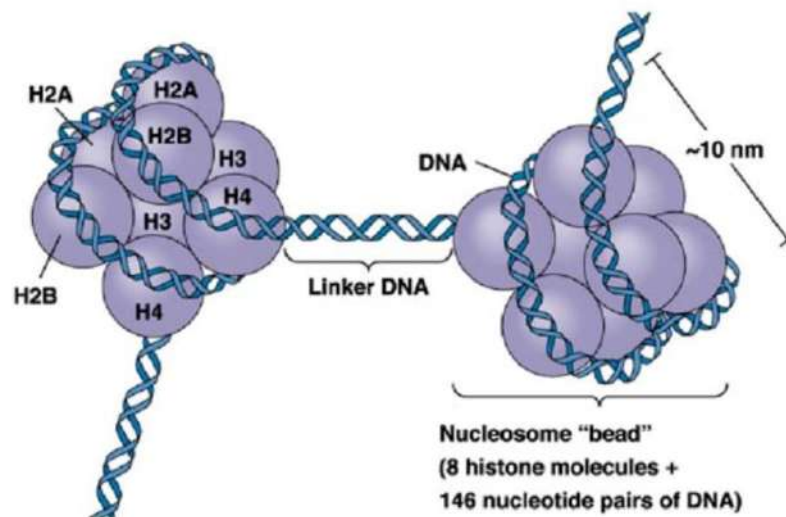


Figure 9 : Représentation du nucléosome : structure formée d'un segment d' ADN de 146 paires de nucléotides enroulés autour d'un complexe d'histone constitué de 8 histones 2x (H3, H4, H2A et H2B). Image tirée du site: http://www.mun.ca/biology/desmid/brian/BIOL2060/BIOL2060-18/18_21.

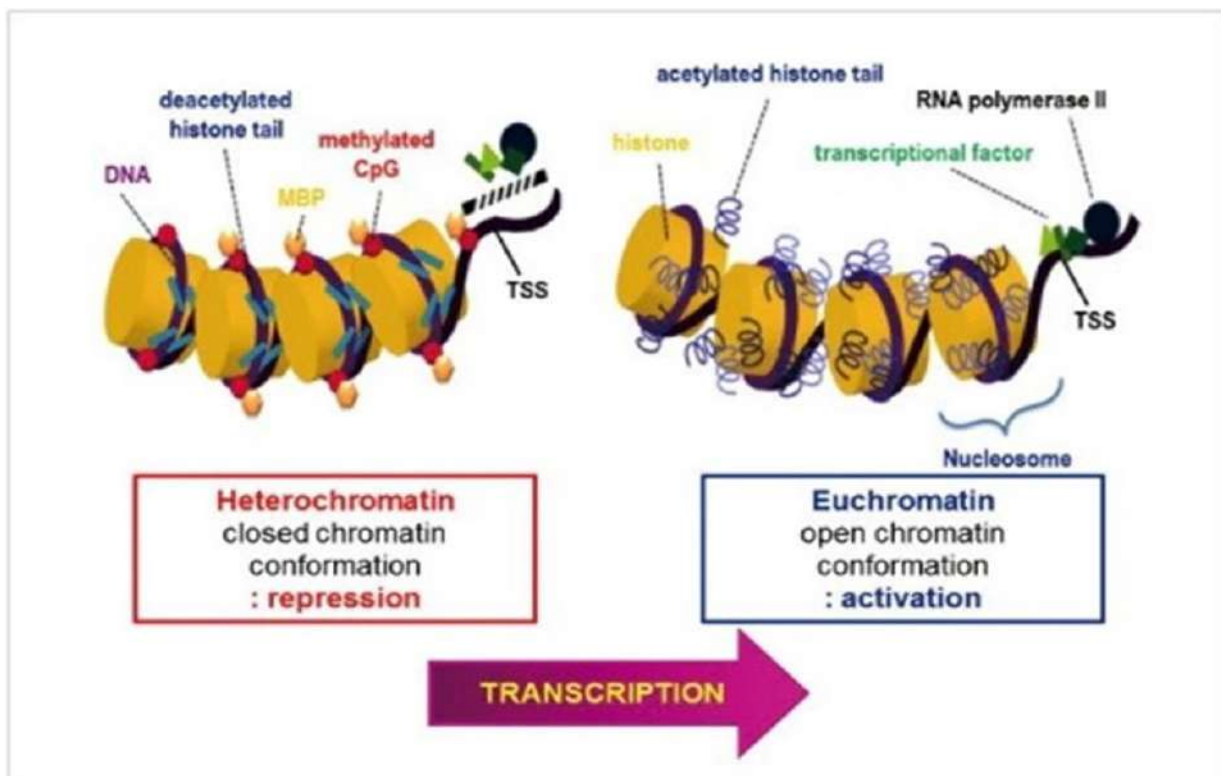


Figure10 : Représentation des différents états de la chromatine (l'euchromatine et l'hétérochromatine) : l'euchromatine qui est de la chromatine relâchée ou ouverte favorable à la transcription des gènes et l'hétérochromatine est de la chromatine compactée ou fermée qui est favorable à la répression des gènes. Image tirée de (Hatzimichael and Crook, 2013; Kim, 2014)

2.1 Epigénétique dans le cancer du sein

a. Les micro-ARN dans les cancers du sein

Les micro-ARN sont des ARN non codants de 21 à 25 nucléotides qui contrôlent l'expression génique au niveau post-transcriptionnel. Plusieurs centaines de gènes codant pour des micro-ARN ont été identifiés. Certains de ces gènes sont conservés entre espèces. Ces micro-ARN règlent l'expression génique en s'appariant avec des ARNm cibles dont ils sont partiellement complémentaires (Jonas and Izaurralde, 2015). Cette hybridation réprime la traduction de la protéine correspondante ou clive l'ARNm cible au milieu du site de fixation du micro-ARN (Figure 11). L'expression des micro-ARN se trouve souvent altérée dans les cancers et ils peuvent jouer un rôle d'oncogène ou de suppresseur de tumeur. L'altération de la régulation de l'expression des gènes via des mécanismes épigénétiques est fortement impliquée dans la genèse et la progression du cancer du sein. Dans le cancer du sein certains micro-ARN sont capables de rendre les cellules cancéreuses plus sensibles à la chimiothérapie (Corsini et al., 2012; Umeh-Garcia et al., 2020).

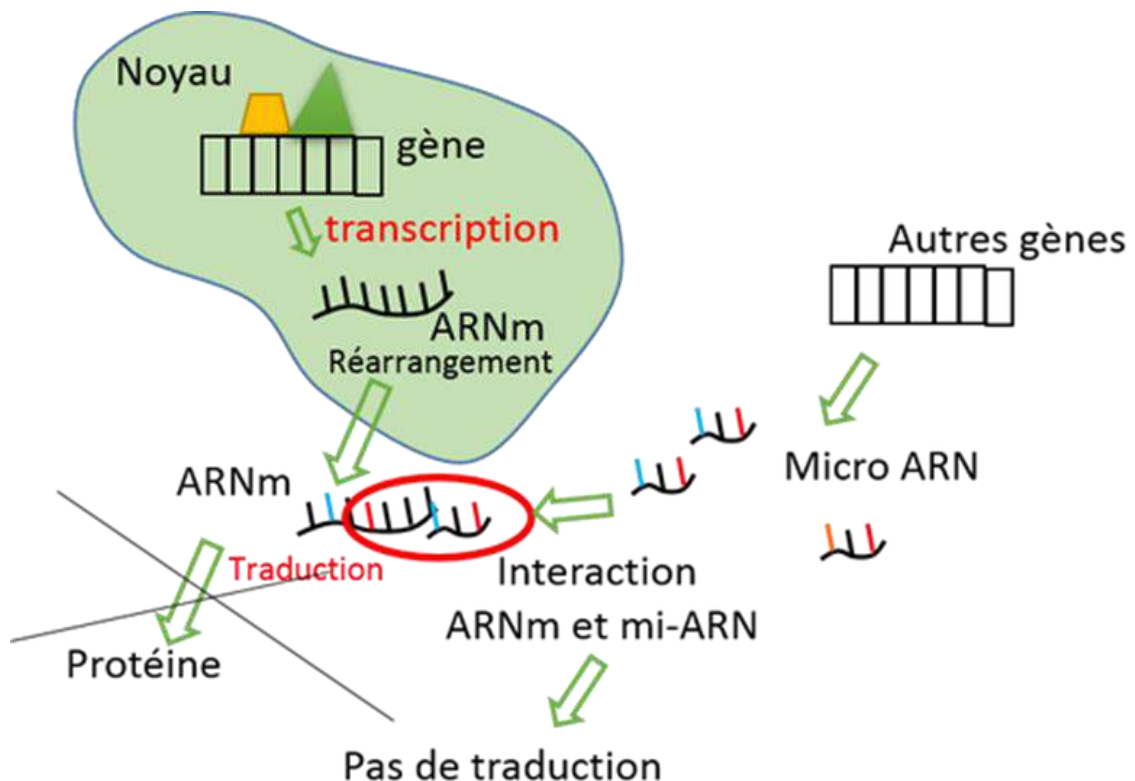


Figure 11 : Illustration de la régulation épigénétique par les Micro-ARN. Inhibition de la traduction des ARNm par interaction entre les micro-ARN et les ARNm. Figure tirée de <https://allaiterbonheuretraiison.wordpress.com/2016/02/22/lait-humain-et-expression-des-genes-2-autres-mecanismes/>.

b. La méthylation de l'ADN dans les cancers du sein

La méthylation de l'ADN joue un rôle crucial dans le contrôle de nombreux processus cellulaires tels que le développement embryonnaire, la transcription, l'inactivation du chromosome X et l'empreinte génomique (Bestor et al., 2015). Elle correspond à l'ajout d'un groupement méthyl-CH₃ sur le carbone 5 des résidus cytosines de l'ADN. Cette modification chimique est assurée par des enzymes que sont les ADN méthyl-transférases ou DNMT (DNMT1, DNMT3A et DNMT3B) à partir d'un donneur de méthyl, la S-adénosyl-méthionine (SAM) (Bestor et al., 2015; Edwards et al., 2010). La réversibilité de cette réaction serait sous la dépendance d'une déméthylase. Ces groupements méthyls forment ainsi un revêtement moléculaire, variable d'un gène à l'autre, d'un type cellulaire à un autre, et confèrent un caractère unique et transmissible à l'ADN. La structure moléculaire de l'ADN a ainsi sa composante génétique, qui est la même dans toutes les cellules, et sa composante épigénétique variable suivant les types cellulaires. Chez les mammifères, ces événements de méthylation ont lieu majoritairement au niveau de dinucléotides CpG (cytosine-phosphate-guanine) (Ioshikhes and Zhang, 2000; Lister et al., 2009). 70 à 90 % des dinucléotides CpG sont méthylés et se localisent dans des régions de chromatine compactée ou hétérochromatine, inaccessibles à la machinerie transcriptionnelle. En revanche, les dinucléotides CpG non méthylés se regroupent en régions riches en CpG ; ce sont les îlots CpG (Lister et al., 2009). Il a été vu que le niveau de ces DNMTs in vitro et in vivo se trouvait augmenté dans les cancers du sein et de la prostate. La méthylation de l'ADN et les micros-ARNs n'explique pas à eux seule l'inactivation des gènes dans les cancers et en particulier dans le cancer du sein. En effet, les modifications post-traductionnelles de la queue des histones se trouvent elles aussi dérégulées.

c. Les modifications post-traductionnelle des histones dans les cancers du sein

La régulation de la transcription des gènes chez les eucaryotes se joue en grande partie au niveau de la chromatine. L'unité de base de ce compactage du matériel génétique est le nucléosome où l'ADN est enroulé autour d'un octamère protéique composé de 2 copies de chacune des histones H2A, H2B, H3 et H4 et verrouillé par l'histone H1 (Tessarz and

Kouzarides, 2014). A l'aide entre autres de l'histone H1, il peut y avoir formation d'états chromatiniens encore plus condensés allant jusqu'au chromosome mitotique. Ces différents états chromatiniens modulent l'accessibilité de l'ADN pour les divers processus biologiques nucléaires. Les mécanismes précis impliqués dans l'ouverture et la fermeture de la chromatine deviennent de plus en plus clairs. Deux types d'activités enzymatiques peuvent être recrutées pour modifier la structure de la chromatine : tout d'abord celles utilisant l'énergie de l'hydrolyse de l'ATP pour altérer les liens histone-ADN à l'intérieur du nucléosome et celles qui modifient de façon covalente certains résidus des histones menant ultimement à une chromatine plus ouverte ou condensée suivant le type de modifications qu'elles subissent.

Les modifications post-traductionnelles des histones ont été proposées comme formant un code qui dicterait l'état chromatiniens et ainsi la manière de réguler un gène ou un locus (Figure 12). Une fois établie, ce code marquerait de façon épigénétique un locus soit d'une manière transitoire ponctuelle soit de façon stable sur de grandes régions. Il est donc devenu évident que les modifications post-traductionnelles des histones sont des acteurs majeurs dans les mécanismes de régulation transcriptionnelle. Il existe quatre types majeurs de modifications des histones : l'acétylation, la méthylation, la phosphorylation et l'ubiquitination (Azuara et al., 2006). Les différentes combinaisons de ces modifications formeraient un code épigénétique qui, une fois lu par des domaines protéiques spécifiques, entraînerait diverses réponses à des endroits précis du génome eucaryote. En effet, l'acétylation posée par des histones acétyltransférases (HATs) et enlevée par des histones déacétylases (HDACs), va diminuer en général l'affinité des histones pour l'ADN en créant un état ouvert de la chromatine donc permissif pour la transcription. Par ailleurs, la méthylation des histones, posée par des méthyl-transférases (HMTs), est associée soit à une activation transcriptionnelle soit à une répression qui va dépendre de la lysine ciblée et du niveau de méthylation (no-, mono- (me1), di- (me2) ou tri- (me3) méthylé) (Azuara et al., 2006; Azzaz et al., 2014). La marque triméthylée de la lysine 27 de l'histone 3 (H3K27me3) et la marque diméthylée de la lysine 9 de l'histone 3 (H3K9me2) sont généralement associées à une répression du gène alors que H3K4me3 est généralement associée à une activation transcriptionnelle (Zhang et al., 2012). Ces méthylations vont, par conséquent, définir le niveau de compaction de la chromatine et l'accessibilité ou non aux gènes par des facteurs de transcription. Plus le niveau de compaction sera important, plus l'activité du gène sera généralement réprimée puisqu'il ne sera pas accessible par ses différents facteurs de régulation. Par conséquent, le bon fonctionnement de ces marques épigénétiques se fait grâce

2.2 Acétylation de l'histone dans les cancers du sein

L'acétylation des histones est l'un des mécanismes post-traductionnel principal du remodelage de la chromatine et de la régulation de l'expression des gènes. Cette acétylation est contrôlée par deux familles de protéines à savoir les histones déacétylases et les histones acétyl-transférases (Figure 13). L'équilibre de ces protéines est indispensable pour la cellule, car elles sont impliquées dans de nombreux mécanismes cellulaires tels que l'équilibre des profils d'acétylation, le cycle cellulaire, l'expression des gènes et de nombreuses voies cellulaires.

Toutefois, dans de nombreuses pathologies telles que les cancers, leur expression se voit modifier créant ainsi un déséquilibre dans le ratio HATs/HDACs et un nouveau profil d'acétylation. Dans de nombreux cancers, le profil d'acétylation et l'expression des HATs et HDACs sont utilisées pour diagnostiquer, stratifier et suivre l'évolution de la pathologie. Au cours de ma thèse, je me suis focalisé sur une HDAC qui est SIRT1 et principalement une HAT qui est TIP60, ces deux protéines sont antagonistes et partagent quasiment les mêmes cibles.

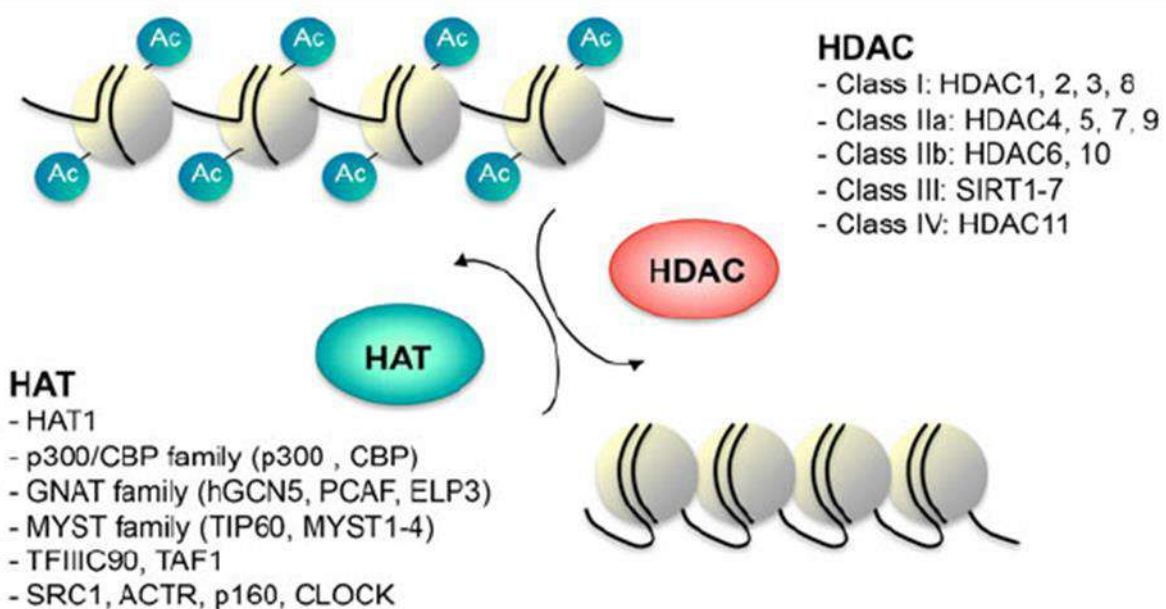


Figure 13 : Représentation de la régulation de l'acétylation des histones par la famille des histone acétyl-transférases (HAT) et des histone déacétylases (HDAC). Illustration de la conformation de la chromatine selon l'équilibre de ces deux familles HAT/HDAC. Figure tirée de (Schneider et al., 2013).

a. Histones déacétylases et cancer du sein

Ces dernières années, le rôle des histones déacétylases dans le processus de carcinogénèse mammaire a été beaucoup étudié. Les histones déacétylases sont formées principalement d'une grande famille appelée la famille des sirtuines qui sont omniprésentes et présentent la particularité d'effectuer la désacétylation de la lysine et la mono-ADP-ribosyltransférase dans les cellules (Figure 14). Plusieurs travaux ont montré l'implication des déacétylases sirtuines tels que SIRT1 dans l'initiation, la croissance et le développement tumoral du cancer du sein. Cependant, peu d'études ont fait le lien entre son rôle de régulateur épigénétique à travers son activité sur les marques d'histones telles que H3K4ac, H3K9ac et H4K16ac et son implication dans la carcinogénèse mammaire.








	Domaines			Localisation	Activité
	N-terminal domain	Core	C-terminal domain		
SIRT1				Noyau	DAC*
SIRT2				Cytoplasme	DAC & ART*
SIRT3				Mitochondrie	DAC & ART
SIRT4				Mitochondrie	ART, DAC
SIRT5				Mitochondrie	DAC, DM * & DS*
SIRT6				Noyau	ART, DAC
SIRT7				Nucléole	DAC ?

Figure 14: Schéma représentant la famille des sirtuines leurs localisations dans la cellule, leurs différents domaines et leurs activités enzymatique. Les 7 sirtuines (SIRT 1-7), sont localisées dans le noyau, le cytoplasme, le nucléus et la mitochondrie. Elles effectuent les activités de déacétylation (DAC), ADP-ribosyl-transférase (ART), démalonylation (DM) et désuccinylation (DS). Image tirée de la présentation du 7ème colloque MeetOchondrie d'Evian-les-Bains , 4 - 7 mai 2014.(Du et al., 2011; Li and Kazgan, 2011)

- *Régulation de l'acétylation des histones H3 et H4 par SIRT1 dans le cancer du sein*

Dans cette étude, on a voulu mettre en évidence le rôle de l'activité déacétylase de SIRT1 sur les marques H3K4ac, H3K9ac et H4K16ac et la progression tumorale du cancer du sein à la fois ex vivo et in vitro. Pour cela, on a observé la variation d'expression protéique de SIRT1 et des différentes marques d'histones dans différents types de tumeur du sein. Nous avons aussi regardé si SIRT1 interagissait avec les histones H3 et H4 au niveau de certains gènes cibles (*ESR1-2*, *AR*, *BRCA1*, *EZH2* et *EP300*) connus pour être dérégulés dans le cancer du sein.

Publication 1:

SIRT1-dependent epigenetic regulation of H3 and H4 histone acetylation in human breast cancer. Rifaï K, Judes G, **Idrissou M**, Daures M, Bignon YJ, Penault-Llorca F, Bernard-Gallon D. *Oncotarget*. 2018 Jul 17;9(55):30661-30678.

SIRT1-dependent epigenetic regulation of H3 and H4 histone acetylation in human breast cancer

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Keywords: breast cancer; epigenetics; SIRT1; histone marks; epigenetic modulation

Received: May 03, 2018

Accepted: June 22, 2018

Published: July 17, 2018

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ABSTRACT

Breast cancer is the most frequently diagnosed malignancy in women worldwide. It is well established that the complexity of carcinogenesis involves profound epigenetic deregulations that contribute to the tumorigenesis process. Deregulated H3 and H4 acetylated histone marks are amongst those alterations. Sirtuin-1 (SIRT1) is a class-III histone deacetylase deeply involved in apoptosis, genomic stability, gene expression regulation and breast tumorigenesis. However, the underlying molecular mechanism by which SIRT1 regulates H3 and H4 acetylated marks, and consequently cancer-related gene expression in breast cancer, remains uncharacterized. In this study, we elucidated SIRT1 epigenetic role and analyzed the link between the latter and histones H3 and H4 epigenetic marks in all 5 molecular subtypes of breast cancer. Using a cohort of 135 human breast tumors and their matched normal tissues, as well as 5 human-derived cell lines, we identified H3k4ac as a new prime target of SIRT1 in breast cancer. We also uncovered an inverse correlation between SIRT1 and the 3 epigenetic marks H3k4ac, H3k9ac and H4k16ac expression patterns. We showed that SIRT1 modulates the acetylation patterns of histones H3 and H4 in breast cancer. Moreover, SIRT1 regulates its H3 acetylated targets in a subtype-specific manner. Furthermore, SIRT1 siRNA-mediated knockdown increases histone acetylation levels at 6 breast cancer-related gene promoters: *AR*, *BRCA1*, *ERS1*, *ERS2*, *EZH2* and *EP300*. In summary, this report characterizes for the first time the epigenetic behavior of SIRT1 in human breast carcinoma. These novel findings point to a potential use of SIRT1 as an epigenetic therapeutic target in breast cancer.

INTRODUCTION

Breast cancer remains the leading cause of cancer death among females in less developed countries, and second leading cause of cancer death in more developed countries after lung cancer [1]. The occurrence of breast cancer is a complex, multifactorial process that is regulated by a number of different genes at different tumor formation stages [2]. Breast cancer is also characterized by its molecular and clinical heterogeneity with variations

in gene expression profiles among women [3]. The St. Gallen molecular classification divides breast tumors into 5 distinct subtypes in ascending order of tumor aggressiveness [4]. Luminal A, luminal B (HER2-) and luminal B (HER2+), these 3 subtypes are included in the Hormone Receptor-positive Breast Cancer (HRBC). HER2-enriched or HER2 Breast Cancer (H2BC). And finally triple-negative breast cancer (TNBC), also known as basal-like, which is characterized as very aggressive compared to the other molecular subtypes [5]. 85 to 90%

of breast tumors are called sporadic or non-hereditary tumors that can spawn due to many environmental risk factors. Sporadic breast tumors are especially characterized by the presence of underlying abnormalities in their epigenome [6].

The complexity of carcinogenesis cannot be represented by genetic mutations alone, but also involves profound epigenetic alterations. The epigenetic regulation of the genome includes among others, histone post-translational modifications (PTMs) [7]. Deregulated histone PTMs or histone marks are considered as biomarkers of cancer prognosis and were shown to predict patient outcome in various human carcinomas [8, 9]. In breast cancer, analysis of human breast tumors revealed a highly significant correlation between global histone marks patterns and tumor molecular phenotypes, prognostic factors, and clinical outcome [10, 11]. Lysine acetylation at the N-terminus tails of histones H3 and H4 is classically associated with increased gene expression. The epigenetic marks (epi-marks) H3 lysine 4 (H3K4ac), lysine 9 (H3K9ac) and H4 lysine 16 (H4K16ac) are well-characterized acetylated marks that are particularly enriched at transcriptionally active gene promoters [12] [13]. H3K9ac and H4K16ac have well-defined roles in regulating chromatin structure. Their deacetylation causes the formation of higher-order chromatin compaction and subsequently transcription repression, as neatly described by Vaquero *et al.* [14, 15]. Global loss of H4K16ac has been shown to be a hallmark of human cancer and associated with early tumor formation stages [16]. Also, H3K9ac has been shown to be underexpressed in breast cancer, as well as other cancers, and its decrease was correlated with tumor progression and poor clinical outcome [10]. On the other hand, few reports studied the role of H3K4ac in cancer. The function of H3K4ac was often related to that of H3K4me3, since both acetylation and methylation of lysine (K4) residue are associated with active transcription [17]. In addition, the epigenetic acyl-lysine ‘eraser’ of H3K4ac histone marker has not been yet identified in humans. In a recent study, Messier *et al.* explored the dynamics of H3K4ac in 2 breast cancer cell lines. They demonstrated the latter as an indicator of deregulated cancer-related pathways. They also uncovered a role of H3K4ac in predicting epigenetic changes associated with early stages of transformation [18].

Histone deacetylases (HDACs) are major actors of epigenetic regulation. Dysfunctional HDACs have been found to be closely related to the tumorigenesis process and cancer metastasis [19]. Due to their deacetylase activity of a broad spectrum of substrates, Sirtuins are considered to be master regulators of several basic cellular mechanisms [20]. Silent mating type information regulation 2 homolog 1 (SIRT1) is a NAD⁺-dependent class III HDAC. The founding member of the Sirtuins family is tightly implicated in the regulation of numerous key cellular processes including apoptosis and cell survival, DNA damage repair, chromatin remodeling,

gene expression regulation, and cancer development and metastasis [20, 21]. It has been shown that SIRT1 regulates genome stability in part through deacetylation of N-terminus tails of acetylated histones: H1K26ac, H3K56ac, H2A variant H2A.Z, in addition to H3K9ac and H4K16ac [14, 15, 22]. SIRT1 also regulates the catalytic activity of a plethora of downstream non-histone targets. For example, SIRT1 can deacetylate and downregulate the activity of tumor suppressor enzymes such as p53 [23], p73 [24], E2F1 [25], and Forkhead box proteins FOXO transcription factors [26], but also oncogenes such as NF-kappaB [27], STAT3 [28], Survivin [29] and β -Catenin [30]. On the contrary, SIRT1 can upregulate the activity of other oncogenes like c-Myc [31] and HIF-1 α [32]. As a result, the critical role of multifaceted SIRT1 in human carcinogenesis remains very controversial due to its contradictory functional roles [33, 34]. In breast cancer, both tumor-suppressive and tumor-promoting functions of SIRT1 have been reported and the controversy regarding SIRT1 role in the disease continues still.

SIRT1 implication in the occurrence and progression of breast cancer pathogenesis have been identified and extensively investigated over recent years. However, SIRT1-dependent epigenetic regulation of H3 and H4 acetylated histone marks, and consequently cancer-related gene expression in human breast cancer, has not been investigated yet. In this study, we examined for the first time the epigenetic mechanisms by which SIRT1 regulates the acetylation patterns of histones H3 and H4 epigenetic marks in sporadic breast cancer, we also investigated the link between SIRT1 and the 3 epi-marks H3K4ac, H3K9ac and H4K16ac in all 5 intrinsic subtypes of the disease. The present report adds a layer of clarity on the ongoing controversy of SIRT1 behavior in human breast carcinoma.

RESULTS

Description of the study cohort characteristics

The breast cancer molecular subtypes studied here spanned luminal A ($n = 36$, 26.7%), luminal B (HER2-) ($n = 34$, 25.2%), luminal B (HER2+) ($n = 25$, 18.5%), HER2- enriched ($n = 15$, 11.1%) and triple-negative breast cancer (TNBC) ($n = 25$, 18.5%) (Table 1). All patients were females aged 40 to 84 years (mean $64.6 \pm SD 5.3$). All tumors were graded according to the modified Scarff-Bloom-Richardson grading system (SBR) as grade 1 ($n = 17$), grade 2 ($n = 69$) and grade 3 ($n = 49$). Tumor size ranged from 0.4 to 7.8 cm (3.1 ± 0.7). Samples were ER-, PR- and HER2-positive in $n = 95$ (70.3%), $n = 58$ (42.9%) and $n = 40$ (29.6%) patients, respectively. We found an insignificant correlation between all intrinsic subtypes and age of patients ($p = 0.643$) and tumor size ($p = 0.079$). Luminal A and B (HER2-) subtypes presented a significant correlation with low SBR grade tumors, whereas luminal B (HER2+), HER2- enriched and triple-negative subtypes

Table 1: Clinico-pathological characteristics of the breast cancer patients included in this study

	Total	Luminal A	Luminal B (HER2-)	Luminal B (HER2+)	HER2-enriched	Triple-negative	P value [†]
Patients, n (%)	N = 135 (100%)	n = 36 (26.7%)	n = 34 (25.2%)	n = 25 (18.5%)	n = 15 (11.1%)	n = 25 (18.5%)	
Age at diagnosis							0.643
45–65	66 (48.8)	21 (58.3)	15 (44.1)	12 (48)	8 (53.3)	10 (40)	
> 65	69 (51.2)	15 (41.6)	19 (55.8)	13 (52)	7 (46.6)	15 (60)	
SBR grade							0.0001
I	17 (12.5)	13 (36.1)	4 (11.7)	0	0	0	
II	69 (51.1)	21 (58.3)	24 (70.5)	11 (44)	6 (40)	7 (28)	
III	49 (36.4)	2 (5.5)	6 (17.6)	14 (56)	9 (60)	18 (72)	
Size of tumor (cm)							0.079
< 1.5	31 (22.9)	11 (30.5)	9 (26.4)	5 (20)	2 (13.3)	4 (16)	
1.5–2.5	61 (45.1)	19 (52.7)	18 (52.9)	11 (44)	5 (33.3)	8 (32)	
> 2.5	43 (31.8)	6 (16.6)	7 (20.5)	9 (36)	8 (53.3)	13 (52)	
ER status (%)							0.0001
Positive	95 (70.3)	36 (100)	34 (100)	25 (100)	0	0	
Negative	40 (29.6)	0	0	0	15 (100)	25 (100)	
PR status (%)							0.0001
0%–50%	20 (14.8)	7 (19.4)	8 (23.5)	5 (20)	0	0	
51%–100%	38 (28.1)	29 (80.5)	7 (20.5)	2 (8)	0	0	
Negative	77 (57)	0	19 (55.8)	18 (78)	15 (100)	25 (100)	
HER2 status (%)							0.0001
Positive	40 (29.6)	0	0	25 (100)	15 (100)	0	
Negative	95 (70.3)	36 (100)	34 (100)	0	0	25 (100)	
Ki67 status (%)							0.0001
≤ 20%	53 (39.2)	29 (80.5)	8 (23.5)	7 (28)	4 (26.6)	5 (20)	
> 20%	82 (60.7)	7 (19.4)	26 (76.4)	18 (72)	11 (73.3)	20 (80)	

Abbreviations: ER: Estrogen Receptor, PR: Progesterone Receptor, HER2: Human Epidermal growth factor Receptor 2, Ki-67: cellular marker for proliferation.

[†]Pearson's Chi-square test.

exhibited high SBR grade tumors ($p < 0.001$). As for the hormonal receptors status, a clear distinction between the 5 molecular subtypes can be noted as per the molecular classification of St. Gallen. The clinico-pathological variables of the 135 breast cancer patients are presented in (Table 1).

Inverse correlation between SIRT1 and H3k4ac, H3k9ac and H4k16ac global expression patterns in breast tumors versus matched normal tissues

In order to investigate the epigenetic role of the histone deacetylase SIRT1 in sporadic breast cancer, we began our studies in *ex-vivo* by assessing the relative

expression levels of SIRT1 and the 3 epigenetic marks H3k4ac, H3k9ac and H4k16ac in all 5 molecular subtypes of breast tumors and their matched normal tissue samples using immunoblot analysis (Figure 1A). The blots showed a significant upregulation of SIRT1 expression levels in luminal and HER2-enriched subtypes and significant downregulation in TNBC subtype, in comparison with their matched normal tissues (Figure 1B). The differential expression pattern of SIRT1 across the 5 molecular subtypes was characterized in our earlier study [35]. In contrast, the expression levels of H3k4ac, H3k9ac, and H4k16ac were significantly reduced in luminal and HER2-enriched subtypes and relatively upregulated in TNBC subtype, all compared to their matched normal tissues

(Figure 1B). This inverse correlation provides a causal link between the expression patterns of SIRT1 and the 3 epi-marks in human breast cancer.

SIRT1 simultaneously colocalizes and physically interacts with histone H3 acetylated marks in human breast cancer

In order to determine whether HDAC SIRT1 interacts with histone H3 acetylated epi-marks in human

breast cancer, we began by performing chromatin immunoprecipitation (ChIP) assays of SIRT1 on 50 breast tumors and their 50 matched normal tissues ($n = 10$ tumors for each of the 5 molecular subtypes). The assays were then analyzed with real-time quantitative PCR (q-PCR) targeting the promoters of a gene panel consisted of 6 genes. The targeted genes are strongly deregulated and directly implicated in the pathogenesis of breast cancer, as follows: *ERS1*, *ERS2* and *AR* genes that code for the Estrogen receptors ($ER-\alpha$), ($ER-\beta$) and the Androgen

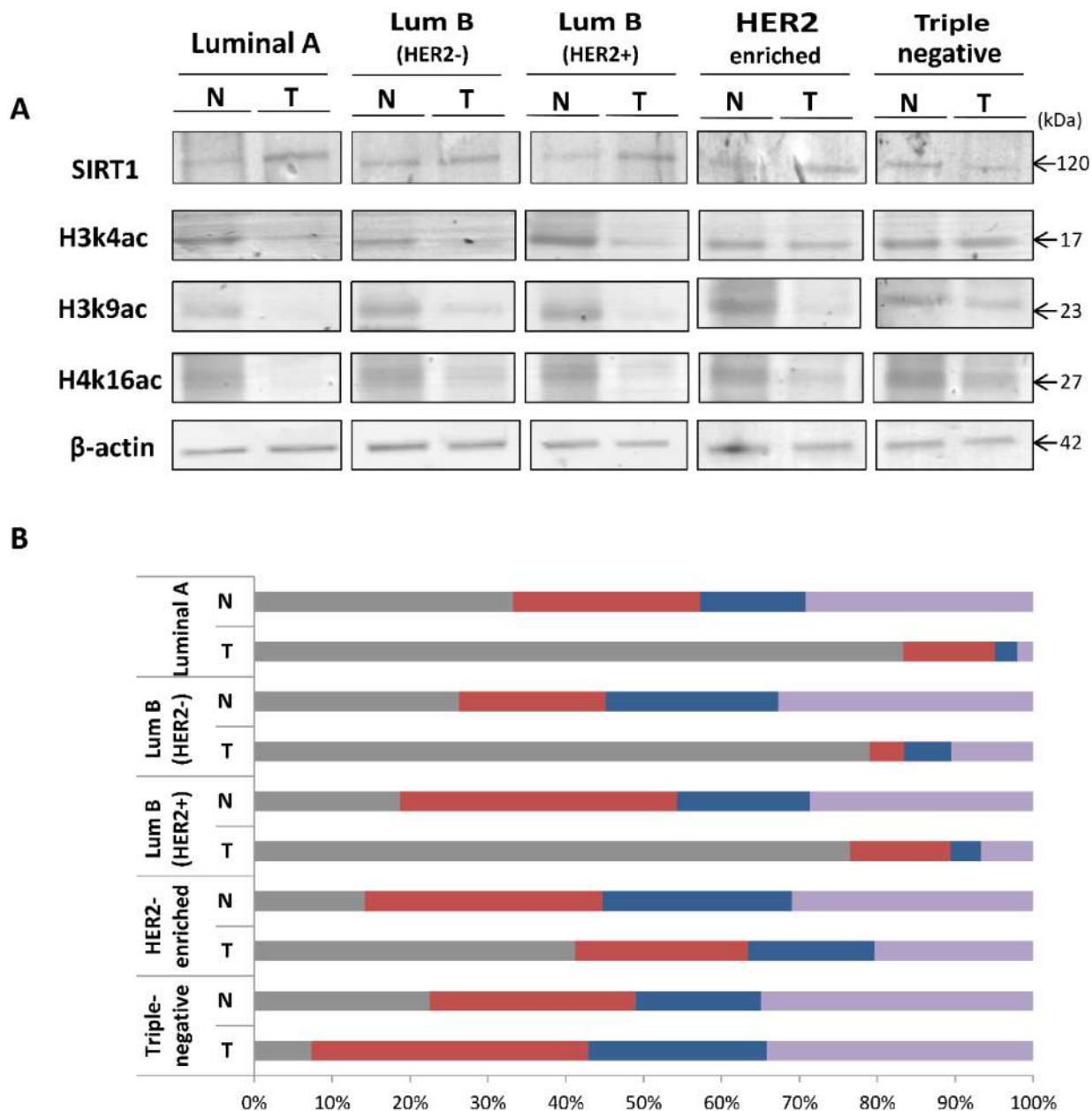


Figure 1: Differential expression patterns of SIRT1, H3k4ac, H3k9ac and H4k16ac in the 5 molecular breast tumor subtypes compared to matched normal tissues. (A) Equal amounts of proteins were immunoblotted with anti-SIRT1 Ab (120 kDa), anti-H3k4ac Ab (17 kDa), anti-H3k9ac Ab (23 kDa) and anti-H4k16ac Ab (27 kDa). β -actin (42 kDa) served as an internal loading control. (B) Relative expression levels were evaluated using Quantity One software and normalized against the internal control β -actin. Each bar represents the percentage contribution of each of the 4 proteins compared to the total set as (100%). All experiments were performed in triplicate fashion. N: Normal, T: Tumor.

receptor (AR) respectively, the tumor suppressor gene *BRCA1*, and *EZH2* and *EP300* genes coding for histone modifying enzymes (EZH2) and (p300) respectively. The results of ChIP assays showed a significant increase of SIRT1 enrichment on promoters of targeted genes in HRBC and H2BC subtypes, and less significantly in TNBC subtype in comparison to matched normal tissues. The data evoke the possibility that SIRT1 plays a role in the epigenetic regulation of these genes expression in breast cancer (Figure 2). A multi-way analysis of variance (ANOVA) test showed a significant difference of SIRT1 enrichment patterns on gene promoters across all subtypes. Tukey's range test was then used for multiple comparisons to identify sample means that are significantly different from each other. Two factors were taken into account when performing the statistical procedures: breast cancer molecular subtype (Group effect) and targeted gene type (Gene effect). The post-hoc analysis distinguished 3 distinct patterns of SIRT1 enrichment depending on human breast tumors subtypes, SIRT1 was found to be most enriched on target gene promoters in luminal B subtypes, then luminal A and HER2-enriched subtypes and finally, least enriched in TNBC subtype (Figure 3A). However, there was no significant discrimination of

SIRT1 enrichment in relation to different types of genes (Figure 3B).

After confirming the presence of SIRT1 on target gene promoters, we proceeded to investigate whether SIRT1 specifically interacts with histone H3 acetylated marks on those promoters by performing ChIP followed by re-ChIP assays on 110 breast tumors from all 5 molecular subtypes and their 110 matched normal tissues ($n = 26$ luminal A, $n = 24$ luminal B (HER2-), $n = 20$ luminal B (HER2+), $n = 15$ HER2-enriched and $n = 25$ triple-negative). Breast tissues were first assayed by ChIP using anti-H3k4ac or anti-H3k9ac Abs. The obtained samples were then re-immunoprecipitated a second time with anti-SIRT1 Ab. Finally, the immunoprecipitates were analyzed by real-time q-PCR targeting the promoters of the breast cancer-related gene panel previously described. The results showed a simultaneous co-occupancy of SIRT1 with H3k4ac and with H3k9ac on all 6 gene promoters across all 5 subtypes in comparison to matched normal tissues (Figure 4), suggesting that SIRT1 could affect the expression of our targeted genes through epigenetic modification of histone H3 lysine 4 (K4) and lysine 9 (K9) on their promoters. The results also showed a great discrepancy of SIRT1-H3k4ac/H3k9ac colocalization profiles that seem to differ depending

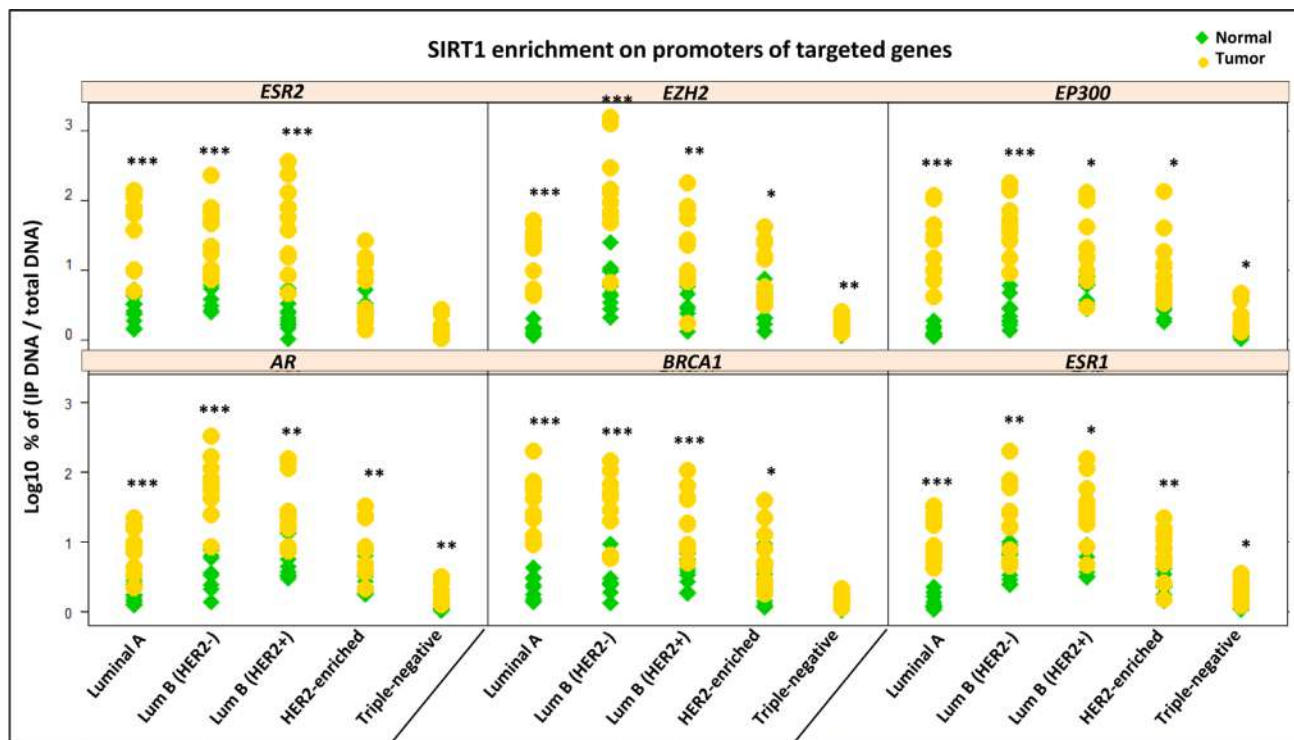


Figure 2: SIRT1 enrichment on promoters of 6 breast cancer-related genes in the 5 molecular breast tumor subtypes versus matched normal tissues. Column scatter plot showing the results of ChIP assays using anti-SIRT1 Ab on 50 breast tumors and their 50 matched normal tissues: $n = 10$ tumors for each of the 5 molecular subtypes. The efficiency of ChIP was calculated by real time quantitative PCR using the primers and probes of 6 targeted genes: *AR*, *BRCA1*, *ERS1*, *ERS2*, *EZH2* and *EP300*. The y-axis represents the log expression percent of (IP DNA/Total DNA) on target genes promoters. Statistically significant difference of SIRT1 enrichment in tumors versus normal tissues was analyzed by Student's *t*-test. *P* values were two-tailed, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ were considered statistically significant.

on molecular subtype, targeted gene type and studied epi-mark, suggesting that SIRT1-epigenetic regulation depends on multiple factors in different molecular subtypes. To further clarify this observation, multiple-

group comparisons ANOVA test followed by Tukey's range test were carried out with three factors taken into account: molecular subtype (Group effect), gene type (Gene effect) and targeted epi-mark (Mark effect). The

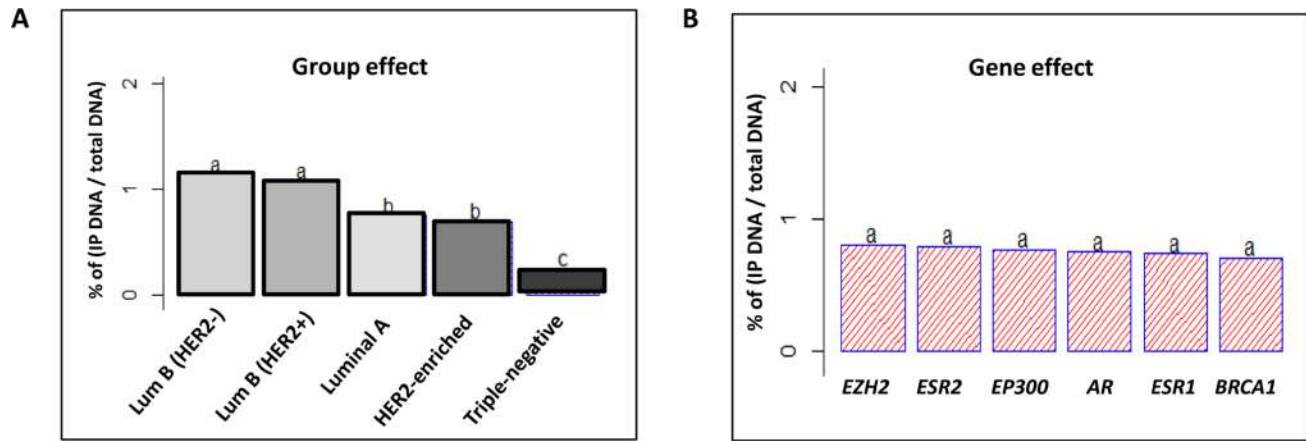


Figure 3: Tukey's post-hoc comparison of the means analyzing Group and Gene effects. ANOVA test followed by Tukey's multiple comparison test were performed on the results of 50 ChIP assays analyzed by q-PCR (A) The statistical analysis discerned 3 different SIRT1 enrichment patterns depending on tumor molecular subtype (Group effect). (B) Insignificant discrimination of SIRT1 enrichment in relation to variable gene types (Gene effect). The letters 'a', 'b' and 'c' indicate statistical significance between groups.

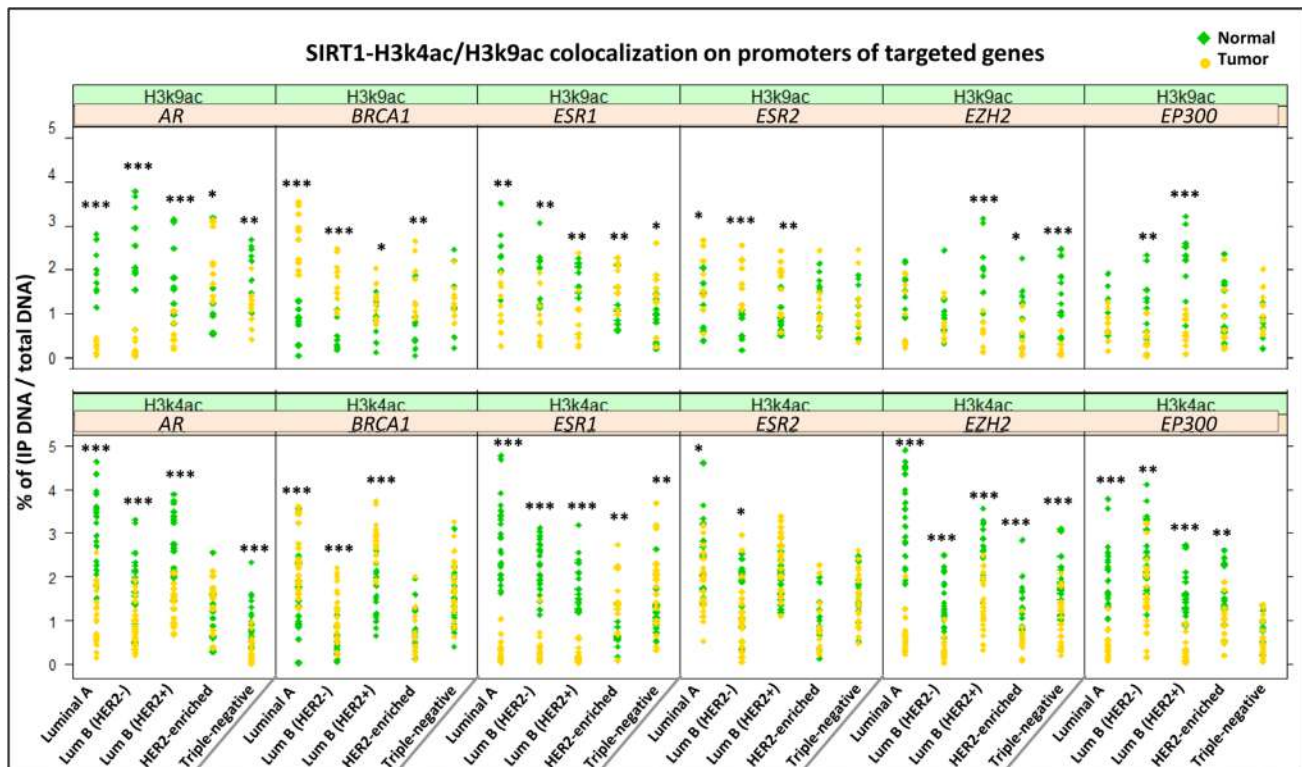


Figure 4: Simultaneous colocalization of SIRT1-H3k4ac and SIRT1-H3k9ac on targeted gene panel promoters across all 5 molecular subtypes versus matched normal tissues. Column scatter plot showing the results of ChIP followed by re-ChIP assays using anti-SIRT1, anti-H3k4ac and anti-H3k9ac Abs on 110 breast tumors and their 110 matched normal tissues. The breast tumors were divided as follows: $n = 26$ luminal A, $n = 24$ luminal B (HER2-), $n = 20$ luminal B (HER2+), $n = 15$ HER2-enriched and $n = 25$ triple-negative breast tumors. The efficiency of ChIP was calculated by real time q-PCR on promoters of 6 targeted genes: *AR*, *BRCA1*, *ESR1*, *ESR2*, *EZH2* and *EP300*. The y-axis represents the percentage of (IP DNA/Total DNA) on target genes promoters. Statistically significant difference of SIRT1 colocalization patterns in tumors versus normal tissues was analyzed by Student's *t*-test. *P* values were two-tailed, $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ were considered statistically significant.

post-hoc analysis discerned 2 different SIRT1-H3k4ac/H3k9ac colocalization profiles depending on tumor subtype (Figure 5A) and 3 different colocalization profiles depending on gene type (Figure 5B). Intriguingly, the statistical analysis showed that SIRT1 significantly colocalizes with H3k4ac over H3k9ac on targeted genes across all tumor subtypes (Figure 5C). Afterward, we proceeded to examine whether there is an actual direct interaction between HDAC SIRT1 and histone H3 epimarks in breast cancer. To do so, we conducted several co-immunoprecipitation assays. Proteins were extracted from breast tumors from each of the 5 molecular subtypes and their matched normal tissues. Extracted proteins were at first immunoprecipitated with anti-H3k4ac or anti-H3k9ac Abs, the immunoprecipitates were then immunoblotted with anti-SIRT1 Ab. The co-immunoprecipitation assays highlighted a global physical interaction between SIRT1 and H3k4ac as well as H3k9ac across all molecular subtypes, implying that SIRT1 could directly deacetylate H3k4ac and H3k9ac in breast cancer. Additionally, the

direct interaction between SIRT1 and both epimarks is significantly increased in breast tumors compared to matched normal tissues (Figure 6).

Active role of SIRT1 in the deacetylation of H3k4 acetylated mark (H3k4ac) in human breast cancer

Unlike H3k9ac and H4k16ac, H3k4ac is not a known histone target of human histone deacetylase SIRT1. However, Silent Information Regulator 2 (SIR2), the highly conserved orthologue of mammalian SIRT1 in yeast, is the major HDAC of H3k4ac [36]. After uncovering an inverse correlation between SIRT1 and H3k4ac expression patterns, a simultaneous co-occupancy on the same genomic locus and a direct physical interaction between the two across all breast tumors subtypes, we hypothesized that HDAC SIRT1 could play an active role in the deacetylation of H3k4ac in human breast cancer.

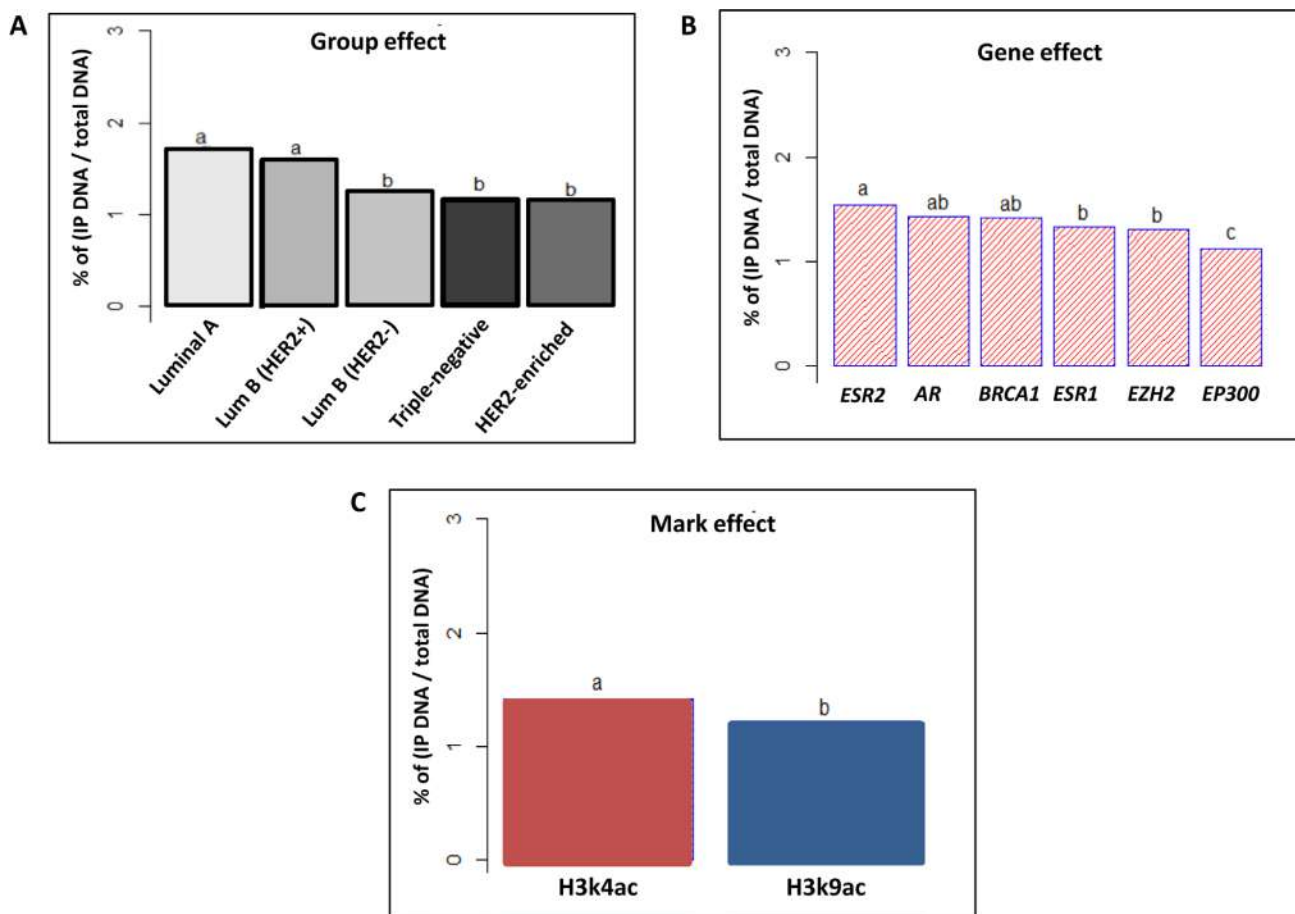


Figure 5: Tukey's post-hoc comparison of the means analyzing Group, Gene and Mark effects. ANOVA test followed by Tukey's multiple comparison test were performed on the results of 110 ChIP assays analyzed by q-PCR (A) The statistical analysis distinguished 2 distinct patterns of SIRT1-H3k4ac/H3k9ac collocation on target promoters depending on tumor molecular subtype (Group effect). (B) 3 distinct patterns of SIRT1-H3k4ac/H3k9ac collocation depending on gene type (Gene effect). (C) Significant collocation of SIRT1 with H3k4ac over H3k9ac across all gene types and tumor subtypes (Mark effect). The letters 'a', 'b' and 'c' indicate statistical significance between groups.

Inverse correlation between SIRT1 and H3k4ac expression patterns in 5 intrinsic subtype breast cancer cell lines

We began our *in-vitro* experiments by assessing the relative expression levels of SIRT1 and H3k4ac in 5 intrinsic subtype breast cancer cell lines using immunoblot analysis. (ER+) breast cancer cell lines: MCF-7 and T-47D were used as representatives of the luminal subtype, whereas (ER-) breast cancer cell lines: MDA-MB 453, MDA-MB 231 and MDA-MB 468 were used as representatives of the triple-negative subtype. The normal breast cell line MCF10A was used as a control. We observed significantly high expression of SIRT1 in MCF-7, T-47D, MDA-MB 453 and MDA-MB 231 cell lines, and relatively lower expression in MDA-MB 468, all compared to MCF10A cell line. At the opposite, significantly low H3k4ac expression levels were observed in all 5 intrinsic cell lines in comparison with the control cell line (Figure 7).

SIRT1-siRNA suppresses SIRT1 expression and induces a global increase in H3k4ac, as well as H3k9ac and H4k16ac expression levels in breast cancer cell lines

To gain insight into the mechanism responsible for the deacetylation of H3k4 acetylated mark (H3k4ac), we silenced SIRT1 expression with SIRT1-siRNA (small interfering RNA) in the 5 human-derived mammary cell lines previously described. We were interested

in determining whether SIRT1 depletion could alter the relative expression patterns of H3k4ac, as well as H3k9ac and H4k16ac in breast cancer. After 48 hours of transfection, extracted proteins were subjected to immunoblot analysis (Figure 8A). The results showed a significant decrease of SIRT1 expression levels in all MCF-7, T-47D, MDA-MB 453, MDA-MB 231 and MDA-MB 468 transfected cell lines compared to non-transfected control cell lines (Figure 8B). More importantly, a significant increase of H3k4ac, as well as H3k9ac and H4k16ac expression levels, were observed in all 5 transfected cell lines compared to control cell lines (Figure 8B). SIRT1 depletion has led to increased H3k4 acetylation in 5 intrinsic subtype breast cancer cell lines, thus, the deacetylation of H3k4ac seems to be mainly dependent on SIRT1 histone deacetylase activity in breast cancer. Furthermore, the inverse correlation between SIRT1 and the 3 epi-marks expression patterns in transfected versus non-transfected cell lines is similar to that found in breast tumors compared to matched normal tissues. Therefore, SIRT1 seems to be directly responsible for the modulation of H3k4ac, as well as H3k9ac and H4k16ac expression patterns, obviously through direct deacetylation, in breast cancer.

SIRT1 knockdown modulates histone acetylation at targeted gene panel promoters in a subtype-specific manner

To further elucidate SIRT1 epigenetic role in human breast cancer, we conducted direct H3k4ac and

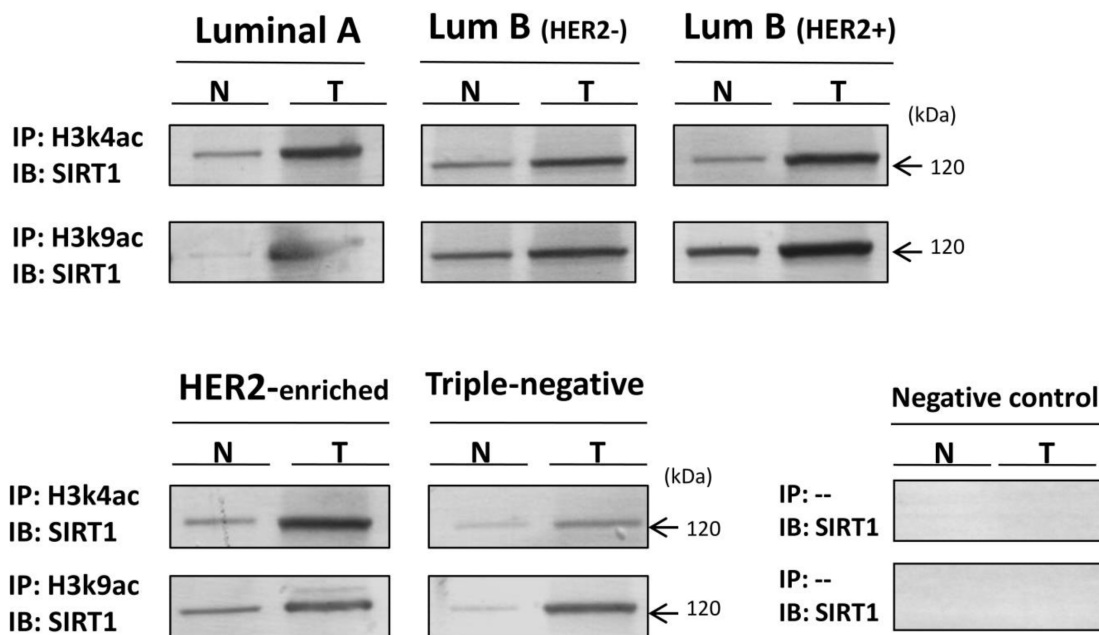


Figure 6: Global physical interaction between SIRT1 and H3k4ac/H3k9ac epi-marks across all molecular breast tumor subtypes compared to matched normal tissues. 100 to 500 μ g of extracted proteins were at first immunoprecipitated using anti-H3k4ac or anti-H3k9ac Abs (IP), or immunoprecipitated without Ab (IP:--) that served as negative control. The immunoprecipitates were then immunoblotted (IB) with anti-SIRT1 Ab. All experiments were performed in triplicate fashion. N: Normal, T: Tumor.

H3k9ac ChIP assays on transfected cell lines. We wanted to investigate whether SIRT1 gene silencing could specifically alter the enrichment patterns of histone H3 acetylated epi-marks on the promoters of our target genes, and consequently, impact the targeted genes expression patterns in breast cancer. To explore this possibility, transfected and non-transfected MCF-7, T-47D, MDA-MB 453, MDA-MB 231 and MDA-MB 468 cell lines were subjected to ChIP assays using anti-H3k4ac or anti-H3k9ac Abs. The samples were analyzed by real-time q-PCR targeting the promoters of the breast cancer-related gene panel: *AR*, *BRCA1*, *ERS2*, *ERS1*, *EZH2*, and *EP300*. The results showed a significant increase of both targeted epi-marks H3k4ac and H3k9ac on all 6 gene promoters across all 5 transfected cell lines in comparison to non-transfected control lines (Figures 9 and 10). Interestingly, 2 distinct patterns of H3k4ac and H3k9ac enrichment can be observed following SIRT1 knockdown, the patterns seem to be predominantly dependent on breast cancer cell line intrinsic subtype. The 2 epi-marks were found to be particularly enriched on *BRCA1* and *ERS2* gene promoters in luminal (ER+) subtype cell lines: MCF-7 (Figure 9A) and T-47D (Figure 9B). In contrast, both epi-marks were especially enriched on *AR*, *EZH2* and *EP300* promoters in triple-negative (ER-) subtype cell lines: MDA-MB 453 (Figure 10A), MDA-MB 231 (Figure 10B) and MDA-MB 468 (Figure 10C), implying that SIRT1 regulates its H3 histone targets principally depending on molecular subtype. In conclusion, SIRT1 siRNA-mediated knockdown has significantly increased the acetylation levels of H3k4ac and H3k9ac at the breast cancer-related gene panel promoters; thus, SIRT1

mediates the deacetylation of histone marks H3k4ac, as well as H3k9ac, in breast cancer. The results also revealed SIRT1 differential regulation of H3 acetylated epi-marks in a subtype-specific manner.

DISCUSSION

Breast cancer is the most commonly diagnosed cancer in women worldwide; it is a multifactorial genetic disease. Sporadic breast tumors represent 85 to 90% of all breast tumors and are especially characterized by an altered epigenome. Deregulated histone epigenome along with other epigenetic alterations play a crucial role in the initiation and progression of breast cancer [9, 37]. Sirtuin-1 is a class III histone deacetylase that can deacetylate both histone and non-histone targets. The mammalian counterpart of yeast SIR2 is deeply implicated in breast cancer development and metastasis. The contradictory functional roles of SIRT1 in breast cancer have been extensively studied over recent years. However, the underlying molecular mechanism by which HDAC SIRT1 regulates its acetylated histone targets, and consequently cancer-related gene expression in breast cancer, is still unknown. In this study, we identified a new prime target of SIRT1 in breast cancer, the acetylated H3k4 histone mark (H3k4ac). We also highlighted a SIRT1-dependent modulation of histones H3 and H4 acetylation patterns in breast cancer. Moreover, we revealed that SIRT1 regulation of its H3 acetylated targets depends greatly on gene type and molecular subtype. Furthermore, we showed that SIRT1 depletion increases histone H3 acetylation levels in a subtype-specific manner

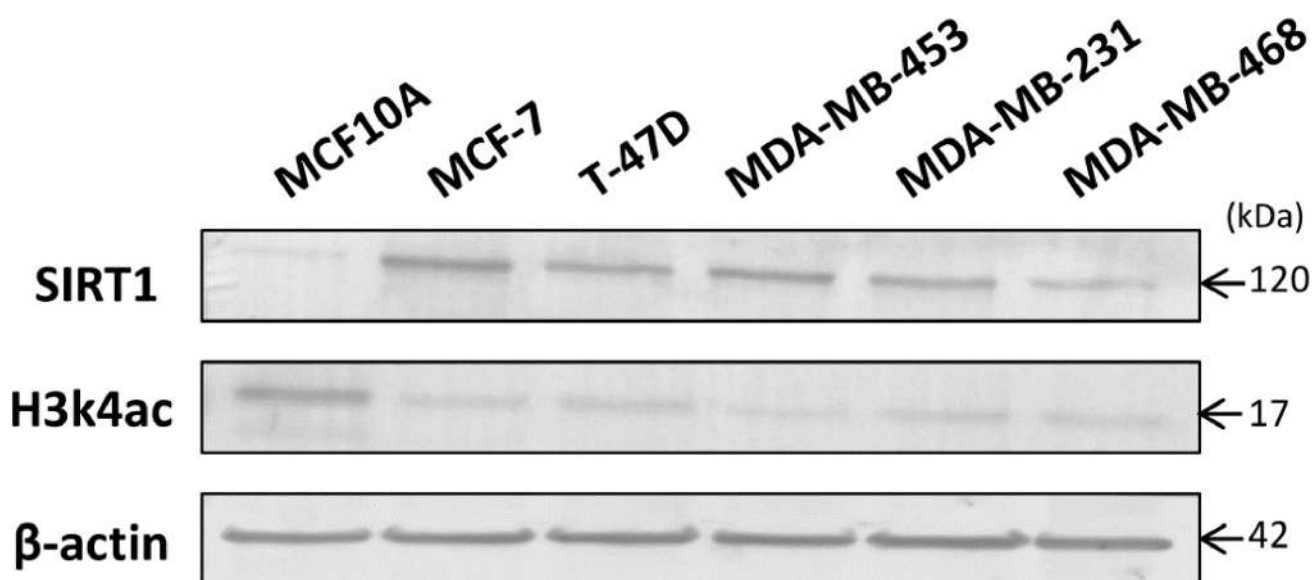


Figure 7: Inverse correlation between SIRT1 and H3k4ac expression levels in 5 intrinsic breast cancer cell lines compared to normal breast cell line. Proteins were extracted from MCF10A, MCF-7, T-47D, MDA-MB 453, MDA-MB 231 and MDA-MB 468 cell lines. Equal amounts of extracted proteins were immunoblotted using anti-SIRT1 Ab (120 kDa) and anti-H3k4ac Ab (17 kDa). β -actin (42 kDa) served as an internal loading control.

at 6 breast cancer-related gene promoters: *AR*, *BRCAL*, *ERS1*, *ERS2*, *EZH2*, and *EP300*, suggesting that SIRT1 could play an active role in regulating their expression in breast cancer pathogenesis. This is the first report that characterizes the epigenetic behavior of SIRT1 in breast cancer and establishes its status as an epigenetic eraser in human breast carcinoma.

Alteration of histone epigenome is one of the earliest steps in oncogenic transformation. Since histone marks have a direct effect on cancer-related gene

expression [7, 38], and since different breast cancer subtypes present distinct gene expression profiles [3] [39], it becomes essential to study the mechanisms of histone epigenome regulation in different subtypes of breast cancer pathogenesis. SIRT1 plays a major role in maintaining genome integrity, largely through regulation of epigenetic mechanisms. SIRT1 epigenetic regulation is realized through direct deacetylation of specific histone markers and controlling the activity of chromatin-modifying enzymes [22]. Histone marks H3k4ac, H3k9ac,

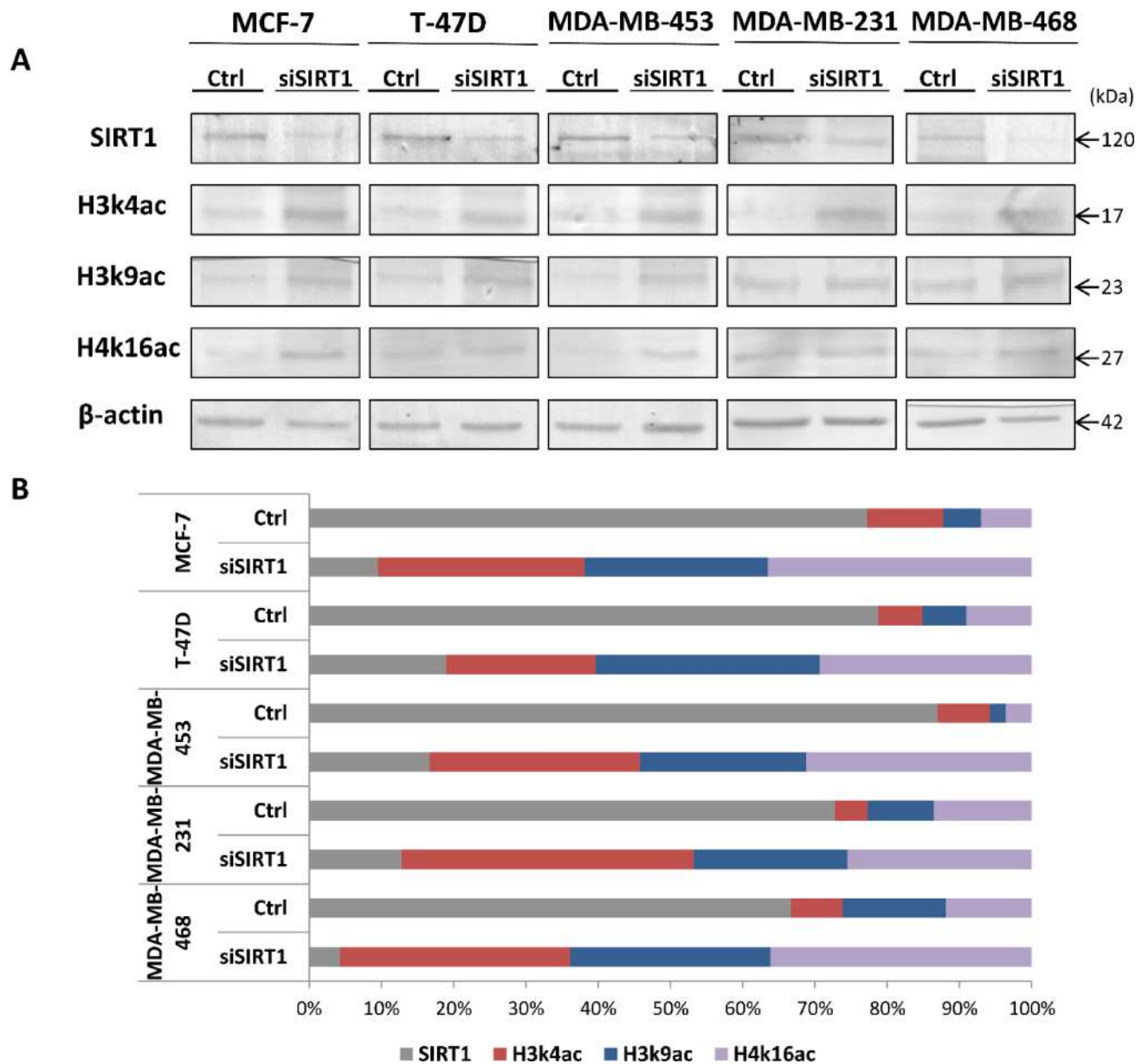


Figure 8: Control of SIRT1 gene silencing with SIRT1-siRNA and its impact on the expression patterns of targeted epi-marks H3k4ac, H3k9ac and H4k16ac *in-vitro*. (A) MCF-7, T-47D, MDA-MB 453, MDA-MB 231 and MDA-MB 468 cells were transfected with SIRT1-siRNA (siSIRT1) or negative control siRNA (Ctrl). After 48 hours of transfection, equal amounts of proteins were immunoblotted with anti-SIRT1 Ab (120 kDa), anti-H3k4ac Ab (17 kDa), anti-H3k9ac Ab (23 kDa) and anti-H4k16ac Ab (27 kDa). β-actin (42 kDa) served as an internal loading control. (B) Relative expression levels were evaluated using Quantity One software and normalized against the internal control β-actin. Each bar represents the percentage contribution of each of the 4 proteins compared to the total set as (100%). All experiments were performed in triplicate fashion.

and H4k16ac are well-established epigenetic markers of active transcription and actively participate in gene expression [12, 13]. In this study, we showed that the 3 epi-marks relative expression patterns were significantly reduced in breast tumors compared to normal tissues, especially in HRBC and H2BC subtypes. Interestingly, SIRT1 is significantly upregulated in those particular subtypes, previously described in our earlier study [35]. This observation prompted us to suggest that SIRT1 is

directly or indirectly responsible for the modulation of the 3 targeted marks in breast cancer. To validate this observation, we silenced SIRT1 expression *in-vitro* via small interfering RNA (siRNA). We opted to use 5 human mammary cell lines that represent the 2 main molecular subtypes of breast cancers: luminal (ER+) and triple-negative (ER-) subtypes. We chose MCF-7 and T-47D cell lines that are classically used as representatives of luminal subtype. Whereas (ER-) cell lines MDA-MB 453, MDA-

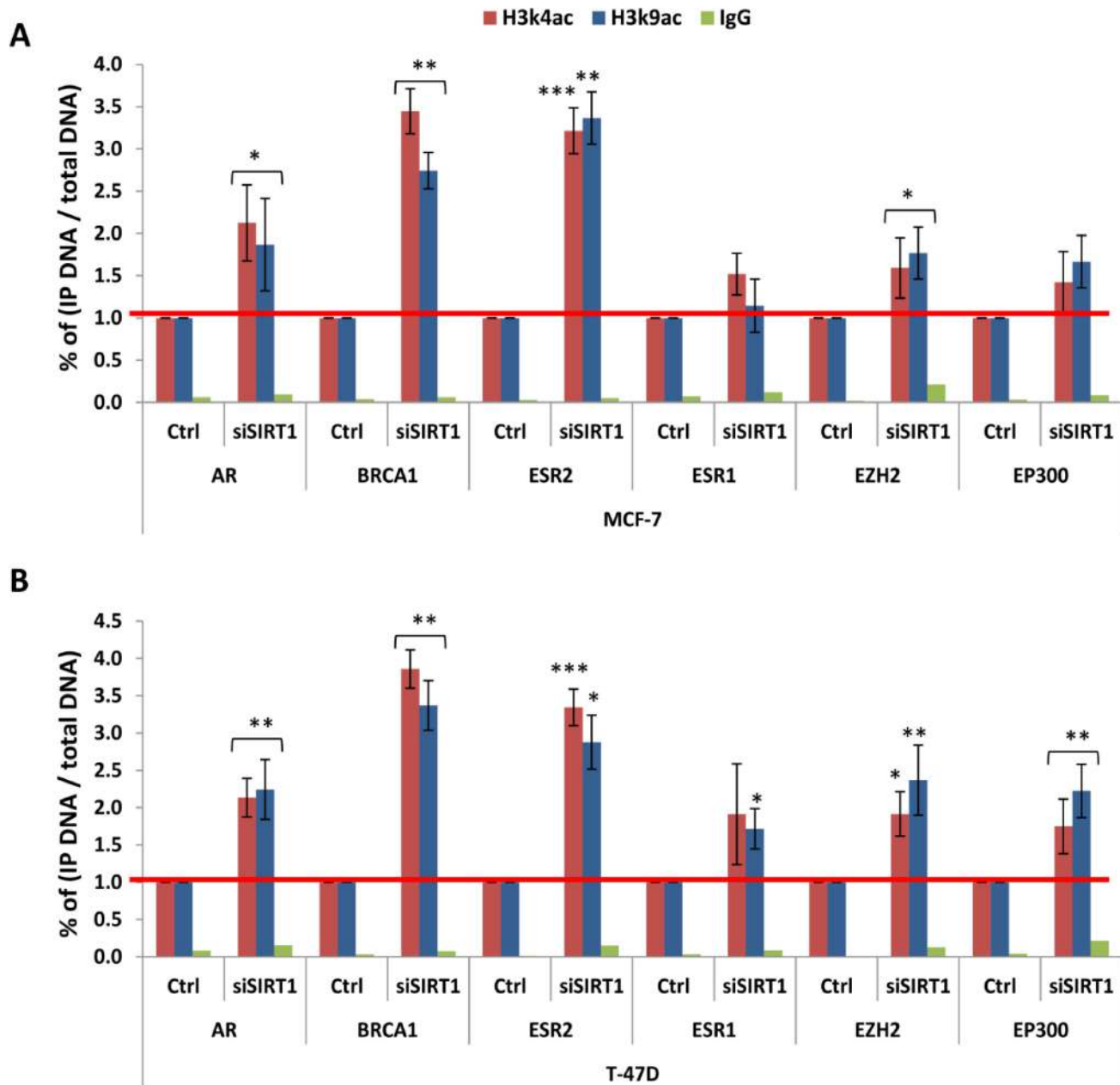


Figure 9: Impact of SIRT1 knockdown on the enrichment of H3k4ac and H3k9ac at targeted gene panel promoters in luminal subtype cell lines. Transfected (siSIRT1) and non-transfected (Ctrl) MCF-7 (A) and T-47D (B) cell lines were subjected to direct ChIP assays using anti-H3k4ac Ab, anti-H3k9ac Ab and non-immune IgG Ab serving as negative control. The efficiency of ChIP was calculated by real time q-PCR on promoters of 6 targeted genes: *AR*, *BRCA1*, *ERS2*, *ERS1*, *EZH2* and *EP300*. All data are presented as fold enrichment of transfected over control cell lines (set as 1). The y-axis represents the percentage of (IP DNA/Total DNA) on target genes promoters. Each column represents the mean \pm SD of 3 replicate experiments. *P* values were two-tailed, **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 were considered statistically significant.

MB 231 and MDA-MB 468 were used as representatives of the 3 main molecular subtypes of triple-negative breast cancers: Luminal Androgen Receptor (LAR),

Mesenchymal-like and Basal-like subtypes respectively, as elegantly characterized by Lehman *et al.* [40]. SIRT1 gene silencing has caused a significant increase of global

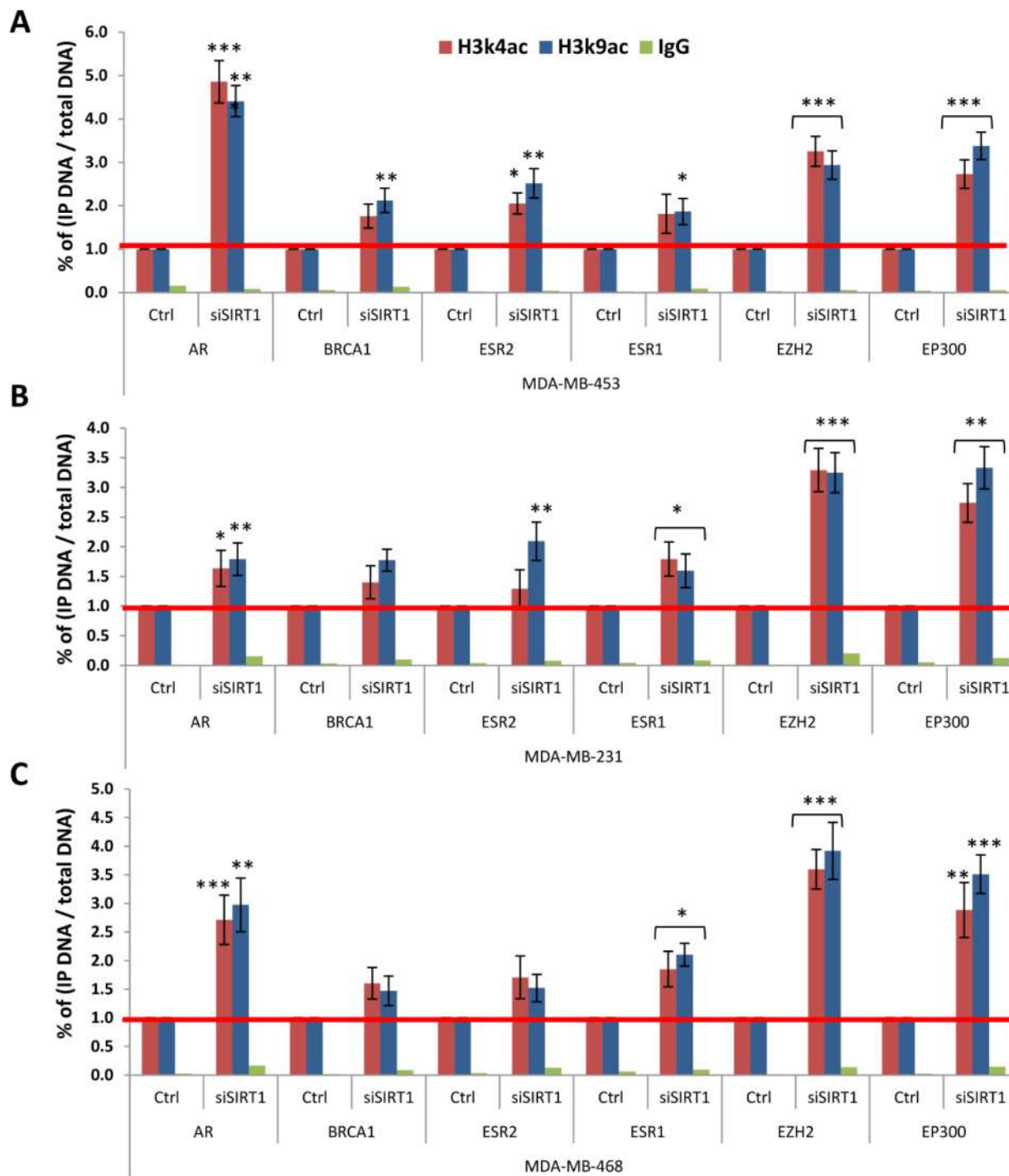


Figure 10: Impact of SIRT1 knockdown on the enrichment of H3k4ac and H3k9ac at targeted gene panel promoters in TNBC subtype cell lines. Transfected (siSIRT1) and non-transfected (Ctrl) MDA-MB 453 (A), MDA-MB 231 (B) and MDA-MB 468 (C) cell lines were subjected to direct ChIP assays using anti-H3k4ac Ab, anti-H3k9ac Ab and non-immune IgG Ab serving as negative control. The efficiency of ChIP was calculated by real time q-PCR on promoters of 6 targeted genes: *AR*, *BRCA1*, *ERS2*, *ERS1*, *EZH2* and *EP300*. All data are presented as fold enrichment of transfected over control cell lines (set as 1). The y-axis represents the percentage of (IP DNA/Total DNA) on target genes promoters. Each column represents the mean \pm SD of 3 replicate experiments. *P* values were two-tailed, **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 were considered statistically significant.

expression levels of the 3 targeted epi-marks in all transfected lines versus non-transfected control lines, we thus concluded that SIRT1 is actively responsible for the modulation of H3k4ac, H3k9ac, and H4k16ac in luminal and triple-negative molecular subtypes of breast cancer.

To further analyze SIRT1 epigenetic role in sporadic breast cancer, we opted to study SIRT1 interaction with its H3 acetylated targets by carrying out ChIP and re-ChIP assays on 6 breast-cancer related genes: *AR*, *BRCA1*, *ERS1*, *ERS2*, *EZH2* and *EP300*. The targeted genes play major roles in breast cancer carcinogenesis, either by stimulating breast tumors development and tumor progression, such is the case for oncogenes *AR* [41], *ERS1* [42], *EZH2* [43] and *EP300* [44], or having anti-proliferative properties such as tumor suppressor genes *BRCA1* [45] and *ERS2* [46]. SIRT1 ChIP data implies that the latter could have an active role in regulating the expression of these genes in breast cancer either by directly modulating the epigenetic histone markers on their promoters and/or recruiting other chromatin-modifying complexes to that genomic area. However, SIRT1 less significant binding in TNBC subtype could be explained by its reduced expression in it. ChIP, re-ChIP as well as co-immunoprecipitation assays confirmed that SIRT1 physically interacts with and regulates its H3 histone targets H3k4ac and H3k9ac across the 5 molecular subtypes. However, SIRT1 epigenetic regulation is significantly discriminated by gene type and molecular subtype. These results are in line with the findings of Li *et al.* [11] who demonstrated widespread subtype-specific histone modifications in different molecular subtypes. In fact, SIRT1 also negatively regulates the activity of epigenetic 'writers' that deposit the histone markers [22]. It has been shown that SIRT1 interacts with and impairs the activity of histone acetyltransferases (HATs) p300 and MOF that are responsible for H3k9 and H4k16 acetylation, [47, 48] respectively. Therefore, SIRT differential epigenetic regulation in breast cancer seems to extend to both histone markers and their epigenetic 'writers'.

SIRT1 widespread regulation of multiple cancer-related enzymes often leads to its multifaceted functions in various cancers. In consequence, the contradictory roles of SIRT1 were demonstrated in various human malignancies. In colorectal cancer (CRC), both confirmed tumor-suppressive and tumor-promoting functions of SIRT1 have been reported [49]. In a previous study, we suggested a bivalent role of SIRT1 in breast cancer based on its differential expression patterns in human breast tumors. We suggested that SIRT1 most probably has an oncogenic role in HRBC subtypes and a tumor-suppressor role in TNBC subtype [35]. To further explore SIRT1 differential epigenetic regulation in breast cancer, we performed *in-vitro* ChIP analysis with H3k4ac and H3k9ac on SIRT1-siRNA transfected cell lines previously described. SIRT1 knockdown has generated 2 distinct profiles of both epi-marks enrichment on targeted gene promoters

that corresponds to the 2 main molecular breast cancer subtypes. The results showed an increase of H3k4ac and H3k9ac expression by 3 to 4-fold on *BRCA1* and *ESR2* genes promoters in both (ER+) cell lines, indicating that SIRT1 contributes to their repression through epigenetic chromatin modification; hence exerting oncogenic properties in breast cancer luminal subtypes. These results are consistent with the findings of Elangovan *et al.* [50] and Ma *et al.* [51] who reported that SIRT1 overexpression in luminal breast cancer subtypes is positively correlated with an oncogenic behavior. At the opposite, in (ER-) cell lines MDA-MB 453, MDA-MB 231 and MDA-MB 468, SIRT1 deficiency induces a 2.5 to 4-fold increase of H3k4ac and H3k9ac expression on *EZH2* and *EP300* promoters, indicating that SIRT1 contributes to the 2 oncogenes repression; hence exerting tumor-suppressive properties in breast cancer triple-negative subtypes. However, a slight increase of H3k4ac and H3k9ac expression on *EZH2* promoter was also observed in the in (ER+) cell lines, implying that *EZH2* expression could be regulated in part by SIRT1 in different subtypes of breast cancer. We also noticed a dramatic increase of H3k4ac and H3k9ac expression by 4.5 to 5-fold on *AR* promoter in MDA-MB 453 cell line representative of the LAR subtype. In fact, LAR subtype or apocrine breast carcinoma is characterized by the expression of *AR* oncogene that contributes to breast tumorigenesis [40]. Therefore, SIRT1 seems to exert tumor-suppressive properties in apocrine breast cancer as well, through epigenetic repression of the *AR* oncogene. These findings are in line with the studies of Yi *et al.* [52] and Simic *et al.* [53] who reported that SIRT1 overexpression suppressed cancer metastasis and tumor cell invasion in triple-negative breast cancer. Based on the above knowledge, we suggest that SIRT1 selectively regulates its histone targets, and consequently gene expression and that SIRT1 epigenetic regulation in breast cancer seems to be predominantly governed by gene type and molecular subtype.

In conclusion, we analyzed an aspect of SIRT1 epigenetic control in breast tumors and established SIRT1 status as an epigenetic eraser in breast cancer. After *ex-vivo* studies on paired breast tumor/normal samples across all molecular subtypes, as well as *in-vitro* experiments on human mammary cell lines, we report that SIRT1 mediates the deacetylation of H3k4ac histone marker in breast cancer. SIRT1 also modulates histones H3 and H4 acetylated marks in different subtypes of breast cancer. In addition, SIRT1 physically interacts with and regulates its H3 acetylated targets in a subtype-specific fashion. Moreover, SIRT1 deficiency is associated with substantial induction of acetylated H3k4 and H3k9 epigenetic marks on 6 breast cancer-related gene promoters: *AR*, *BRCA1*, *ERS1*, *ERS2*, *EZH2*, and *EP300*. We postulate that SIRT1 plays a differential role in breast cancer development depending on molecular subtype, in part through its epigenetic action. This study thus further consolidates the

potential use of SIRT1 as a druggable epigenetic target in human breast cancer.

MATERIALS AND METHODS

Patients' selection and collection of tissue samples

This study included a total of 135 patients admitted to the Centre Jean Perrin from June 2010 to December 2016 for cancer treatment, and diagnosed with breast cancer carcinoma. Patients were informed about the study and gave informed consent prior to inclusion. All 135 tumors and their adjacent normal breast tissues came from the Centre Jean Perrin tumor bank, Biological Resource Center (CRB), accredited under No.BB-0033-00075, where they were stored in liquid nitrogen at -196°C . Patients who received chemotherapy, hormonal therapy and/or radiotherapy for cancer in other parts of the body were excluded from the study, as were patients with predisposition to breast cancer and/or family members with breast cancer.

Molecular breast cancer subtype classification

Based on estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki-67 proliferative index; breast tumors were classified into 5 intrinsic subtypes according to the St. Gallen Consensus Conference guidelines [4] as follows: Hormone Receptor-positive Breast Cancer (HRBC) comprising luminal A [ER+, PR+, HER2- and Ki-67 <14%], luminal B (HER2-) [ER+, PR+/-, HER2- and Ki-67 \geq 14%] and luminal B (HER2+) [ER+, PR-, HER2+ and any Ki-67]. HER2 Breast Cancer (H2BC): [ER-, PR- and HER2 overexpressed], and lastly Triple-Negative Breast Cancer (TNBC): [ER-, PR-, and HER2-].

Breast cancer cell lines and cell culture

All 6 human cell lines used in this study were purchased from the ATCC (American Type Culture Collection, Manassas, VA, USA). (ER+) breast cancer cell lines: MCF-7 and T-47D were used as representatives of the luminal subtype. (ER-) breast cancer cell lines: MDA-MB 453, MDA-MB 231 and MDA-MB 468 were used as representatives of the triple-negative subtype. MCF10A, a normal breast cell line, was included as a control. MCF-7 and T-47D cells were cultured in RPMI-1640 medium (Gibco Grand Island, NY) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 1% L-glutamine (Invitrogen, Carlsbad, CA, USA), 0.1% gentamycin (Panpharma, Luitré, France) and insulin (1–4 mg/ml, Novo Nordisk, Denmark) in a humidified atmosphere at 37°C containing 5% CO_2 . MDA-MB 453, MDA-MB 231 and MDA-MB 468 cells were cultured in Leibovitz's L-15 medium (Gibco) containing 10% fetal

bovine serum, 1% L-glutamine and 0.1% gentamycin in a 37°C humidified atmosphere without CO_2 . MCF10A cells were grown in DMEM/F-12 medium (Gibco) containing 10% fetal bovine serum, 1% L-glutamine, 0.1% gentamycin and completed with insulin (10 $\mu\text{g}/\text{ml}$), cholera toxin (100 ng/ml), epidermal growth factor (20 ng/ml), hydrocortisone (500 ng/ml) (Sigma) in a humidified atmosphere at 37°C containing 5% CO_2 .

SIRT1-siRNA transfection

Breast cancer cell lines were transfected with human SIRT1 Silencer® Pre-designed and Validated siRNAs and Silencer® Negative Control #1 siRNA (Ambion, Life technologies). The sense and antisense RNA sequences are as follows: 5'-GCUGUACGAGGAGAUUUtt-3' and 5'-AAAUAUCUCCUCGUACAGCtt-3', respectively. According to the manufacturer's instructions, cells were transfected at 80% confluence level with 30–60 nM of SIRT1-siRNA or negative control siRNA using the Lipofectamine® RNAiMAX transfection reagent (Invitrogen, Life technologies), that was diluted in Opti-MEM™ Medium (Gibco). SIRT1 knock-down was verified 48 hours after transfection by immunoblotting.

Chromatin immunoprecipitation (ChIP) and re-ChIP assays

ChIP assays were performed on chromatin extracted from tumors and their matched normal tissues, as well as from transfected cell lines using the Auto iDeal ChIP-seq kit for Histones (C01010171, Diagenode, Seraing, Belgium) according to manufacturer's instructions. The extracted chromatin was later sonicated for 30 min (30 cycles, 30 s ON/ 30 s OFF) at 4°C with Bioruptor™ sonicator (Diagenode). 3 μg of the following antibodies (Abs) were used: anti-H3k4ac Ab (C15410322), anti-H3k9ac Ab (C15410004) (Diagenode) and non-immune rabbit IgG (Kch-504-250, Diagenode) serving as a negative control. The ChIP was carried out by SX-8X® IP-Star® Compact Automated System (Diagenode). The samples were incubated for 3 h for antibody coating with protein A-coated magnetic beads, then for 10 h at 4°C for immunoprecipitation reaction. Later on, 4 μl of elution buffer iE2 was added to the samples and the input was prepared with 2 μl of extracted chromatin in 100 μl of elution buffer iE1/iE2. The reverse cross-linking was performed for 45 min at 65°C . For Re-ChIP assays, the immunoprecipitated DNA from the first ChIP assay was eluted with elution buffer iE1 containing 10 mM dithiothreitol (DTT) for 30 min at 37°C . The second ChIP assay (re-ChIP) was then carried out using 3 μg of anti-SIRT1 Ab (C15200063, Diagenode). At the end, Immunoprecipitated DNA (IP DNA) and total DNA (input) from both ChIP and Re-ChIP assays were purified by MicroChIP DiaPure Columns (C03040001, Diagenode) and analyzed by real-time qPCR. The quality control and

efficacy of all Chip assays performed in this study were verified using positive and negative controls provided in the manufacturer's kit and according to their instructions (Diagenode). Control of ChIP analysis was performed prior to direct SIRT1 ChIP assays (Supplementary Figure 1) and prior to SIRT1 and H3k4ac/H3k9ac ChIP and re-ChIP assays (Supplementary Figure 2).

Quantitative real-time PCR method and data analysis

5 µl of IP DNA or total DNA were amplified by real-time qPCR using Taqman Universal PCR Master Mix as per the manufacturer's protocol using the ABI Prism 7900HT real-time PCR system (AB Applied Biosystems, ThermoFisher Scientific). Real-time PCR was performed in triplicate using 96-well MicroAmp Optical plates (AB Applied Biosystems) with optical adhesive film, at a final reaction volume of 25 µl containing 1X TaqMan Universal PCR Master Mix, 250 nM of probe (AB Applied Biosystems) and 400 nM for each of the forward and reverse primers (Sigma-Aldrich). Primer and probe sequences for *AR*, *BRC1*, *ERS2*, *ERS1*, *EZH2*, and *EP300* genes were selected with the help of Primer Express software (ABI), and are as follows:

AR gene, forward primer: 5'-TGCGCCAGCACTTGTTC-3'; reverse primer: 5'-CACCGCGCGCTAACG-3'; probe: 5'-6FAM-CCAAAGC CACTAGGCAG-MGB-3'; *BRC1* gene, forward primer: 5'-CCCCGTCCAGGAAGTCTCA-3'; reverse primer: 5'-GCGCGGGAATTACAGATAAATT-3'; probe: 5'-6FAM-C GAGCTCACGCCGCGCAG-TAMRA-3'; *ERS2* gene, forward primer: 5'-GAGAGGCTTTGGGTTTGCAAA T-3'; reverse primer: 5'-CCTCTAGTCCACGGCTTGC-3'; probe: 5'-6FAM-CAGCAAACGTAACCTCGGGCCCTG-TAMRA-3'; *ERS1* gene, forward primer: 5'-CCCTAC ATTGGCTTAAACATCA-3'; reverse primer: 5'-TCTTTG GATCGCTCCAAAT-3'; probe: 5'-6FAM-TCCAGGCAC AACTC-MGB -3'; *EZH2* gene, forward primer: 5'-CC CTCCAGAAACACAATCAATAGA-3'; reverse primer: 5'-CCGCCTGGTCTGGCTTTAT-3'; probe: 5'-6FAM-CA GAGCAGCTCGACTCT TCCCTCAAACCT-TAMRA-3'; *EP300* gene, forward primer: 5'-CGATGGCACAGG TTAGTTTCG-3'; reverse primer: 5'-GCGCACCGAGTA GAAAGATTAA-3'; probe: 5'-6FAM-CAGCCCCGGC CTCCACGTT-TAMRA-3'.

The thermal reaction cycles used were 50° C for 2 min, 95° C for 10 min, then 40 cycles of 95° C for 15 sec and 60° C for 1 min. The signal was collected at the endpoint of each cycle (Ct) using an AB Prism 7900 Sequence Detector System (AB Applied Biosystems). ChIP efficiency was calculated and reported as a percentage using the formula: % (IP DNA/Total DNA) = $2^{\Delta [(Ct(X\% \text{ total DNA}) - \log(X\%)/\log 2) - Ct(\text{IP DNA})]}$ × 100%. 2 is the amplification efficiency, Ct (input) and Ct (ChIP) are threshold values obtained from exponential

phase of qPCR for the immunoprecipitated DNA sample and input sample respectively, and $\log(X\%)/\log 2$ accounted for the dilution 1/X of the input (Diagenode).

Protein extraction and immunoblot analysis

Whole protein extracts from frozen tissues and cultured cells were obtained using T-PER™ Tissue Protein Extraction Reagent and RIPA buffer (ThermoFisher Scientific) respectively, containing protease and phosphatase inhibitors cocktail (Sigma Aldrich). 25–40 µg of extracted proteins were resolved by electrophoresis on 8–15% SDS-PAGE sodium dodecyl sulfate polyacrylamide gel (Bio-Rad, Hercules, USA), then electro-transferred onto polyvinylidene difluoride membranes (Immobilon-P, PVDF, 0.45 µm, Merck Millipore) in transfer buffer (25 mM Tris-HCL (pH 7.6), 192 mM glycine, 10% methanol). The membranes were blocked with 5% non-fat milk in 0.1% TBS-tween and later immunoblotted with the following primary Abs: anti-SIRT1 Ab (1/500, C15200063), anti-H3k4ac Ab (1/750, C15410322), anti-H3k9ac Ab (1/1000, C15410004), anti-H4k16ac Ab (1/500, C15200219), all purchased from Diagenode and anti-β-actin Ab (1/5000, CP01, Merck Millipore). Membranes were then washed and incubated with alkaline phosphatase-conjugated secondary Abs: anti-mouse IgG (1/2000, S3721) and anti-rabbit IgG (1/2000, S3738) (Promega, Madison, USA). Immunolabeling was detected using Western Blue® Stabilized substrate for Alkaline Phosphatase (Promega) at room temperature.

Co-immunoprecipitation assays

Total proteins were extracted from tumors and matched normal tissues using digestion buffer containing protease and phosphatase inhibitors cocktail (Nuclear complex Co-IP kit, Active Motif, CA, USA) according to the manufacturer's instructions. 100 to 500 µg of protein lysates were incubated in 500 µl of IP Incubation Buffer overnight at 4° C with 5 µg of the following primary Abs: anti-H3K4ac Ab (C15410322) and anti-H3k9ac Ab (C15410004) (Diagenode). Ab/Extract mixture was then incubated with Ab-binding agarose beads for 1 h at 4° C (Protein G Agarose Columns, Active Motif). Afterwards, the Ab/bead complexes were washed with 500 µl of IP Wash Buffer solution supplemented with or w/o BSA, before being eluted with 25 µl of Reducing Buffer. The immunoprecipitates were then immunoblotted with anti-SIRT1 Ab (1/500, C15200063, Diagenode) as previously described.

Statistical analysis

Correlation between the clinical parameters of our study groups were examined by chi-square test (χ^2 test) using SPSS statistics software (SPSS Inc., Chicago, IL). Relative expression levels of SIRT1 protein assayed by

immunoblotting were assessed numerically using Quantity One software (Bio-Rad, CA). Multiple-group comparisons were performed by ANOVA using R software (version 3.0.3). Post-hoc comparison of the means was performed using Tukey's multiple comparison test when the *F*-test was significant ($p < 0.05$). Groups were compared using two-tailed Student's *t*-test carried out after Fisher's exact test. All experiments were done at least in triplicate and the results were expressed as mean \pm SD. In all cases, statistical significance was set at the following *P*-values: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

ACKNOWLEDGMENTS

The authors would like to thank "La ligue régionale contre le cancer-Comité du Puy-de-Dôme" for research support. The authors also thank the Hariri Foundation for providing grant support to Khaldoun Rifai.

CONFLICTS OF INTEREST

The authors declare no existing conflicts of interest.

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Conclusion de la publication 1:

Les résultats de cette étude nous ont permis de mieux comprendre le rôle épigénétique de SIRT1 dans le cancer du sein.

On a pu montrer que SIRT1 agit sur les histone H3 et H4 à travers la déacétylation des marques H3K4ac, H3K9ac et H4K16ac et ceci dans les différents types moléculaires de cancer du sein. Grâce à la déplétion de SIRT1 par SiRNA et sa variation d'expression dans les différents types moléculaires de tumeur du sein, on a pu montrer que SIRT1 jouent un rôle dans la régulation des gènes impliqués dans le cancer du sein en régulant les marques H3K4ac et H3K9ac sur les promoteurs des gènes.

Toutes ces observations ont permis de faire un pas en avant dans la compréhension du fonctionnement de SIRT1 dans le cancer du sein mais aussi de pouvoir envisager l'utilisation de ce dernier comme cible thérapeutique ou encore biomarqueur dans la lutte contre le cancer du sein.

- *L'expression de SIRT1 comme biomarqueur dans les cancers du sein*

Connaissant mieux le rôle de SIRT1 dans le remodelage de la chromatine et le développement du cancer du sein suite à notre étude précédente, on s'est focalisé sur l'expression transcriptionnelle et traductionnelle de SIRT1. Dans la littérature, de nombreuses études se sont déjà penchées sur ce sujet dans différents cancers, cependant, les résultats qui en ressortent sont contradictoires surtout dans le cancer du sein où l'on qualifie souvent SIRT1 de suppresseur de tumeur ou au contraire d'oncogène. Pour nous faire notre propre opinion sur le sujet, 50 tumeurs de sein et leurs tissus sains adjacents ont été utilisés dans l'étude. L'expression transcriptionnelle et traductionnelle de SIRT1 a été observée dans les différents types moléculaires de tumeur de sein à savoir les TNBCs, HER2-enriched et les Luminaux A et B.

Publication 2:

Dual SIRT1 expression patterns strongly suggests its bivalent role in human breast cancer. Rifaï K, Judes G, **Idrissou M**, Daures M, Bignon YJ, Penault-Llorca F, Bernard-Gallon D. *Oncotarget*. 2017 Dec 6;8(67):110922-110930.

Dual SIRT1 expression patterns strongly suggests its bivalent role in human breast cancer

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Keywords: breast cancer; molecular subtypes; SIRT1; expression levels; statistical analysis

Received: July 08, 2017

Accepted: November 13, 2017

Published: December 06, 2017

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ABSTRACT

Breast cancer is the most common cancer in women, and the leading cause of cancer death in women worldwide. SIRT1 (silent mating type information regulation 2 homolog) 1 is a class-III histone deacetylase involved in apoptosis regulation, DNA repair and tumorigenesis. However, its role in breast carcinoma remains controversial, as both tumor-suppressive and tumor-promoting functions have been reported. Also, there are very few reports available where expression of SIRT1 is comprehensively analyzed in breast tumors classified by molecular subtype. Here, using a cohort of 50 human breast tumors and their matched normal tissues, we investigated SIRT1 expression levels in the 5 molecular subtypes of breast cancer according to the St Gallen classification (2013). Tumors and their corresponding normal tissue samples were collected from all patients, and SIRT1 mRNA and protein expression levels were then examined by real-time quantitative polymerase chain reaction and immunoblotting, respectively. After statistical analysis, the results showed a dual expression profile of SIRT1 in human breast carcinoma, with significant overexpression in luminal and HER2-enriched subtypes and significantly reduced expression in the triple-negative subtype. We also found an inverse correlation between SIRT1 expression and breast cancer aggressivity. These novel findings suggest that SIRT1 plays a dual role in breast tumors depending on its expression rate and the molecular subtype of the cancer. Our data also point to a potential role for SIRT1 as a prognostic biomarker in breast cancer.

INTRODUCTION

Breast cancer is the most common cancer in women, and the leading cause of cancer death in women worldwide [1]. It is a multifactorial genetic disease with different prognoses for different subtypes. According to the St Gallen breast cancer classification [2], there are five distinct molecular subtypes of breast cancer classified in ascending order of tumor aggressiveness, from luminal A, relatively the least aggressive with the most favorable prognosis and survival rate [3], to luminal B (HER2-) and luminal B (HER2+), these 3 subtypes are included in

the Hormone Receptor-positive Breast Cancer (HRBC), HER2-enriched or HER2 Breast Cancer (H2BC), and finally triple-negative breast cancer (TNBC), also known as basal-like, which is characterized as very aggressive and associated with poor prognosis and a higher death rate compared to the other molecular subtypes [3]. The process of subtyping breast cancer based on gene expression patterns has clarified differences in biological behavior between subgroups, allowing individualized treatment and better prognosis for each subtype [4].

Sirtuins (SIRT) are NAD⁺-dependent class-III histone deacetylases, a highly-conserved gene family

from yeast to mammals that have drawn increasing attention in recent years due to their action in various pathophysiological processes. In mammals, there are seven known SIRT homologs that localize to different subcellular compartments, and they primarily possess histone deacetylase activity (SIRT1, SIRT2, SIRT3 and SIRT5) or monoribosyltransferase activity (SIRT4 and SIRT6). These sirtuin isoforms can alter a wide variety of substrates involved in cell differentiation, viability, senescence, inflammation, and cellular survival, and thus control diverse key functions ranging from cellular survival to chromatin remodeling. Sirtuins are also closely involved in aging process, lifespan, and various pathologies including cancer, inflammation, immune dysfunction, cardiovascular disorders and neurodegeneration [5, 6].

Silent mating type information regulation 2 homolog 1 (SIRT1), the mammalian counterpart of yeast silent information regulator 2 (Sir2), is the most extensively studied protein in the SIRT family. SIRT1 is involved in key cellular processes such as apoptosis, DNA repair, chromatin remodeling and cancer development [7, 8], but its role in carcinogenesis is controversial, as it can have both tumor-suppressive and tumor-promoting functions, mainly depending on cancer type [9]. For instance, SIRT1-mediated deacetylation of the tumor suppressors p53 [10] and p73 [11] inactivates them, preventing cellular growth arrest, senescence and apoptosis, hence exerting oncogenic functions. On the other hand, SIRT1 is also reported to mediate BRCA1 signaling and inhibit tumor growth through downregulation of oncogenes or by repressing the activity of oncoproteins such as β -catenin [12] and survivin [13]. Furthermore, knockout mice models of SIRT1 are prone to tumor development, which points to a tumor-suppressive SIRT1 action [13]. These seemingly opposite functions might reflect a highly context-specific role of SIRT1 as a tumor-suppressor versus tumor-promotor.

The clinical significance of sirtuins in various human cancers has mostly been evaluated based on sirtuin expression patterns in tumors and non-tumor samples. Generally, overexpression of a protein in tumors indicates its oncogenic properties, whereas reduced expression of a protein indicates its tumor-suppressive properties. Studies using this approach report that SIRT1 is upregulated in a spectrum of cancers including, but not limited to, liver cancer [14], acute myeloid leukemia [15], bone cancer [16], thyroid cancer [17] and skin cancer [18], but downregulated in other cancers including colon cancer [12], oral squamous cell carcinoma [19], glioblastoma and ovarian cancer [20]. Studies in breast cancer have confirmed that SIRT1 is involved in tumorigenesis, metastasis [21] and chemoresistance [22]. However, there have been relatively few studies investigating SIRT1 expression levels to identify its function, and the results are contradictory. A limitation of these studies is that they

did not take into account the heterogeneity of various intrinsic breast cancer subtypes, and most of them did not use tissue samples from breast cancer patients but relied on breast cancer cell lines instead. Here, we evaluated both the mRNA and protein expression patterns of SIRT1 using human breast tumors and their corresponding normal breast tissues, in all 5 molecular subtypes of breast cancer. This research brings key insight to the ongoing controversy of SIRT1 behavior in breast cancer carcinoma.

RESULTS

Study population characteristics

The breast cancer molecular subtypes studied here spanned luminal A ($n = 10$, 20%), luminal B (HER2-) ($n = 10$, 20%), luminal B (HER2+) ($n = 10$, 20%), HER2-enriched ($n = 10$, 20%) and triple-negative ($n = 10$, 20%). All patients were females aged 45 to 82 years (mean $63.8 \pm SD 7.1$). Tumor size ranged from 0.5 to 7 cm (2.3 ± 0.5). All tumors were graded according to the modified Scarff-Bloom-Richardson grading system (SBR) as grade 1 ($n = 3$), grade 2 ($n = 25$) and grade 3 ($n = 22$). Samples were ER-, PR- and HER2-positive in $n = 30$ (60%), $n = 16$ (32%) and $n = 20$ (40%) patients, respectively. Table 1 gives the clinico-pathological variables of the 50 breast cancer patients.

SIRT1 is upregulated in (HRBC) and (H2BC) subtypes and downregulated in (TNBC) subtype

To assess SIRT1 expression at transcriptional/post-transcriptional level, SIRT1 messenger RNA (mRNA) was extracted from $N = 50$ tumors and their matched normal tissues ($n = 10$ for each of the 5 molecular subtypes), reverse-transcribed into complementary DNA (cDNA), then quantified by real-time quantitative PCR (RT-qPCR). Compared to matched normal tissues, relative SIRT1 mRNA expression was significantly higher in luminal A (mean $7.8 \pm SD 2.5$, $p < 0.001$; Figure 1A), luminal B (HER2-) (5.7 ± 1.7 , $p < 0.001$; Figure 1B), luminal B (HER2+) (6.5 ± 2.1 , $p < 0.001$; Figure 1C) and HER2-enriched (2.7 ± 1 , $p < 0.001$; Figure 1D), but significantly lower in the triple-negative subtype (0.35 ± 0.2 , $p < 0.001$; Figure 1E).

Positive correlation between SIRT1 expression and the St Gallen molecular classification

The differences between SIRT1 mRNA expression levels among the 5 molecular subtypes were further investigated using multi-way analysis of variance (ANOVA) followed by post-hoc analysis. Tukey's range test was then used for multiple comparisons among mean SIRT1 mRNA expression levels. The statistical procedures distinguished 3 distinct patterns of SIRT1

Table 1: Clinico-pathological characteristics of the breast cancer patients included in this study

	Total	Luminal A	Luminal B (HER2-)	Luminal B (HER2+)	HER2-enriched	Triple-negative	P value
Patients, n (%)	N = 50 (100%)	n = 10 (20%)	n = 10 (20%)	n = 10 (20%)	n = 10 (20%)	n = 10 (20%)	
Age							0.809
45–65	25 (50)	6 (60)	4 (40)	5 (50)	6 (60)	4 (40)	
>65	25 (50)	4 (40)	6 (60)	5 (50)	4 (40)	6 (60)	
SBR grade							0.001
I	3 (6)	3 (30)	0	0	0	0	
II	25 (50)	7 (70)	8 (80)	4 (40)	3 (30)	3 (30)	
III	22 (44)	0	2 (20)	6 (60)	7 (70)	7 (70)	
Size (cm)							0.265
<1.5	10 (20)	2 (20)	3 (30)	2 (20)	1 (10)	2 (20)	
1.5–2.5	21 (42)	7 (70)	4 (40)	5 (50)	2 (20)	3 (30)	
>2.5	19 (38)	1 (10)	3 (30)	3 (30)	7 (70)	5 (50)	
ER							0.0001
Positive	30 (60)	10 (100)	10 (100)	10 (100)	0	0	
Negative	20 (40)	0	0	0	10 (100)	10 (100)	
PR							0.0001
0%–50%	5 (10)	1 (10)	2 (20)	2 (20)	0	0	
51%–100%	11 (22)	9 (90)	2 (20)	0	0	0	
Negative	34 (68)	0	6 (60)	8 (80)	10 (100)	10 (100)	
HER2							0.0001
Positive	20 (40)	0	0	10 (100)	10 (100)	0	
Negative	30 (60)	10 (100)	10 (100)	0	0	10 (100)	
Ki-67							0.0001
≤20%	19 (38)	10 (100)	2 (20)	3 (30)	2 (20)	2 (20)	
>20%	31 (62)	0	8 (80)	7 (70)	8 (80)	8 (80)	

ER: Estrogen Receptor, PR: Progesterone Receptor, HER2: Human Epidermal growth factor Receptor 2, Ki-67: cellular marker for proliferation.

expression in human breast cancer tumors that correspond to the 3 molecular subtypes: overexpression in (HRBC) subtypes, slight overexpression in the (H2BC) subtype, and underexpression in the (TNBC) subtype (Figure 2).

Consistency between SIRT1 mRNA and protein expression patterns

In order to determine whether SIRT1 transcription levels are equally translated into functional proteins, SIRT1 protein levels were assessed in breast tumors and their matched normal tissue samples using immunoblot analysis. We found that SIRT1 protein expression pattern differs amongst the 5 molecular subtypes, as shown in (Figure 3A). In comparison with normal breast tissue, SIRT1 protein expression was significantly higher in (HRBC) subtypes and in the (H2BC) subtype, but significantly reduced in the (TNBC) subtype (Figure 3B). These results are consistent with the mRNA expression level data.

DISCUSSION

In order to clarify the biological behavior of SIRT1 and evaluate its role in breast carcinoma, we evaluated SIRT1 expression patterns at the transcriptional and translational levels in human breast tumors and their corresponding normal breast tissues, according to St Gallen molecular subtype class. Although some studies have investigated SIRT1 expression in breast cancer, however, this is the first study to extensively examine SIRT1 mRNA and protein expression levels according to intrinsic subtypes with a sample size that satisfies statistical power requirements.

SIRT1 is a class-III histone deacetylase critically involved in the occurrence and development of a multitude of tumors, and reported to be involved in regulating a multitude of biological processes including apoptosis, cell survival, proliferation and stress response. SIRT1 expression levels have been extensively investigated in many malignancies in order to assess its

role. SIRT1 expression and function are found to vary drastically depending on cell and tumor types, making it a multifaceted enzyme with contradictory functions depending on its upstream regulators and downstream targets [23]. SIRT1 overexpression has been reported in several human cancers, it was generally associated with poor prognosis and poor overall survival [24], whereas reduced SIRT1 expression was consistent with a tumor-suppressor role [12, 20].

Several studies have investigated SIRT1 expression in breast cancer, but while some studies found upregulated SIRT1 expression, others did not concur. There are multiple reasons that could explain this discrepancy between studies: the fact that SIRT1 expression was evaluated only at transcriptional level [25], or using only breast cancer cell lines [26, 27], and/or using human breast tissue samples but without accounting for the various molecular subtypes [25, 28–30] or without having a statistically sufficient sample size [28, 31]. This unclear picture promoted us to conduct the study here. The results found here revealed different

SIRT1 expression patterns among different breast cancer molecular subtypes. We report significant overexpression of SIRT1 mRNA and protein levels in HRBC and H2BC subtypes, and a significant underexpression in the TNBC subtype. This dual expression pattern of SIRT1 in tumors points to a differential role of SIRT1 in human breast cancer. Based on its expression patterns, SIRT1 most probably has an oncogenic role in the HRBC and H2BC subtypes, in line with Elangovan *et al.* [32] and Ma *et al.* [33], who reported that SIRT1 overexpression in luminal breast cancer subtypes is correlated with an oncogenic behavior. In contrast, SIRT1 may play a tumor-suppressor role in the TNBC subtype, in line with Yi *et al.* [34] who reported that the activation of SIRT1 by a SIRT1-specific activator YK-3-237 induced deacetylation of the mutant form of p53 (mtp53), suppressing the proliferation and arresting the cell growth of triple-negative breast cancer cell lines. Furthermore, Simic *et al.* [35] showed that ectopic expression of SIRT1 suppresses cancer metastasis and tumor cell invasion. Moreover, our findings showed a positive correlation between SIRT1 expression and St

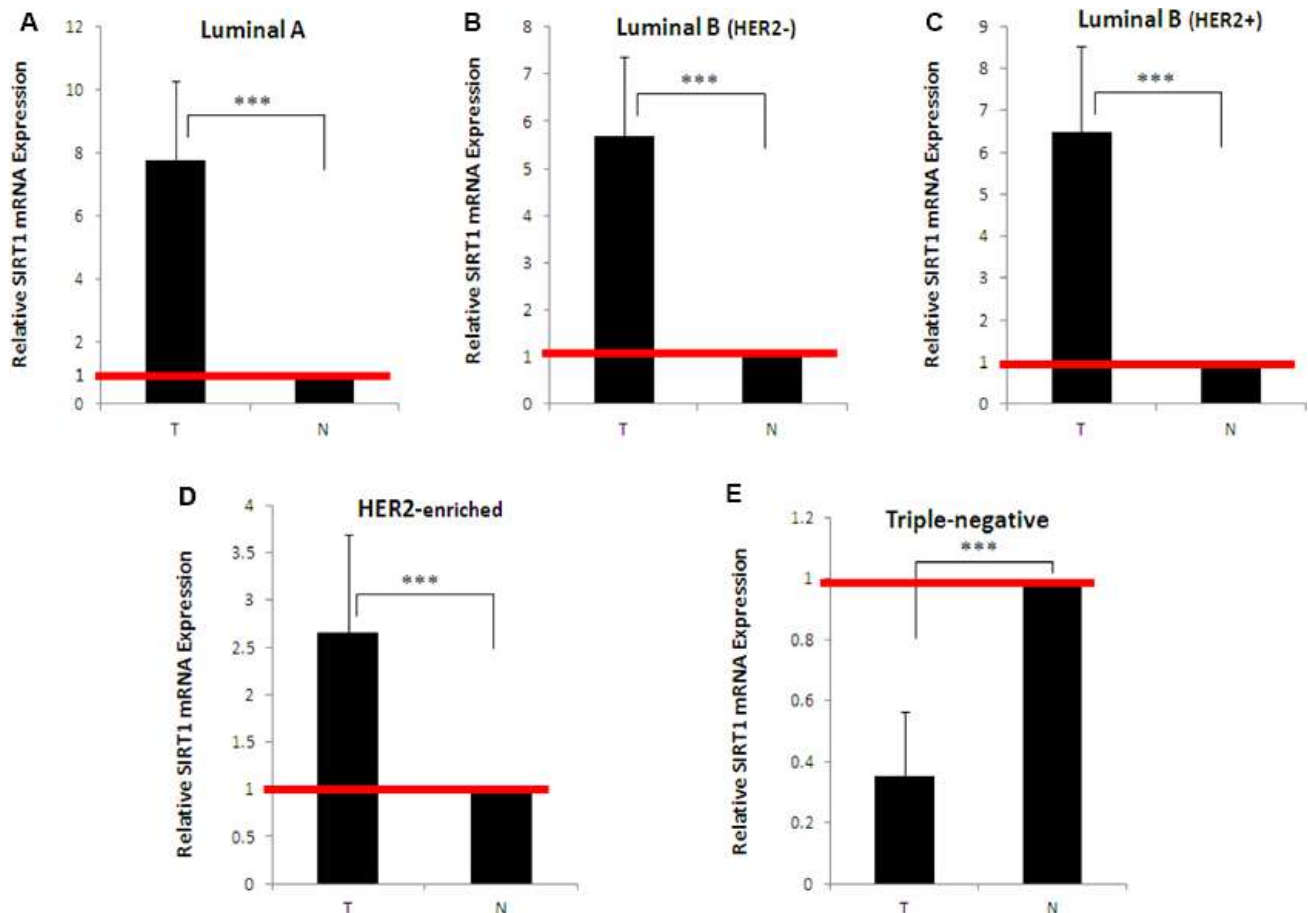


Figure 1: Quantitative expression levels of SIRT1 in different breast tumor subtypes and their matched normal tissue samples. SIRT1 expression levels were quantified by real-time quantitative PCR using mRNA extracted from (A) $n = 10$ luminal A, (B) $n = 10$ luminal B (HER2-), (C) $n = 10$ luminal B (HER2+), (D) $n = 10$ HER2-enriched, (E) $n = 10$ triple-negative breast tumors, and their adjacent normal tissues. SIRT1 mRNA expression was normalized against 18S rRNA levels. SIRT1 expression in breast tumors was expressed as fold-change compared to normal breast tissues (defined as 1). Each real-time PCR reaction was performed in triplicate, the results are expressed as mean \pm SD, P values were two-tailed and $***P < 0.001$ was considered statistically significant. T: Tumor, N: Normal.

Gallen molecular subtype classification. After classifying the breast tumors used in ascending order of aggressivity, decreased SIRT1 expression was found to correlate with increased breast cancer aggressivity and poor prognosis. We conclude that SIRT1 may serve as a prognostic biomarker in breast cancer carcinomas.

In conclusion, this study demonstrated for the first time a differential pattern of SIRT1 expression in breast cancer at both transcriptional and protein level using human breast tumors and their uninvolved benign counterparts, it also established an association between SIRT1 expression and St Gallen classification. Taken together, these results suggest that SIRT1 plays a bivalent subtype-dependent role in breast carcinoma, and that SIRT1 could also be a potential prognostic marker in breast cancer. Given that SIRT1 regulates a wide range of substrates directly involved in the tumorigenesis process, it could make a novel and potentially promising anticancer therapeutic target, especially if results from clinical trials currently testing specific SIRT1 inhibitors are deemed good.

MATERIALS AND METHODS

Study population selection and collection of tissue samples

This study included a total of 50 patients admitted to the Centre Jean Perrin from October 2012 to September

2016 for cancer treatment, and diagnosed with breast cancer carcinoma. Patients were informed about the study and gave informed consent prior to inclusion. All 50 tumors and their adjacent normal breast tissues came from the Centre Jean Perrin Biological Resource Center, where they were put in cryotubes and stored in liquid nitrogen at -196°C . Patients who received chemotherapy, hormonal therapy and/or radiotherapy for cancer in other parts of the body were excluded from the study, as were patients with predisposition to breast cancer and/or family members with breast cancer.

Intrinsic breast cancer subtype classification

The breast carcinomas were classified into 5 molecular subtypes according to St Gallen breast cancer conference guidelines [2] based on estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and Ki-67 proliferative index, as follows:

Luminal A: [ER- and/or PR-positive, HER2-negative, and Ki-67 <14%]

Luminal B (HER2-): [ER- and/or PR positive, HER2-negative and Ki-67 \geq 14%]

Luminal B (HER2+): [ER- and/or PR-positive, HER2-positive, and any Ki-67]; these 3 subtypes are included in the hormone receptor-positive breast cancer (HRBC) group.

HER2-enriched/HER2 breast cancer (H2BC): [ER- and/or PR-negative, HER2 overexpressed]

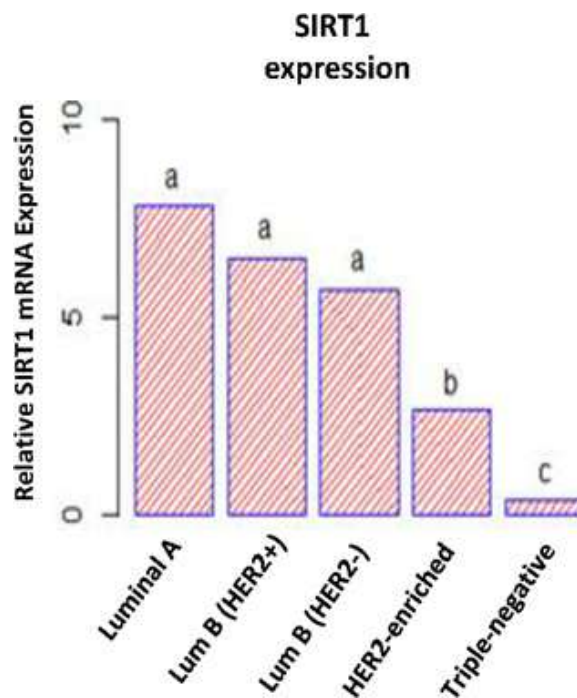


Figure 2: Differential SIRT1 mRNA expression patterns in breast tumors. ANOVA followed by Tukey's multiple comparison test performed on SIRT1 mRNA expression levels. This statistical analysis discerned 3 different SIRT1 expression patterns. The letters 'a', 'b' and 'c' indicated statistical significance between groups.

Triple-negative breast cancer (TNBC): [ER-, PR-, and HER2-negative].

Total RNA isolation from tissues and reverse transcription (RT)

Tumoral and non-tumoral tissue samples were cut into pieces and homogenized with TissueRuptor® (Qiagen, Hilden, Germany). Total RNA was isolated using TRIzol Reagent (Ambion, Life Technologies, CA) then extracted using a PureLink RNA Mini Kit (Invitrogen, Thermo Fisher Scientific, CA). RNA samples purity was verified

using NanoDrop ND-8000 spectrophotometer. cDNA was then obtained using the high-capacity cDNA reverse transcription kit (AB Applied Biosystems, Foster City, CA) according to the manufacturer's protocol.

RT-qPCR methods and data analysis

Synthesized cDNA was amplified using TaqMan Gene expression PCR Master Mix (AB Applied Biosystems) as per the manufacturer's protocol. Each duplex PCR was assembled using 96-well MicroAmp Optical plates (AB Applied Biosystems) with 25 ng of

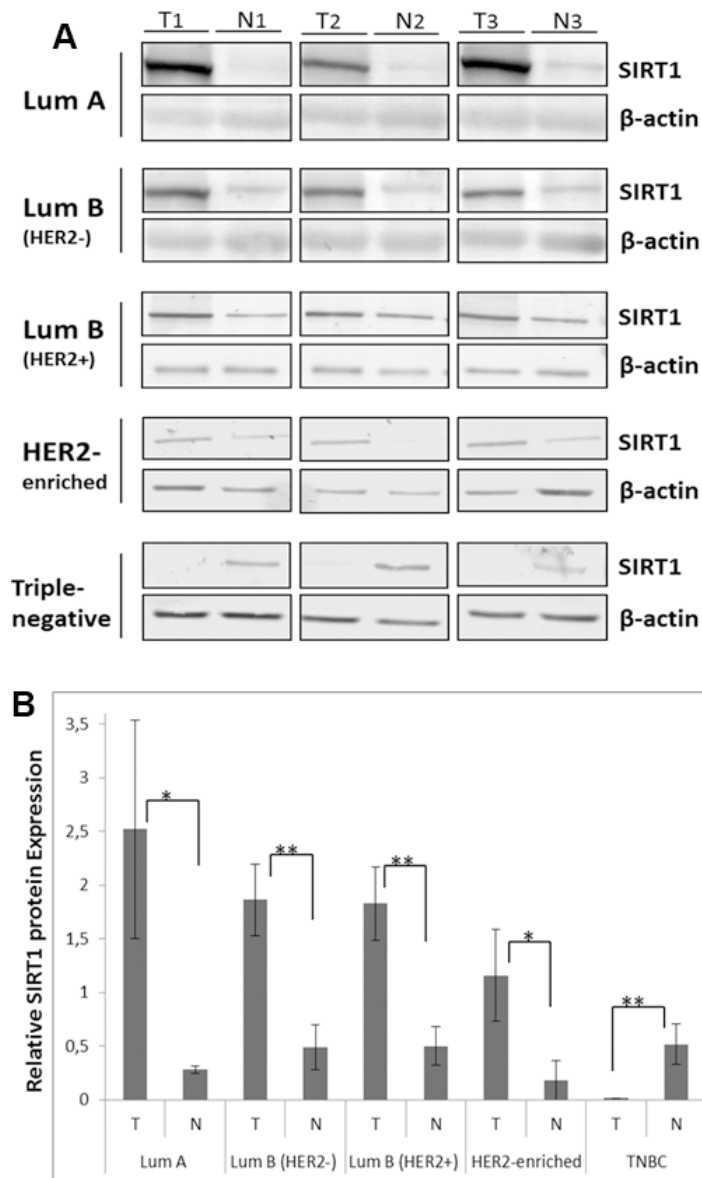


Figure 3: Differential SIRT1 protein expression patterns in breast tumors. (A) Representative immunoblots of 3 independent experiments showing SIRT1 protein expression in the 5 molecular subtypes of breast cancer. Equal amounts of proteins were immunoblotted with SIRT1 antibody (110 kDa). β-actin (42 kDa) served as loading control. (B) Relative SIRT1 protein expression was evaluated using Quantity One software with SIRT1 expression normalized against β-actin as loading control. Each bar represents the mean ± SD of 3 replicate experiments. For the statistical analysis, *P*-values were two tailed, **P* < 0.05 and ***P* < 0.01 were considered statistically significant. T: Tumor, N: Normal.

template cDNA in a total volume of 25 μ L containing 12.5 μ L TaqMan Gene Expression Master Mix (2X), 1.25 μ L TaqMan Gene Expression assay-on-demand SIRT1 [Hs01009006_m1] (200 nM), 0.25 μ L endogenous control 18S rRNA primers (10 μ M) and 0.25 μ L 18S rRNA probe (5 μ M). Primer sets for specific reverse transcription of SIRT1 and endogenous control 18S rRNA were all obtained from (AB Applied Biosystems), and are as follows: SIRT1 forward 5-CCTGTGAAAGTGATGAGGAGGATAG-3; reverse 5-TTGGATTCCCGCAACCTG-3. 18S forward: 5'-CGG CTA CCA CAT CCA AGG AA-3', reverse: 5'-GCT GGA ATT ACC GCG GCT-3', probe: 5'-TGCTGG CAC CAG ACT TGC CCT C-3'. The thermal reaction cycles used were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. The signal was collected at the endpoint of each cycle using an AB Prism 7900 Sequence Detector System (AB Applied Biosystems). Relative gene expression was determined by normalizing to reference gene *18S* and according to the relative quantitative ($\Delta\Delta$ Ct) method. Fold change in SIRT1 expression was then calculated using the ($2^{-\Delta\Delta$ Ct) method. SIRT1 mRNA expression in breast tumors was calculated relative to the matched normal breast tissues. All experiments were done in triplicate, and results were expressed as means \pm SD.

Protein extraction and immunoblot analysis

Frozen tissues were homogenized before being lysed using T-PER™ Tissue Protein Extraction Reagent (ThermoFisher Scientific) containing protease inhibitor cocktail (Sigma Aldrich). Whole protein extracts were resolved by electrophoresis on 8% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), then electro-transferred onto polyvinylidene difluoride membranes (Immobilon-P, PVDF, 0.45 μ m, Merck Millipore) in transfer buffer (25 mM Tris-HCL (pH 7.6), 192 mM glycine, 10% methanol). The membranes were blocked with 5% non-fat milk in 0.1% TBS-tween and later immunoblotted with monoclonal anti-SIRT1 antibody (1/500, MAb-063-050, Diagenode) or monoclonal anti- β -actin antibody (1/5000, CP01, Merck Millipore). Membranes were then washed and incubated with alkaline phosphatase-conjugated secondary antibody anti-mouse IgG (1/2000, S3721, Promega). Immunolabeling was detected using Western Blue® Stabilized substrate for Alkaline Phosphatase (Promega) at room temperature.

Statistical analysis

Correlation between the clinical parameters of our study groups were examined by chi-square test (χ^2 test) using SPSS statistics software (SPSS Inc., Chicago, IL). Multiple-group comparisons were performed by ANOVA using R software (version 3.0.3). Post-hoc comparison of the means was performed using Tukey's multiple

comparison test when the F-test was significant ($p < 0.05$). Relative expression levels of SIRT1 protein assayed by immunoblotting were assessed numerically using Quantity One software (Bio-Rad, CA). Groups were compared using a two-tailed unpaired Student's *t*-test carried out after a Fisher's exact test. All experiments were done in triplicate and the results were expressed as mean \pm SD. In all cases, statistical significance was set at the following *P*-values: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

ACKNOWLEDGMENTS

The authors would like to thank La ligue régionale contre le cancer-Comité du Puy-de-Dôme for research support. The authors also thank the Hariri Foundation for providing grant support to Khaldoun Rifai.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Conclusion de la publication 2:

Dans l'optique de se faire notre propre idée du rôle de SIRT1 et de son niveau d'expression dans les différents types moléculaires de cancer du sein. L'expression de SIRT1 au niveau ARNm et protéique a été analysée par RT-qPCR et western blot.

Ces analyses nous ont permis de voir que l'expression de SIRT1 était corrélée à l'agressivité du cancer.

- Dans les cancers luminaux A, B et les HER2-enriched, on a une surexpression de SIRT1 par rapport aux tissus sains correspondants aux tumeurs.

- Dans les cancers TNBCs on observe plutôt une sous-expression de SIRT1 par rapport aux tissus sains correspondants aux tumeurs.

- Enfin on a pu constater que l'expression de SIRT1 diminuait plus on allait dans les tumeurs les plus agressives. Ainsi, on a une forte expression dans les tumeurs lumineales et très faible expression dans les TNBCs.

En conclusion, SIRT1 jouerait un rôle ambivalent dans le cancer du sein à savoir un rôle oncogène dans les cancers Luminaux et HER2-enriched et supresseur de tumeur dans les TNBCs. Cette étude permet aussi de valider le fait que SIRT1 pourrait être utilisé comme biomarqueur du cancer du sein car son expression diffère dans les différents types cancers du sein.

- *Rôle bivalent de SIRT1 dans le cancer du sein*

Les résultats obtenus lors de nos études sur SIRT1 et les connaissances récoltées dans la littérature nous ont poussés à creuser encore plus loin dans la compréhension du rôle de SIRT1 dans le cancer du sein.


Dans cette revue, les fonctions contradictoires de SIRT1 dans l'initiation et le développement du cancer du sein ont été notre centre d'intérêt. De ce fait, nous avons rassemblé les informations démontrant que SIRT1 peut être une protéine oncogène, mais aussi une protéine suppressive de tumeur. Toutefois, on s'est focalisé sur les mécanismes dérégulés dans le cancer du sein et l'implication de SIRT1 dans ces mécanismes.

Publication 3:

Breaking down the Contradictory Roles of Histone Deacetylase SIRT1 in Human Breast Cancer. Rifaï K, Idrissou M, Penault-Llorca F, Bignon YJ, Bernard-Gallon D. *Cancers (Basel)*. 2018 Oct 30;10(11). pii: E409.

Review

Breaking down the Contradictory Roles of Histone Deacetylase SIRT1 in Human Breast Cancer

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Received: 21 September 2018; Accepted: 26 October 2018; Published: 30 October 2018



Abstract: Breast cancer (BC) is the most common type of cancer in women worldwide; it is a multifactorial genetic disease. Acetylation and deacetylation are major post-translational protein modifications that regulate gene expression and the activity of a myriad of oncoproteins. Aberrant deacetylase activity can promote or suppress tumorigenesis and cancer metastasis in different types of human cancers, including breast cancer. Sirtuin-1 (SIRT1) is a class-III histone deacetylase (HDAC) that deacetylates both histone and non-histone targets. The often-described ‘regulator of regulators’ is deeply implicated in apoptosis, gene regulation, genome maintenance, DNA repair, aging, and cancer development. However, despite the accumulated studies over the past decade, the role of SIRT1 in human breast cancer remains a subject of debate and controversy. The ambiguity surrounding the implications of SIRT1 in breast tumorigenesis stems from the discrepancy between studies, which have shown both tumor-suppressive and promoting functions of SIRT1. Furthermore, studies have shown that SIRT1 deficiency promotes or suppresses tumors in breast cancer, making it an attractive therapeutic target in cancer treatment. This review provides a comprehensive examination of the various implications of SIRT1 in breast cancer development and metastasis. We will also discuss the mechanisms underlying the conflicting roles of SIRT1, as well as its selective modulators, in breast carcinogenesis.

Keywords: breast cancer; SIRT1; deacetylation; epigenetic silencing; tumor promoter; tumor suppressor; SIRT1 modulators

1. Introduction

Breast cancer is a genetically heterogeneous disease that remains the most commonly diagnosed malignancy amongst women worldwide. It is also the second leading cause of cancer death among females in developed countries after lung cancer [1]. Epigenetic alterations of proteins, histones, and chromatin play a fundamental role in gene expression regulation and ultimately, cancer formation. Reversible protein acetylation and deacetylation are amongst those alterations [2]. Histone deacetylases (HDACs) are major actors in gene expression regulation. By removal of acetyl groups from N-terminus tails of histones, HDACs repress the expression of genes implicated in the carcinogenesis process, such as oncogenes and tumor suppressor genes (TSGs). In addition to histones, HDACs regulate the expression and activity of a myriad of proteins involved in both cancer initiation and progression.

Furthermore, aberrant expression of HDACs in various human cancers, and consequently their dysfunctional deacetylase activity, is deeply involved in the carcinogenesis process [3].

Sirtuins (SIRT1) are nicotinamide adenine dinucleotide (NAD⁺)-dependent class-III HDACs or lysine deacetylases (KDACs) that belong to the silent information regulator 2 (SIR2) family. The seven mammalian sirtuins (SIRT1–7) are key regulators in major biological processes, including cell death and survival, regulation of genomic stability, cellular senescence, metabolic regulation and inflammation [4,5]. Therefore, sirtuins have gained tremendous attention in the past decade in cancer research and numerous studies have demonstrated their direct implication in the carcinogenesis process of multiple human cancers [6]. Silent mating type information regulation 2 homolog 1 (Sirtuin-1) is the founding member of the sirtuin family and the most extensively studied. SIRT1 is expressed ubiquitously and is mainly found in the nucleus, but can shuttle between the nucleus and cytoplasm using its two nuclear localization signals and two nuclear export signals [7]. Due to its deacetylase activity, SIRT1 regulates a wide variety of fundamental cellular processes including apoptosis, DNA damage response and repair, cell differentiation and proliferation, chromatin remodeling and gene expression, endocrine signaling, aging, metabolism, stress response, and cancer development and metastasis [4,5,8–10]. Similar to most HDACs, aberrant SIRT1 expression is identified in numerous human malignancies and is directly linked to the tumorigenesis process and metastasis.

The implications of SIRT1 in breast cancer occurrence and development have been reported and largely studied over recent years, but its exact role in breast cancer remains very controversial and paradoxical so far. In fact, bifurcated SIRT1 can act as either a tumor suppressor or promoter in cancer cells. This highly context-specific role of SIRT1 in breast carcinoma seems to depend mainly on its upstream regulators or downstream substrates, as well as on its spatial distribution, cellular and molecular context, and tumor types. In this review, we summarize available data and give a general overview of the multiple implications of SIRT1 in breast tumorigenesis. We also explore the mechanisms underlying SIRT1 opposite functions in breast carcinogenesis.

2. The Multifaceted Functions of HDAC SIRT1 in Cancer Biology

Other than histone deacetylation, the functional roles of SIRT1 are fulfilled by directly interacting with and deacetylating a wide range of downstream non-histone substrates, resulting in activation or repression of their catalytic activity. SIRT1 deacetylase activity regulates:

1. Tumor suppressors, including p53 [11], p73 [12], Forkhead transcription factors (FoxO) [13], E2F1 [14], and Rb (Retinoblastoma) [15].
2. Tumor promoters, including c-Myc [16], N-Myc [17], cortactin (CTTN) [18], NF- κ B [19], β -catenin [20], and HIF-1 α [21].
3. Chromatin-related enzymes, including p300 [22], hMOF and TIP60 [23], PCAF [24], HDAC1 [25], DNMT1 [26], SUV39H1 [27], and EZH2 (enhancer of Zest 2) [28].
4. Nuclear receptors and related factors, including estrogen receptor-alpha (ER- α) [29], androgen receptor (AR) [30], liver X receptor (LXR) [31], PPAR γ [32], and PPAR γ coactivator 1 α (PGC-1 α) [33].
5. DNA damage repair enzymes, such as Ku70 [34], XPC [35], XPA [36], APE1 [37], and WRN [38].

As a result of its diverse biological functions, multifaceted SIRT1 is critically implicated in the occurrence and progression of numerous human malignancies. Yet researchers have long been baffled by SIRT1 contradictory actions in the carcinogenesis process, and its involvement in cancer biology remains an open question.

3. SIRT1-Dependent Epigenetic Silencing via Histone Modification in Breast Carcinogenesis

SIRT1 lysine deacetylase activity regulates chromatin structure and transcription through epigenetic mechanisms [9]. Lysine acetylation of histones H3 and H4 is classically associated with transcriptional activation and increased gene expression. On the contrary, their deacetylation is

generally associated with inactive chromatin and repression of gene expression [39]. In carcinogenesis, histone deacetylation leads to epigenetic silencing of various cancer-related genes; thus, HDACs could exert either tumor-promoting or tumor-suppressive roles depending on whether the repression happens in the genomic region of a tumor suppressor or a tumor promoter respectively. HDAC SIRT1 embodies these properties, and orchestrates the regulation of multiple cancer-related genes through histone deacetylation. Indeed, SIRT1 contributes to the epigenetic silencing by deacetylating H3 and H4 acetylated markers such as histones H3 lysine 4 (H3K4) [40], lysine 56 (H3K56) [41], lysine 9 (H3K9), lysine 14 (H3K14), and histone H4 lysine 16 (H4K16) [42,43]. In breast cancer, SIRT1-dependent epigenetic silencing of both oncogenes and TSGs is reported.

Pruitt et al. demonstrated that SIRT1 deficiency re-activates aberrantly silenced TSGs by increasing the acetylation of H3K9 and H4K16 epigenetic markers at their promoters in two breast cancer (BC) cell lines, indicating SIRT1-mediated epigenetic repression of TSGs through histone modifications in BC [44]. In contrast, Wang et al. revealed a tumor suppressor role of SIRT1 in BC. They reported that SIRT1 inhibits tumor growth *in vivo* by suppressing the expression of survivin, a member of the inhibitor of apoptosis (IAP) family that drives cell proliferation and viability [45]. SIRT1-mediated epigenetic silencing of survivin occurs through deacetylating the H3K9 marker on the survivin promoter, consequently suppressing its transcription in mammary tumors [46]. To address the confusion regarding SIRT1-dependent epigenetic regulation in BC pathogenesis, we characterized in recent studies an aspect of SIRT1 epigenetic behavior in human breast carcinoma. We showed that the opposite functions of SIRT1 in breast cancer are closely related to the molecular subtype. By modulating the acetylation status of key H3 and H4 epigenetic markers in a subtype-specific fashion, SIRT1 is more likely to exert an oncogenic role in luminal molecular subtypes and a tumor suppressor role in the triple-negative subtype (TNBC), also known as basal-like, both *in vitro* and *ex vivo*. Furthermore, we revealed that SIRT1 deficiency is associated with substantial induction of acetylated markers on six breast cancer-related gene promoters: *AR*, *BRCA1*, *ERS1*, *ERS2*, *EZH2*, and *EP300*, suggesting an active role of SIRT1 in regulating the expression of these genes in BC. We concluded that SIRT1 differential epigenetic regulation in breast cancer is predominantly governed by gene type and molecular subtype [40,47]. Other than BC, the duality of SIRT1 epigenetic regulation was also highlighted in colorectal cancer [48].

In fact, SIRT1-mediated epigenetic regulation extends to histone acetyltransferases (HATs), and other histone modifiers involved in transcription repression. SIRT1 fine-tuning of gene expression regulation is partly manifested through the repression of acetyltransferase activity of major HATs. Remarkably, these HATs acetylate the same histone targets as SIRT1. For instance, SIRT1 downregulates and blocks the activity of p300 of the p300/CBP family [22], hMOF and TIP60 of the MYST family [23], and PCAF of the GNAT family [24]. It stabilizes and stimulates the activity of HDACs, e.g., HDAC1 [25], DNA methyltransferases, e.g., DNMT1 deacetylated at Lys1349 and Lys1415 [26], and histone methyltransferases (HMTs), e.g., SUV39H1, deacetylated at Lys266 [27]. Furthermore, SIRT1 interacts and has a close functional relationship with EZH2, an essential HMT that constitutes the core catalytic subunit of polycomb repressive complex 2 (PRC2). SIRT1 and EZH2 form part of the PRC4 complex, along with other polycomb group proteins which are found overexpressed in breast cancer tumors. The two enzymes recruit SUV39H1 and DNMT1 to promote transcriptional repression at targeted genes [27,28,49,50]. Collectively, the data indicated that SIRT1 epigenetic regulation of gene expression is implemented by the means of regulation both, histone markers and their epigenetic 'writers' and 'erasers'.

4. SIRT1 Assuming the Role of Tumor Promoter in Breast Carcinogenesis

The 'guardian of the genome' p53, a vital TSG that is frequently mutated in human tumors, was one of the first identified non-histone substrate of SIRT1 and the first evidence of SIRT1 implication in tumorigenesis. SIRT1 deacetylation of p53 at its Lys382 residue (p53K382) results in repression of p53-dependent apoptosis in response to DNA damage and promotes cell survival [11]. Upon DNA

damage stimuli, SIRT1-mediated deacetylation of p53 is optimized by breast cancer metastasis suppressor 1 (BRMS1) [51]. BRMS1 potentiates SIRT1 activity through physically interacting with deleted in breast cancer 1 (DBC1), a negative regulator of SIRT1 [52]. SIRT1 also deacetylates and represses the activity of other damage-response enzymes, the mammalian forkhead transcription factors FoXO3a and FoXO4, inhibiting forkhead-dependent cell death [13,53]. In addition, SIRT1 binds to and inhibits the activity of E2F1, a tumor suppressor and apoptosis regulator, impairing its apoptotic functions [14]. SIRT1 also downregulates the activity of the tumor-suppressing retinoblastoma protein (Rb). Deacetylation of Rb by SIRT1 formed a domain similar to the SIRT1-targeted domain of p53, resulting in inhibiting Rb-dependent apoptosis [15]. Furthermore, SIRT1 overexpression in tumors is associated with upregulation of various oncoproteins. For example, SIRT1-dependent deacetylation of prototypic Myc oncogenes, c-Myc and N-Myc, enhances their stability and transcriptional activity, resulting in cancer cell survival and proliferation [16,17], respectively.

On the premise that SIRT1 is upregulated in various human cancers, SIRT1 could act as a tumor promoter. Since an abundance of SIRT1 expression is observed in breast tumors, many studies assert an oncogenic role of SIRT1 in breast carcinogenesis, and clinical studies demonstrated SIRT1 as a prognostic factor that significantly correlates with unfavorable clinicopathological factors. Actually, SIRT1 overexpression in breast tumors and mammary BC cell lines is significantly associated with lymph node metastasis, advanced TNM stage, low grade as per the modified Bloom–Richardson system, lymphovascular invasion, shorter disease-free survival (DFS), and overall survival (OS), luminal subtype, ER and PR expression, and is marginally associated with p53 loss [54–56]. Hence, SIRT1 upregulation is strongly correlated with breast tumorigenesis.

Xu et al. reported an upregulation of SIRT1 in breast tumors and (ER+) luminal BC cell line MCF-7. The authors revealed that SIRT1 upregulation promotes the proliferation, migration, and invasion of MCF-7 cells, whereas SIRT1 knockdown inhibits those effects. They showed that SIRT1 overexpression positively correlates with decreased expression of p53 and increased expression of DNA polymerase delta1 (*POLD1*) gene, an oncogene involved in genomic instability and cell proliferation; whilst the result of SIRT1 silencing is opposite. They concluded that SIRT1 is involved in breast carcinogenesis by inhibiting p53 and activating *POLD1* [57]. This was in line with a study by Jin et al. who revealed that SIRT1 upregulation significantly promotes breast cancer growth both in vitro and in vivo, whereas SIRT1 deficiency inhibits cancer cell proliferation. The authors showed that SIRT1 has effects on breast cancer cell growth through promoting the activity of oncogenic PI3K/Akt signaling pathway in vitro, and that SIRT1 is positively correlated with the expression of P-Akt in vivo [56]. SIRT1 is also involved in breast cancer progression and metastasis. Ota et al. demonstrated that SIRT1 inhibition by Sirtinol, a selective SIRT1 inhibitor, induces a senescence-like growth arrest in luminal cell line. The cellular senescence induced by SIRT1 inhibition co-occurs with impaired activation of oncogenic Ras–MAP kinase signaling pathways, implicated in cell growth and proliferation. These findings suggest an active role of SIRT1 in driving cell proliferation through Ras–MAP kinase signaling pathways [58]. Meanwhile, Zhang et al. found that SIRT1 and Cortactin; an oncogene associated with breast cancer metastasis, are more abundant in breast tumors than in their normal adjacent tissues. They showed that SIRT1-mediated deacetylation of cortactin promotes cell migration and breast tumorigenesis [18].

Meanwhile, SIRT1 oncogenic activity in BC is downregulated by different subclasses of miRNAs [59]. MiRNAs are small non-coding microRNAs that regulate the expression of many cancer-related genes. A recent study by Zou et al. reported that SIRT1 is negatively regulated by miR-22, a subclass of miRNAs, in the ER+ MCF-7 cell line. The authors showed that an ectopic expression of miR-22 reduces the proliferation, migration and invasion of MCF-7 cells, whereas SIRT1 overexpression eliminates the suppressive effects of miR-22. They concluded that miR-22 inhibitory effects are partly fulfilled by downregulating SIRT1 expression in vitro [60]. A similar study by Zhang et al. confirmed SIRT1 as a direct target of miR-22 in both (ER+) and (ER–) cell lines. The authors showed that SIRT1 knockdown induces apoptosis, inhibits tumorigenesis, and enhances radiosensitivity of breast cancer cells. In addition, miR-22 overexpression suppresses tumorigenesis

and improves radiosensitivity of breast cancer cells by targeting SIRT1 in vitro. They concluded on the same note as Zou et al. [61].

SIRT1 is a confirmed target of another subclass of miRNAs. MiR-34a represses SIRT1 expression through a miR-34a-binding site within the 3'UTR of SIRT1. MiR-34a-mediated inhibition of SIRT1 leads to an increase of acetylated p53 and consequently, increased expression of pro-apoptotic genes *p21* and *PUMA* in colon cancer cells [62]. In breast cancer, an ectopic expression of miR-34a inhibits the growth of breast cancer cells by inducing apoptosis and suppressing cell migration in both ER+ and ER– cell lines. It was revealed that miR-34a tumor-suppressive role is partly implemented by the means of suppressing SIRT1 expression in vitro [63]. Another study showed that SIRT1 downregulation or miR-34a upregulation inhibits cell proliferation and colony formation ability in the MCF-7 cell line, as well as in CD44+/CD24– breast cancer stem cells (BCSCs). SIRT1 knockdown in BCSCs positively correlates with decreased expression of BCSCs markers: ALDH1, BMI1, and NANOG. In addition, a stable expression of miR-34a or silencing of SIRT1 reduces tumor growth in nude mice xenografts. SIRT1 downregulation also positively correlates with decreased ALDH1 in vivo. It is postulated then, that miR-34a upregulation suppresses the proliferative potential of BCSCs in vitro and in vivo by partially downregulating SIRT1 [64]. The diverse tumor-promoting properties of SIRT1 in breast cancer are resumed in Table 1.

Table 1. Mechanisms of action of SIRT1 tumor-promoting functions in breast carcinogenesis. SIRT1: sirtuin-1; TSG: tumor suppressor gene; POLD1: DNA polymerase delta1; BC: breast cancer; BCSC: breast cancer stem cell.

Mechanism of Action	References
SIRT1 represses TSG expression through epigenetic silencing	[44]
SIRT1 upregulation positively correlates with p53 downregulation and POLD1 upregulation	[57]
SIRT1 stimulates the activation of PI3K/Akt signaling pathway	[56]
SIRT1 downregulation co-occurred with impaired activation of Ras-MAPK signaling pathway	[58]
SIRT1-mediated deacetylation of cortactin promotes cell migration	[18]
SIRT1 upregulation eliminates the tumor-suppressive effects of miR-22	[60]
SIRT1 downregulation induces apoptosis and enhances radiosensitivity of BC cells	[61]
SIRT1 downregulation by miR-34a suppresses proliferation and migration of BC cells	[63]
SIRT1 downregulation in BCSCs positively correlates with decreased expression of BCSCs markers and reduces tumor growth in nude mice xenografts	[64]

5. SIRT1 Assuming the Role of Tumor Suppressors in Breast Carcinogenesis

Alternatively, there is much convincing evidence supporting a tumor suppressive role of SIRT1 in carcinogenesis, considering its implication in maintaining genome integrity via chromatin regulation and DNA damage response. Following DNA damage, SIRT1 regulates and optimizes DNA repair pathways, and is required for efficient single-strand and double-strand DNA breaks (DSB) repair [65]. SIRT1 stabilizes and upregulates the activity of DNA damage repair enzymes including Ku70 [34], XPC [35], XPA [36], APE1 [37] and WRN [38]. Aside from regulating genome stability, SIRT1 represses the expression of oncogenes through epigenetic silencing, and downregulates the activity of oncoproteins through direct deacetylation. For example, SIRT1 downregulates the transcriptional activity of the NF-kappaB-dependent cell survival pathway through physically interacting and deacetylating the RelA/p65 subunit of NF-kappaB at lysine 310 (NF-κB K310) [19]. SIRT1 also impairs the oncogenic activity of the Wnt/β-catenin signaling pathway. Aberrant activation of this pathway in various cancers promotes the transcription of many oncogenes through the transcriptional activity of β-catenin. SIRT1-mediated deacetylation of β-catenin suppresses its ability to activate transcription and drive cell proliferation [20].

In breast cancer, Wang et al. asserted a tumor suppressor role of SIRT1 through its implication in DNA damage response and genome integrity. The authors revealed that SIRT1 haploinsufficiency in SIRT1^{+/-} p53^{+/-} mice facilitates tumorigenesis, whereas SIRT1 activation by resveratrol, a bona fide activator of SIRT1 [66], reduces tumorigenesis in vivo. Moreover, by mutating the *SIRT1*

gene, they found that SIRT1-null mice embryos die during embryonic development and that SIRT1 deficiency causes genetic instability and impaired DNA damage repair. The authors also found an increased expression of anti-apoptotic oncoproteins Bcl-2 and survivin in SIRT1-null embryos [67]. To investigate this observation, Wang et al. conducted another study on human BRCA1-associated breast cancers. The authors noticed that lack of BRCA1 in BRCA1-mutant breast tumors is associated with reduced expression of SIRT1 and high levels of survivin, and showed BRCA1 to positively regulate SIRT1 expression in vitro. They also demonstrated that SIRT1 activation by Resveratrol blocks cell proliferation and antagonizes tumor growth through downregulating survivin expression in vivo [46]. Paradoxically, survivin is also repressed by wild-type p53 [45], the latter being a certified target of SIRT1 [11].

The interplay between SIRT1 and BRCA1 in BC is uncovered in another study. Zhang et al. revealed that BRCA1 induction suppresses AR-dependent tumor growth through SIRT1 activation in both (ER⁺) and (ER⁻) cell lines. They showed that resveratrol inhibits AR-stimulated proliferation by activating SIRT1 in vitro, and that SIRT1 overexpression in xenograft model BALB/c mice represses tumor growth in vivo. They concluded on the note that SIRT1 inhibits breast cancer development through diverse cellular processes [68], further establishing SIRT1 tumor-suppressive properties in breast cancer. In fact, the direct functional link of SIRT1 with AR was previously characterized by Fu et al. who revealed that SIRT1 binds to and downregulates AR activity in vitro. They showed that SIRT1-mediated repression of AR activity inhibits androgen-induced cell proliferation in prostate cancer [30]. A recent study by Yu et al. showed that an ectopic expression of SIRT1 in mesenchymal stem cells (MSCs) effectively suppresses breast tumor growth by inhibiting proliferation and inducing apoptosis in vivo. The authors found that SIRT1-induced antitumor activity in MSCs is achieved by increasing CXCL10 expression, a chemotactic factor necessary for the recruitment of the antitumor natural killer (NK) cells. They showed that breast tumor suppression is carried out through the actions of CXCL10-recruited NK cells [69].

In addition, SIRT1 reduces drug-resistance in breast cancer. A well-structured study by Shi et al. reported that SIRT1 deficiency induces chemo-resistance to paclitaxel (PTX), a chemotherapy drug used to treat BC, by disrupting the SIRT1-PRRX1-KLF4 axis which regulates chemo-resistance. The authors found that SIRT1 depletion destabilizes PRRX1 and leads to KLF4 upregulation, a core stemness factor that promotes carcinogenesis. KLF4 subsequently promotes transcription of ALDH1, which induces BCSCs, confers cellular resistance to chemotherapy, and promotes distant metastasis [70]. SIRT1 was also shown to reduce drug-resistance to tamoxifen (TAM), a widely used drug in the treatment of luminal BC. Li et al. revealed that SIRT1 silencing leads to TAM-resistance in luminal MCF-7 cell line (TAMR-MCF-7 cells), whereas SIRT1 restoration compromised brachyury-mediated TAM-resistance. The authors demonstrated that the overexpression of brachyury, a molecular mediator of resistance to tamoxifen, enhances TAM-resistance by increasing cell viability, reducing cell apoptosis, and downregulating SIRT1 expression in vitro. They concluded that brachyury mediates TAM-resistance by downregulating SIRT1 expression [71]. However, a study by Choi et al. postulated that SIRT1 overexpression contributes to TAM-resistance in MCF-7 cells by activating FoxO1 (Forkhead box-containing protein, O subfamily1), which in turn upregulates the expression of MRP2 (multidrug resistance protein 2) in TAMR-MCF-7 cells [72]. The diverse tumor-suppressive properties of SIRT1 in breast cancer are resumed in Table 2.

Table 2. Mechanisms of action of SIRT1 tumor-suppressive functions in breast carcinogenesis. AR: androgen receptor; MSC: mesenchymal stem cell; NK: natural killer.

Mechanism of Action	References
SIRT1 upregulation by resveratrol reduces breast tumorigenesis in vivo SIRT1 loss causes genetic instability and impaired DNA damage repair SIRT1 loss positively correlates with an increased expression of oncoproteins Bcl-2 and survivin	[67]
SIRT1 upregulation antagonizes tumor growth by downregulating survivin expression in vivo SIRT1 represses survivin expression through epigenetic silencing	[46]
SIRT1 upregulation inhibits AR-stimulated proliferation in vitro SIRT1 upregulation represses tumor growth in xenograft BALB/c mice	[68]
SIRT1 upregulation in MSCs suppresses tumor growth in vivo through CXCL10-recruited NK cells	[69]
SIRT1 downregulation causes chemo-resistance by impairing SIRT1-PRRX1-KLF4 axis	[70]
SIRT1 downregulation induces brachyury-mediated tamoxifen-resistance in the luminal cell line	[71]

6. The Functional Duality of SIRT1 in Breast Cancer

Conflicting studies concerning SIRT1 ambiguous involvement in breast cancer extend to many aspects of the disease.

6.1. SIRT1 Role in ER- α -Positive Luminal BC Molecular Subtypes

The oncogenic estrogen/ER- α -mediated signaling pathways stimulate cell proliferation and tumor growth in luminal hormone-dependent subtypes, through the activation of estrogen-responsive genes by ER- α transcriptional activity. Yu et al. revealed that SIRT1 binds to and inhibits the transcriptional activity of ER- α by regulating its acetylation status. They showed that SIRT1 represses the co-activator synergy between DBC1 and CCAR1, ER- α co-activators that enhance its transcriptional activity. They asserted SIRT1 as a major regulator of ER- α activity and co-activator synergy [29]. Meanwhile, Moore et al. reported that SIRT1 inhibits tumor cell reaction to estrogen in vitro. The authors showed that SIRT1 represses basal and inducible expression of estrogen-responsive genes, while inhibition of SIRT1 activity results in transcriptional activation of estrogen-responsive genes and consequently, cancer cell proliferation. They demonstrated that SIRT1-mediated repression of the proliferative response to estrogens is ER- α -dependent. They concluded that SIRT1 downregulates the ER-mediated signaling pathway in BC cells [73]. A more recent study by Xu et al. showed that SIRT1-mediated deacetylation of ER- α represses the transactivation of ER- α and consequently, inhibits the proliferation of BC cells in vitro. The authors showed that checkpoint suppressor 1 (CHES1) interacts with ER- α and enhances the recruitment of SIRT1, thus enabling SIRT1-mediated repression of ER- α transactivation and impairing ER- α transcriptional activity [74]. Furthermore, the SIRT1 activator resveratrol has been reported to suppress estrogen-dependent growth of luminal BC cells [75]. These studies demonstrated an anti-tumor role of SIRT1 in luminal subtypes through impairing ER-mediated signaling pathways (Figure 1).

On the other hand, alternative studies reported an oncogenic role of SIRT1 in luminal breast tumors. Elangovan et al. revealed that SIRT1 is activated and upregulated by ER- α in response to estrogens. They showed that ER- α physically binds to and functionally cooperates with SIRT1 toward the stimulation of breast tumor cells. In addition, SIRT1 inactivation eliminates estrogen/ER- α -induced cell growth and tumor development, triggering apoptosis and cell growth arrest. The authors concluded that SIRT1 is required for estrogen-induced breast cancer growth [76]. Another study by Yao et al. demonstrated that SIRT1 deficiency suppresses ER- α expression and leads to inhibition of estrogen-responsive gene expression in vitro. They showed that SIRT1 deficiency downregulates ER- α -mediated estrogen response genes in vivo, impairing ER- α -mediated signaling pathways in breast tumors. They postulated that SIRT1 may be a co-activator of ER- α in breast cancer [77]. In accordance with these findings, Santolla et al. investigated the expression and function of SIRT1 by

estrogens in ER-negative BC cells and cancer-associated fibroblasts (CAFs). The authors showed that estrogens upregulate SIRT1 expression through GPER (G protein-coupled ER) along with subsequent activation of the oncogenic EGFR/ERK/c-fos/AP-1 transduction pathway *in vitro*. They demonstrated that SIRT1 and GPER promote tumor growth both *in vitro* and *in vivo*. The authors then asserted a pro-survival role of SIRT1 and its implication in the prevention of apoptosis and cell cycle arrest [78].

6.2. SIRT1 Role in Non-Hormone-Dependant Triple-Negative Subtype (TNBC)

There are also contrasting studies concerning SIRT1 biological role in the TNBC subtype. Yi et al. reported that SIRT1 activation by a SIRT1 specific activator YK-3-237, induces the deacetylation of mt-p53, the oncogenic mutant form of p53. Deacetylation of mt-p53 upregulates the expression of wild-type p53-targets the *PUMA* and *NOXA* pro-apoptotic genes, suppressing cell proliferation and arresting cell growth of TNBC cell lines [79]. On the other hand, Wu et al. asserted an oncogenic role of SIRT1 in TNBC subtype. They revealed that an increased expression of SIRT1 is associated with poor prognosis, shorter DFS and OS, and distant metastasis in both TNBC and non-TNBC subtypes [55]. These findings are in agreement with those of Chung et al. who reported that SIRT1 upregulation positively correlates with tumor invasion and lymph node metastasis. They also showed that *SIRT1* gene silencing with SIRT1-siRNA significantly reduces the invasion ability of transfected versus non-transfected TNBC cell lines. The authors suggested the potential role of SIRT1 as a prognostic indicator, as well as a novel therapeutic target in triple negative BC [80]. Interestingly, a recent study by Urrea et al. showed that SIRT1-mediated activation of AMPK selectively inhibits fibronectin-dependent migration of TNBC cells. However, the activation of SIRT1/AMPK axis has a cyto-protective effect in TNBC cells, promoting cell survival and proliferation but suppressing their ability to migrate. The authors demonstrated that SIRT1/AMPK activation impairs cell migration by reducing β 1-integrin, a key protein involved in fibronectin-stimulated cell migration, on the cell surface and in turn, reduces cellular adhesion to the extracellular matrix [81].

6.3. SIRT1 Implication in the Epithelial-to-Mesenchymal Transition (EMT) Process, and Breast Cancer Invasion and Metastasis

The EMT process refers to the transformation of an epithelial cell to a mesenchymal cell; the process results in repressed E-cadherin expression and loss of cell-adhesive properties of epithelial cells. It also prevents apoptosis, and is critically implicated in cancer invasion and metastasis [82]. Using a xenograft mouse model, Simic et al. analyzed the metastatic potential of BC cells with or without SIRT1 *in vivo*. They found that SIRT1 upregulation suppresses cancer metastasis by reducing EMT, consequently maintaining E-cadherin expression; whereas SIRT1 repression promotes metastasis of breast epithelial cells in an orthotopic model of breast cancer. The authors also demonstrated that SIRT1 restrains the transforming-growth-factor (TGF)- β -signaling pathway that drives EMT. They postulated that SIRT1 suppression leads to E-cadherin degradation from the cell surface, thereby releasing β -catenin from the cadherin junctions to the nucleus, which is the characteristic of mesenchymal cells [83], thus asserting SIRT1 tumor-suppressive properties in the EMT process of BC. In contrast, Eades et al. reported that SIRT1 is overexpressed upon EMT-like transformation of human mammary cells *in vitro*, and that TGF- β -induced EMT leads to SIRT1 overexpression in epithelial cells. They also observed an increased SIRT1 recruitment to the E-cadherin promoter, resulting in SIRT1-mediated epigenetic silencing of E-cadherin, while SIRT1 knockdown restores E-cadherin expression. The authors also showed that SIRT1 deficiency prevents transformation of mammary epithelial cells by decreasing anchorage-independent growth and cell migration *in vitro*, hence indicating SIRT1 role in maintaining EMT-like transformation of the mammary epithelium [84]. Another study by Jin et al. revealed that SIRT1 expression is significantly correlated with increased expression of EMT-related proteins, vimentin and snail-1, and reduced expression of E-cadherin in triple-negative breast tumors; whereas inhibition of SIRT1 has opposite effects *in vitro*. They showed that SIRT1 inhibition also reduces the

invasion ability of TNBC cell lines in vitro. The authors then suggested an oncogenic role of SIRT1 in association with EMT in tumor invasion of TNBC subtype [85].

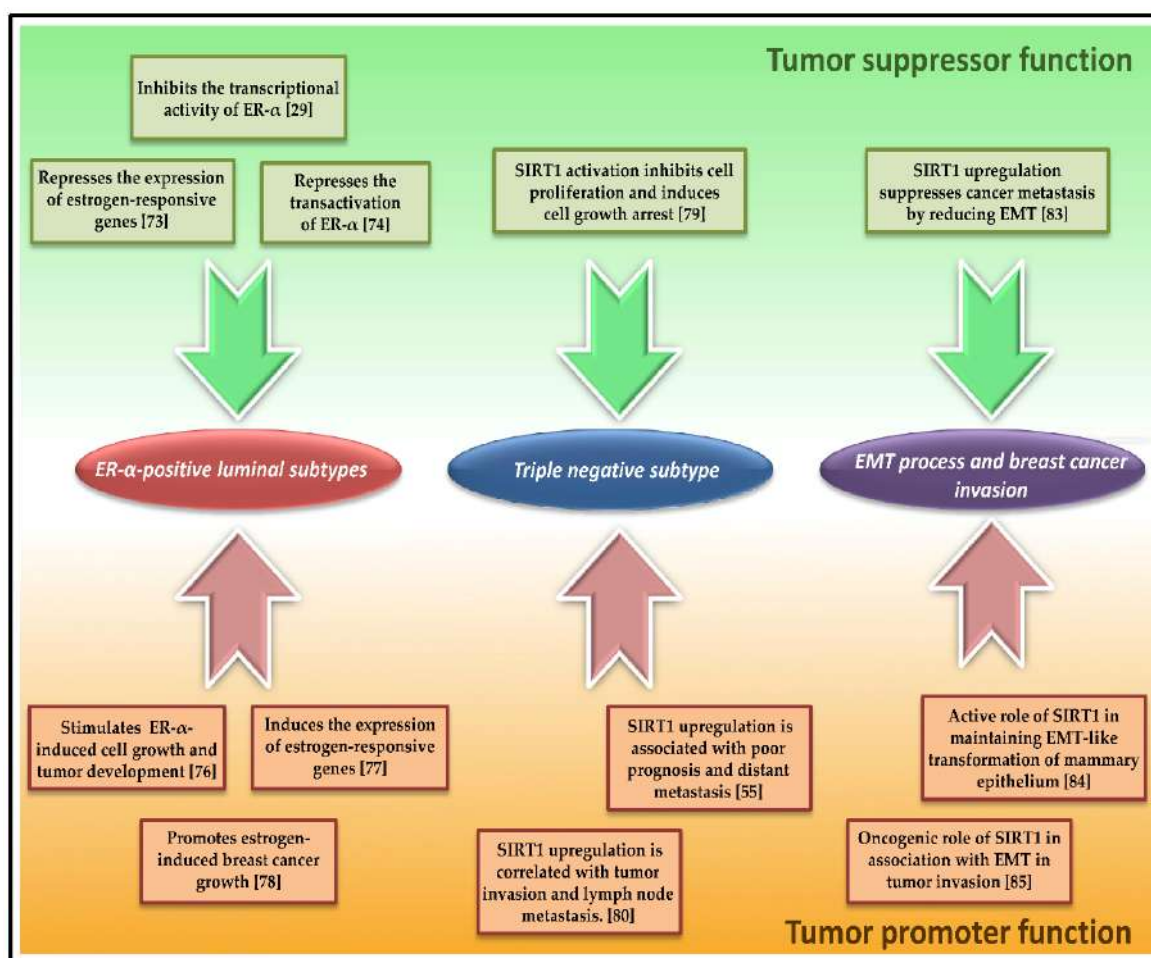


Figure 1. Bifurcated functions of SIRT1 in breast carcinogenesis. ER: estrogen receptor; EMT: epithelial-to-mesenchymal transition.

7. SIRT1 Modulators towards Breast Cancer Treatment

Being a key regulator of numerous cancer-associated processes, SIRT1 has been the subject of intense research in recent years. As a consequence, countless studies investigated/reviewed the therapeutic potential of SIRT1 in cancer treatment, and a plethora of small chemical compounds that modulate SIRT1 activity were discovered and patented [86–89]. These modulators (i.e., activators/inhibitors) not only enabled researchers to have a greater understanding of SIRT1 biological function and regulatory mechanisms, but also showed promising therapeutic applications in clinical trials for various human diseases, such as metabolic disorders, cardiovascular and neurodegenerative diseases, endothelial dysfunctions, inflammation, and cancer [90–92]. Although SIRT1 modulators have proven their efficiency in cancer cells by reducing cell viability and inducing apoptosis, their therapeutic functions remain utterly related to the role and expression rate of SIRT1 in a specific cancer, which in turn may vary drastically as we previously described.

While SIRT1 activators were initially favored as calorie restriction mimetics, researchers demonstrated their beneficial effects in delaying age-related decline in heart function and neuronal loss, also in preventing tumorigenesis. Resveratrol, a polyphenol described as an anti-aging drug and calorie restriction mimetic, was amongst the first characterized activators of SIRT1 [66,93]. In breast cancer, we previously showed that SIRT1 activation by resveratrol in SIRT1^{+/-} p53^{+/-} mice reduces tumorigenesis in vivo [67], as well as AR-stimulated proliferation [68]. Also, resveratrol was

shown to repress estrogen-dependent growth by impairing ER- α -mediated signaling pathways [75]. Due to shortage in resveratrol bioavailability, synthetic compounds that are structurally unrelated to resveratrol but 1000-fold more potent were synthesized and collectively named SIRT1-activating compounds (STAC) [94]. These STACs are currently being used as SIRT1 activators in breast cancer studies; they include among others SRT1460, SRT1720, SRT2104, and SRT2183 [86,87,91].

SIRT1 inhibitors have shown their therapeutic potentials in the treatment of various pathologies such as immunodeficiency virus infections, parasitic diseases, Parkinson's disease, and cancer therapy. Since SIRT1 is upregulated in multiple types of cancer, anticancer studies were more focused on SIRT1 inhibitors compared to SIRT1 activators [87,92]. As a result, a wide range of pharmacological inhibitory molecules were designed and tested such as sirtinol, salermide, splitomicin, cambinol, suramin, tenovin, nicotinamide, indole derivatives, and their structurally similar analogs. In breast cancer, in vitro and in vivo studies on ER⁺ and ER⁻ cell lines showed that SIRT1 inhibition by these molecules suppresses cancer cell proliferation and induces p53-mediated apoptosis through increasing the acetylation of its Lys382 (p53K382), or in some cases, induces p53-independent apoptosis by reactivating proapoptotic genes (such as *CASP* genes that encode for caspase-3/8/9) that were epigenetically repressed by SIRT1 [95–100], thus proving the antitumor activity of SIRT1 inhibitors in BC.

8. Conclusions and Future Directions

In conclusion, regardless of whether SIRT1 has a pro-survival role by repressing TSGs, upregulating the expression of oncogenes, and activating oncogenic signaling pathways such as PI3K/Akt and Ras-MAP kinase, or whether it has a proapoptotic role by reducing tumorigenesis and AR-mediated proliferation, downregulating the expression of oncogenes, and participating in ER- α -mediated signaling pathways and the EMT process, there is no doubt as to its significant role in breast carcinogenesis. Studies showed that SIRT1 plays different roles according to different BC molecular subtypes. Since BC is characterized by its molecular and clinical heterogeneity, with variations in gene expression profiles compared to intrinsic subtypes, one might argue that researchers should take into account the molecular classification of used human mammary tumors and cell lines in their future studies. Further investigations should also include a statistically sufficient sample size, and use of multiple cell lines in the same study. Nonetheless, considerable progress has been made in this research area in the last 10 years. SIRT1 modulators have been discovered or designed, and clinical studies investigating the therapeutic potential of SIRT1 in cancer treatment hold promising results. Thus, this research field should be prioritized and more large-scale studies are needed in order to decipher the code of the enzymatic duality of SIRT1 in breast carcinogenesis.

Funding: This research received no external funding.

Acknowledgments: The authors thank the Hariri Foundation for providing grant support to K.R.

Conflicts of Interest: The authors declare no existing conflicts of interest.

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Conclusion de la publication 3:

Cette revue nous a permis de synthétiser le rôle contradictoire de SIRT1 dans le cancer du sein. En effet, SIRT1 peut agir dans des mécanismes pro-tumoraux en activant ou désactivant certaines protéines. Dans cette revue on a pu voir que SIRT1 est capable de déacétyler la protéine pro apoptotique P53 ou E2F1 qui a pour effet de d'inhiber la voie de la mort programmée des cellules lors d'une anomalie cellulaire tel qu'une mutation. D'autre part, SIRT1 est aussi capable d'inhiber la transition épithélio-mésenchymateuse grâce à la déacétylation d'ATM, ce qui la rend inactive. Ce double mode de fonction de SIRT1 fait de lui une protéine indispensable dans le cancer du sein mais aussi une cible thérapeutique de choix pour lutter contre le cancer du sein.

- *Sirt1 dans d'autres cancers*

Le rôle de SIRT1 est de mieux en mieux connus dans le cancer du sein, on a alors voulu voir si son fonctionnement était le même dans les autres cancers. Dans cette mini-revue, on a voulu voir le fonctionnement de SIRT1 dans le cancer colorectal pour avoir un comparatif avec les observations obtenues dans le cancer du sein. Pour cela on a suivi le même cheminement que dans le cancer du sein à savoir récolter les informations dans la littérature sur le rôle de SIRT1 dans les mécanismes impliqués dans l'installation et la progression du cancer.

Publication 4:

SIRT1 in Colorectal Cancer: A Friend or Foe? Rifaï K, **Idrissou M**, Daures M, Bignon YJ, Penault-Llorca F, Bernard-Gallon D. OMICS. 2018 Apr;22(4):298-300.

SIRT1 in Colorectal Cancer: A Friend or Foe?

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IT HAS BEEN WELL ESTABLISHED THAT SIRT1 plays a key role in the regulation of various crucial biological processes such as apoptosis, DNA damage response, cell senescence, and metabolism. However, its role in carcinogenesis is still subject to debate. SIRT1 is the human ortholog of yeast Sir2 and the protomember of sirtuins family, it is a NAD⁺ dependent class III deacetylase that can deacetylate both histone and nonhistone proteins. On one hand, studies report that SIRT1 activation reduces tumorigenesis in various human cancers. SIRT1 was also associated with the down-regulation and inactivation of several oncogenes or oncoproteins such as NF- κ B and β -catenin. Thus, SIRT1 can serve as a tumor suppressor.

On the other hand, it has been demonstrated that SIRT1 activates various oncogenes such as c-MYC and RAS oncoproteins. SIRT1 has also been shown to contribute to the tumorigenesis process by inducing inhibition and epigenetic silencing of tumor suppressor genes such as p53 and members of the FoxO family. Hence, SIRT1 can exert tumor promoter properties as well. Therefore, depending on its regulation of a multitude of substrates, SIRT1 may play different roles in different types of cancer.

Regulated by various different genes, the pathogenesis of colorectal cancer (CRC) is a multifactorial process that involves the deacetylation capability of SIRT1. Numerous studies have revealed the clinical relevance of SIRT1 expression and its role in the tumor formation, prognosis, and overall survival (OS) of CRC. However, there is a wide discrepancy between these studies concerning SIRT1 expression rate and subsequently its exact role in the disease. The inconsistency between these studies comes from the fact that SIRT1 may play opposing roles in colorectal carcinogenesis.

Consistent with the hypothesis that SIRT1 can act as a tumor suppressor, some studies have shown a reduced SIRT1 expression in human colorectal adenocarcinoma in comparison with normal colorectal tissues. These studies also evaluated the association between SIRT1 expression and clinicopathological parameters of CRC patients, they concluded that decreased SIRT1 expression was associated with colorectal tumor progression and frequent regional lymph node metastasis. Accordingly, SIRT1

expression was associated with better prognosis and OS of CRC patients.

Sun et al. (2017) gave a likely explanation for the decreased SIRT1 expression in CRC patients with advanced stages. They reported that SIRT1 activation by a selective SIRT1 activator SRT1720 reduced the invasiveness of CRC cells *in vitro*. Although the inhibition of SIRT1 by a selective SIRT1 inhibitor Ex-527 promoted their migration, they postulated that SIRT1 suppresses the invasion and migration of CRC cells. The authors also focused on the impact of SIRT1 on CRC metastasis. They uncovered a functional role of the SIRT1/miR-15b-5p/ACOX1 axis in suppressing CRC metastasis *in vitro*, using human CRC continuous cell lines, as well as *in vivo* using BALB/c nude mice.

They stated that SIRT1 negatively regulates miR-15b-5p transcription through deacetylation of AP-1, a transcriptional activator, and consequently impairing its ability to bind to the miR-15b-5p promoter (Fig. 1A). MicroRNAs are small noncoding RNAs that can modulate protein expression. It has been shown that miR-15b-5p regulation of certain targets in tumorigenesis leads to an increased proliferation and migration of cancer cells. In their study, the authors highlighted that miR-15b-5p can directly target and downregulate ACOX1 expression in CRC, an enzyme of the fatty acid oxidation pathway. The authors showed that ACOX1-impaired expression by miR-15b-5p promotes CRC metastasis. The authors also revealed that SIRT1 overexpression in CRC cells significantly suppressed miR-15b-5p expression and increased ACOX1 expression. When tested *in vivo*, CRC cells with stable SIRT1 expression inhibited the expression of miR-15b-5p and enhanced ACOX1 expression, resulting in reduced CRC metastasis. Hence, the authors provide evidence of a suppressive role of SIRT1/miR-15b-5p/ACOX1 axis in CRC metastasis (Sun et al., 2017).

In contrast, many studies asserted an oncogenic role of SIRT1 in CRC. They have reported an abundance of SIRT1 expression in human CRC tissues, especially in advanced-stage tumors and tumors with lymph node (Lv et al., 2014). That overexpression was strongly correlated with metastasis and tumor invasion depth. It was also associated with poor prognosis, shorter OS, and disease-free survival of CRC patients. Chen et al. (2014) reported an overexpression of

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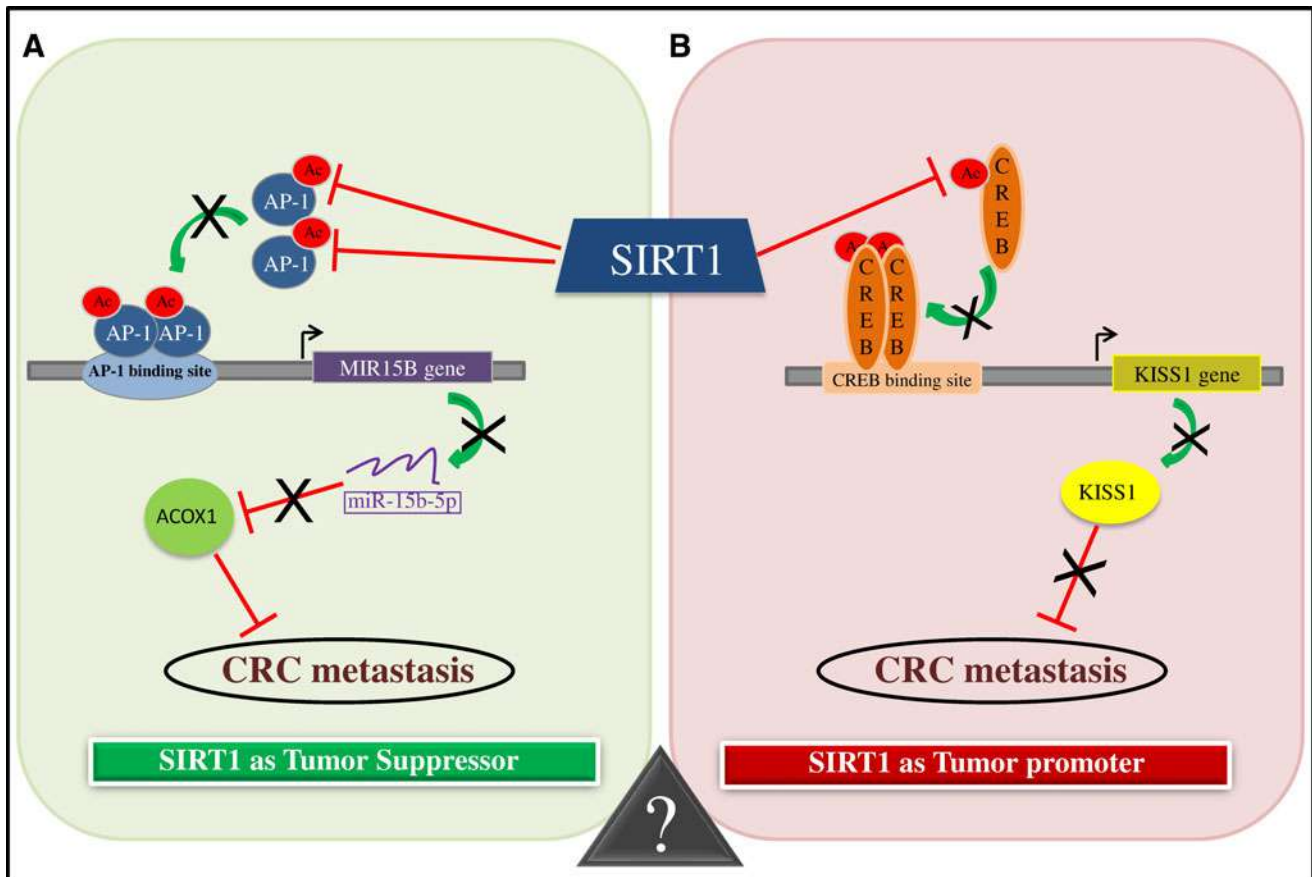


FIG. 1. Contradictory functions of SIRT1 in human colorectal tumorigenesis process. (A) SIRT1 deacetylates and inactivates AP-1, unactivated AP-1 is incapable of binding and promoting miR-15b-5p transcription, which, in turn, prevents miR-15b-5p from binding and inactivating ACOX1, that leads to CRC metastasis inhibition (Sun et al., 2017). (B) SIRT1 deacetylates and inactivates KISS1 transcriptional activator CREB, which prevents the expression of the tumor metastasis suppressor KISS1 and subsequently promoting CRC metastasis (Shen et al., 2016). CRC, colorectal cancer.

SIRT1 in cancer stem-like cells of CRC, cancer stem cells (CSCs) that have the ability of self-renewal and differentiation. The authors also highlighted an underlying association between SIRT1 and cancer stem-like cells. They found that SIRT1 inhibition in CRC cells led to an increased p53 expression. SIRT1 deficiency also led to a decrease in CD133+ percentage, a common stem cell marker that characterizes colorectal CSCs. Furthermore, SIRT1 inhibition led to decreased expression of several stemness-associated genes such as *Oct4*, *Nanog*, *Cripto*, *Tert*, and *Lin28*. Moreover, the authors observed a significant reduction in colorectal tumorigenicity by knocking down SIRT1 expression using SIRT1 small hairpin RNA *in vivo*. They postulated that SIRT1 plays a prosurvival role in CRC by keeping the stemness of cancer stem-like cells, as well maintaining the ability of colony and sphere formation in CRC cells (Chen et al., 2014).

Whereas Shen et al. (2016) were interested in the role of SIRT1 in distant metastasis, the authors revealed an SIRT1/CREB/KISS1 signaling pathway. They showed that SIRT1 knockdown decelerated the progress of CRC metastasis by functionally reducing the invasion and migration of CRC cells both *in vivo* and *in vitro*. SIRT1 knockdown also enhanced the chemosensitivity of CRC cells to 5-fluorouracil

and oxaliplatin, two chemotherapeutic drugs used for clinical treatment of CRC. Furthermore, the authors demonstrated that SIRT1 upregulates the metastasis of CRC by down-regulating KISS1 expression, a major tumor metastasis suppressor. KISS1 downregulation was realized through deacetylating and subsequently inactivating CREB transcriptional activity, a direct SIRT1 target that binds and triggers KISS1 transcription (Fig. 1B). Therefore, the authors concluded that SIRT1 actively contributes to the distant metastasis of CRC through SIRT1/CREB/KISS1 signaling pathway (Shen et al., 2016).

In conclusion, it appears that the controversy surrounding SIRT1 role in colorectal tumorigenesis has not been completely resolved. Consistent with recent findings, SIRT1 seems to have a bivalent role in the pathogenesis of breast cancer as well as CRC (Rifai et al., 2017). Therefore, more in-depth studies are needed to be done to pin down the functional role of SIRT1 in the pathogenesis of CRC.

Acknowledgment

The authors thank the Hariri Foundation for a grant to Khaldoun Rifai.

Author Disclosure Statement

The authors declare that no competing financial interests exist.

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Abbreviations Used

ACOX1	= peroxisomal acyl-coenzyme A oxidase 1
AP-1	= activator protein 1
CRC	= colorectal cancer
CREB	= cAMP response element binding
CSCs	= cancer stem cells
KISS1	= kisspeptin-1
OS	= overall survival
SIRT1	= silent mating type information regulation 2 homolog 1

Conclusion de la publication 4:

Dans cette min-revue, nous avons pu constater que SIRT1 joue un rôle contradictoire dans le cancer colorectal comme observé dans le cancer du sein. De nombreuses études ont montré une inhibition du développement tumoral dans le cancer colorectal grâce à SIRT1. D'autres ont plutôt montré son implication dans la prolifération et la migration des cellules cancéreuses du côlon. En effet, SIRT1 est capable d'inhiber la transcription du micro-ARN oncogène mir-15b-5p en déacétylant la protéine AP-1 qui est un Co-activateur du micro-ARN, cette inhibition a pour effet de stopper la métastase des cellules cancéreuses du colon. D'autre part, elle est capable d'empêcher la transcription de KISS1 qui est un suppresseur de tumeur, cette inhibition a pour effet d'entraîner la métastase des cellules cancéreuses.

Ces informations recueillies sur le cancer colorectal nous suggèrent que SIRT1 pourrait fonctionner de la même façon dans tous les cancers en agissant comme suppresseur de tumeur ou comme oncogène.

b. Histones acétyl-transférases et cancer du sein

L'acétylation des histones est la modification post-traductionnelle d'histone la plus importante. Cette acétylation est régie par un groupe de protéines appelées histones acétyl-transférases (HATs) qui sont généralement impliquées dans la décondensation de la chromatine et l'activation de la transcription des gènes. Leurs activités leur confèrent un rôle important dans plusieurs pathologies, dont principalement le cancer. En effet, leurs capacités à moduler l'état de la chromatine et à activer la transcription des gènes, fait d'eux des éléments importants dans l'installation et la progression tumorale. Ces HATs sont divisées en 3 grandes familles en fonction de leurs structures et de leurs similarités de séquences (Figure 15 et 16).

La famille des p300/CREB-binding protein (CBP) est constituée de l'acétyl-transférase p300 et de CBP qui sont de grandes protéines à plusieurs domaines. Ces domaines leur permettent d'interagir avec de nombreux autres protéines cellulaires et ainsi agir dans plusieurs voies cellulaires (Bedford et al., 2010).

La famille des Gcn5-relat N-acétyl-transférases ou GNAT est une famille constituée de deux protéines partageant une grande similarité de séquence à savoir, lysine acétyl-transférase 2A et 2B (KAT2A et KAT2B). KAT2A et KAT2B ou encore p300/CBP-associated factor (PCAF) qui sont des protéines des complexes SptAda-Gcn5-acétyltransférase (SAGA) et Ada2-containing (ATAC) (Nagy et al., 2010), ils partagent des fonctions communes mais ont chacun des fonctions bien spécifiques (Nagy and Tora, 2007). Ces fonctions et celle d'acétyl-transférase les impliquent dans le processus de carcinogénèse du sein.

La famille de MYST (MOZ, Ybf2, Sas2, Sas3 et TIP60) est constituée des protéines formant son nom. C'est sans doute l'une des familles les plus étudiées avec ses protéines qui jouent des rôles importants dans le remodelage de la chromatine et la transcription des gènes en acétylant les histones ou encore en agissant comme Co-activateur du processus de réparation de l'ADN (Avvakumov and Cote, 2007; Doyon and Cote, 2004; Utley and Cote, 2003). Ces acétyl-transférases possèdent plusieurs domaines y compris leurs domaines catalytiques tels que des bromo-domaines et les chromo-domaines leur permettant de se fixer sur les marques d'histones ou sur la chromatine. L'une de ces acétyl-transférases TIP60 fera l'objet d'une attention particulière dans ma thèse.

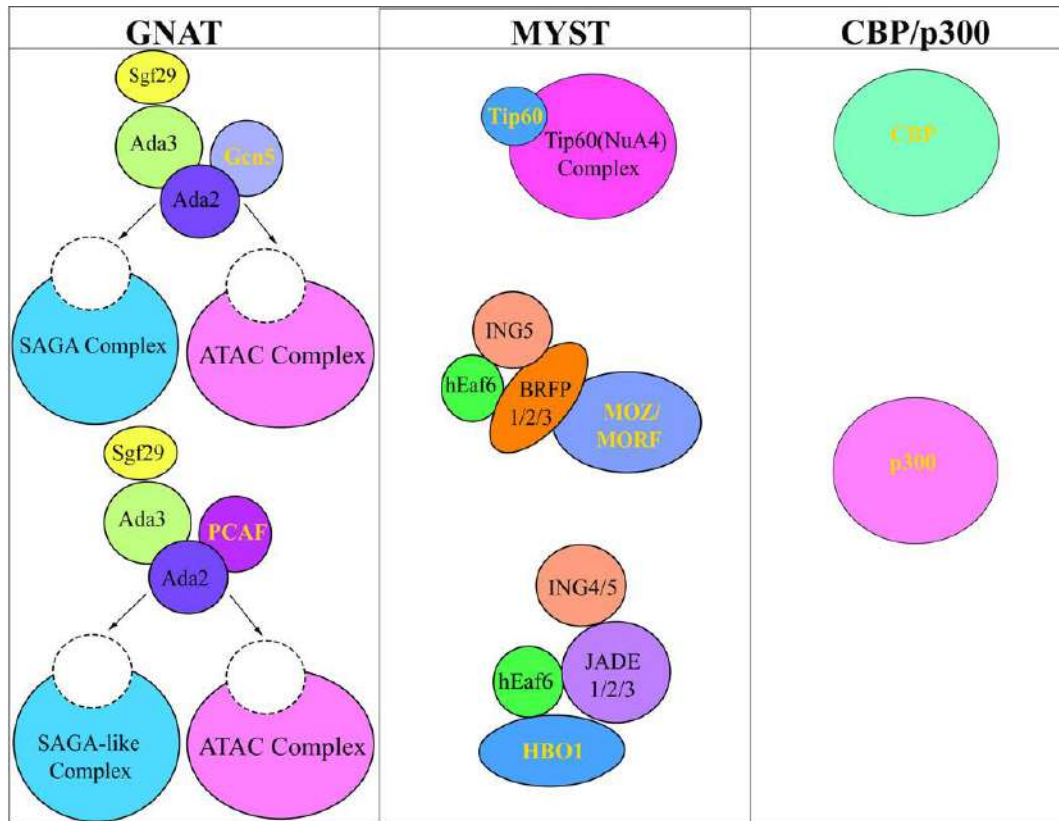


Figure 15 : Représentation des trois grandes familles de HAT. La famille des GNAT, MYST et CBP/p300 est représentée avec les protéines qui les constituent. Image tirée de (Farria et al., 2015).

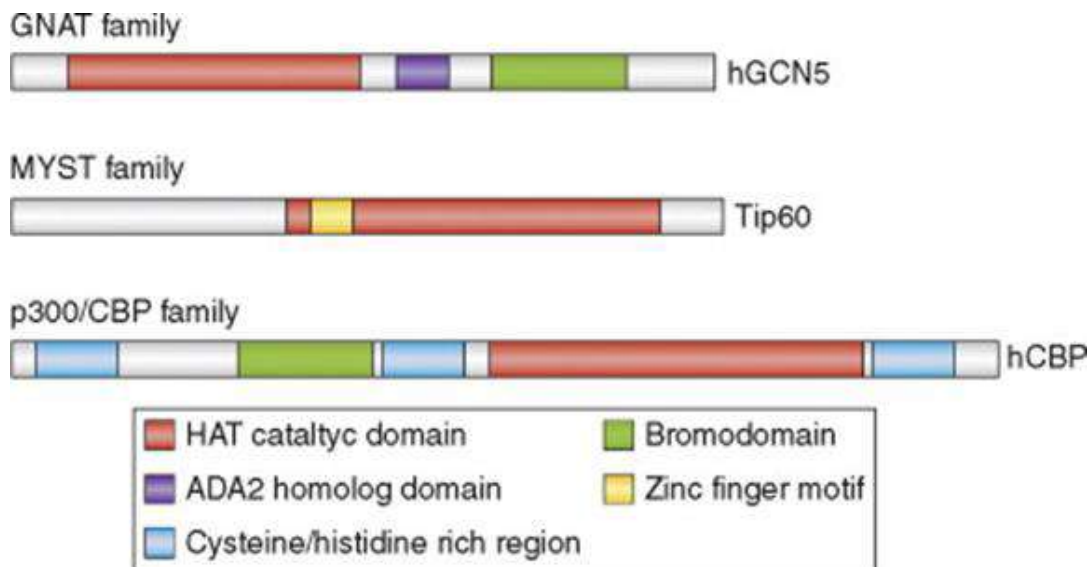


Figure 16 : Prototypes de structure des différents HATs. La famille des GNAT présente généralement un domaine histone acétyl-transférase (HAT), un domaine ADA2 et un Bromodomain. La famille des MYST présente un domaine HAT et des motifs en doigts de zinc. La famille des CBP/ p300 présente un domaine HAT, un bromodomain et des régions riches en cystéine et histidine.

- *TIP60 et cancer du sein*

TIP60 ou lysine acétyl-transférase 5 (KAT5) est l'acétyl-transférase de la famille des MYST la plus étudiée de nos jours. Elle a été découverte en 1996 par Kamine et son équipe qui l'ont clonée et caractérisée grâce à son interaction avec la protéine Tat du virus HIV-1 (Kamine et al 1996). Le gène codant pour cette protéine est appelé HTATIP et est situé sur le chromosome 11 en position 11q13.1. Ce gène code pour trois isoformes (TIP60 α isoforme 2 dominant avec 513 acides aminés, TIP60 β isoforme 3 avec 461 acides aminés et l'isoforme 1 avec 546 acides aminés) grâce à l'épissage alternatif (Figure 17). TIP60 tire son nom de la protéine Tat du virus HIV-1 et de son poids moléculaire 60 KDa. Elle est constituée de plusieurs domaines dont son domaine catalytique en position N-terminale appelé domaine MYST capable de lier l'acétyl-CoA à son substrat, d'un chromo-domaine en position C-terminale permettant de se fixer sur la chromatine, une boîte d'interaction pour récepteurs nucléaires et enfin un doigt de zinc Cys-Cys-His-Cys permettant les liaisons avec d'autres protéines (Nordentoft and Jorgensen, 2003; Xiao et al., 2003) (Figure 17).

TIP60 et plus particulièrement l'isoforme β est localisée en grande partie dans le noyau cellulaire, mais aussi dans le cytoplasme associé à des récepteurs nucléaires grâce à sa boîte d'interaction aux récepteurs nucléaires (Cao and Sudhof, 2001; Gavarravaru and Kamine, 2000; Ran and Pereira-Smith, 2000). TIP60 est une protéine ubiquitaire donc exprimée dans tout l'organisme. Cependant, l'expression de cette dernière est tissu dépendante, dans le cerveau, l'ovaire et la glande thyroïdienne où on a une forte expression de TIP60, dans le sein et le poumon son expression est moyenne, dans le pancréas on a une faible expression de TIP60. Toutefois, dans la prostate on a une expression protéique faible et une expression d'ARNm forte (McAllister et al., 2002) (Figure 18).

TIP60 est une protéine instable, car elle a une demi-vie courte de 30 à 190 min en fonction du tissu et du type cellulaire (Eymin et al., 2006; Legube et al., 2002). Dans les conditions normales, TIP60 est régulée par la voie du protéasome à travers les protéines MDM2 E3 ubiquitine qui se fixent directement sur TIP60 ou la Cullin3 qui est associée avec ATF2 pour dégrader TIP60 (Bhoumik et al., 2008).

Grâce à sa fonction acétyl-transférase, TIP60 est considéré comme une protéine pléiotropique qui agit dans plusieurs voies cellulaires. TIP60 est capable en effet d'acétyler plusieurs types de protéines telles que les histones (H3, H4 et H2AX), des facteurs de transcription (STAT3, C-Myc et P53) et elle est même capable de s'auto-acétyler. Cette auto-acétylation se fait sur les lysines (76, 80, 104, 150, 187, 327 et 383) et permet d'activer sa fonction acétyl-transférase (Figure 19)(Tang et al., 2006; Yang et al., 2012).

TIP60 fait partie d'un complexe de protéine appelé le complexe NUA4 ou complexe TIP60 constitué de 13 protéines chacune jouant un rôle bien spécifique (Figure 20). TIP60 est la sous-unité acétyl-transférase de ce complexe tandis que P400 est la sous-unité ATPase (Doyon and Cote, 2004; Doyon et al., 2004). Avec ce complexe, TIP60 est impliquée dans plusieurs activités cellulaires comme la réponse aux dommages de l'ADN ou P400 recrute TIP60 pour acétyler H2AX sur sa lysine 5 et lancer le signal de réparation de l'ADN (Scherthan, 2003; Yamagata and Kitabayashi, 2009) (Figure 21). Comme avec H2AX, TIP60

et son complexe acétylent aussi des histones tels que l'histone H4 sur les lysines (5, 8, 12 et 16) ou encore l'histone H3 sur les lysines (4 et 14) permettant ainsi le remodelage de la chromatine. D'autre part avec le complexe NUA4, TIP60 peut acétyler des protéines telles qu'ATM. L'acétylation d'ATM entraîne sa phosphorylation et l'activation de son activité (Bhoumik et al., 2008). ATM acétylée et phosphorylée activera p53 en la phosphorylant et à son tour elle entrainera l'arrêt du cycle cellulaire. TIP60 peut aussi acétyler p53 phosphorylée et dans ce cas entrainer l'apoptose de la cellule. Certains récepteurs nucléaires tels que le récepteur aux androgènes AR peut être acétylé entrainant leurs stabilisations (Fu et al., 2004a).

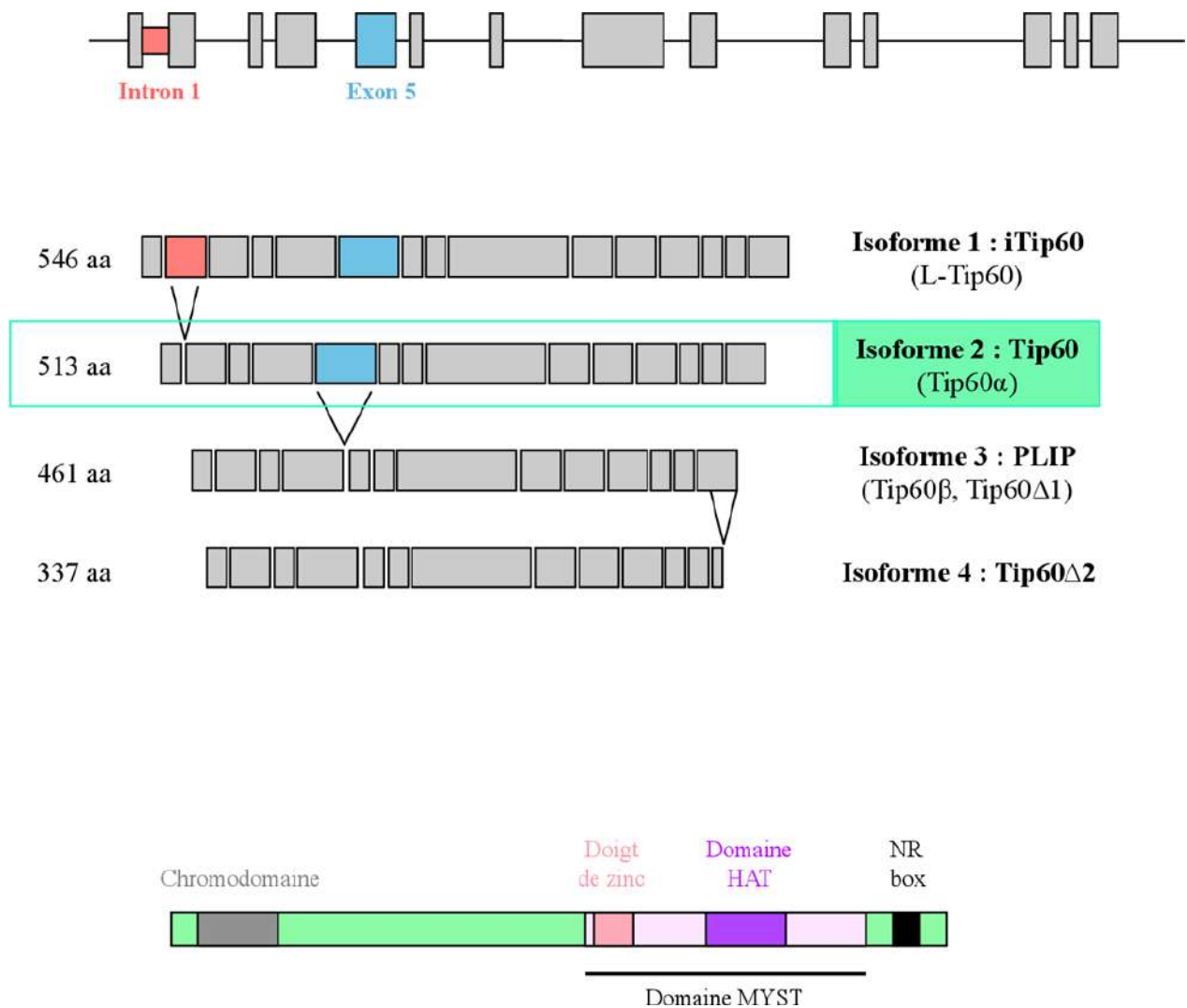


Figure 17 : Représentation de la structure et des différents isoformes de TIP60. Image tirée de (Mattera et al., 2009).

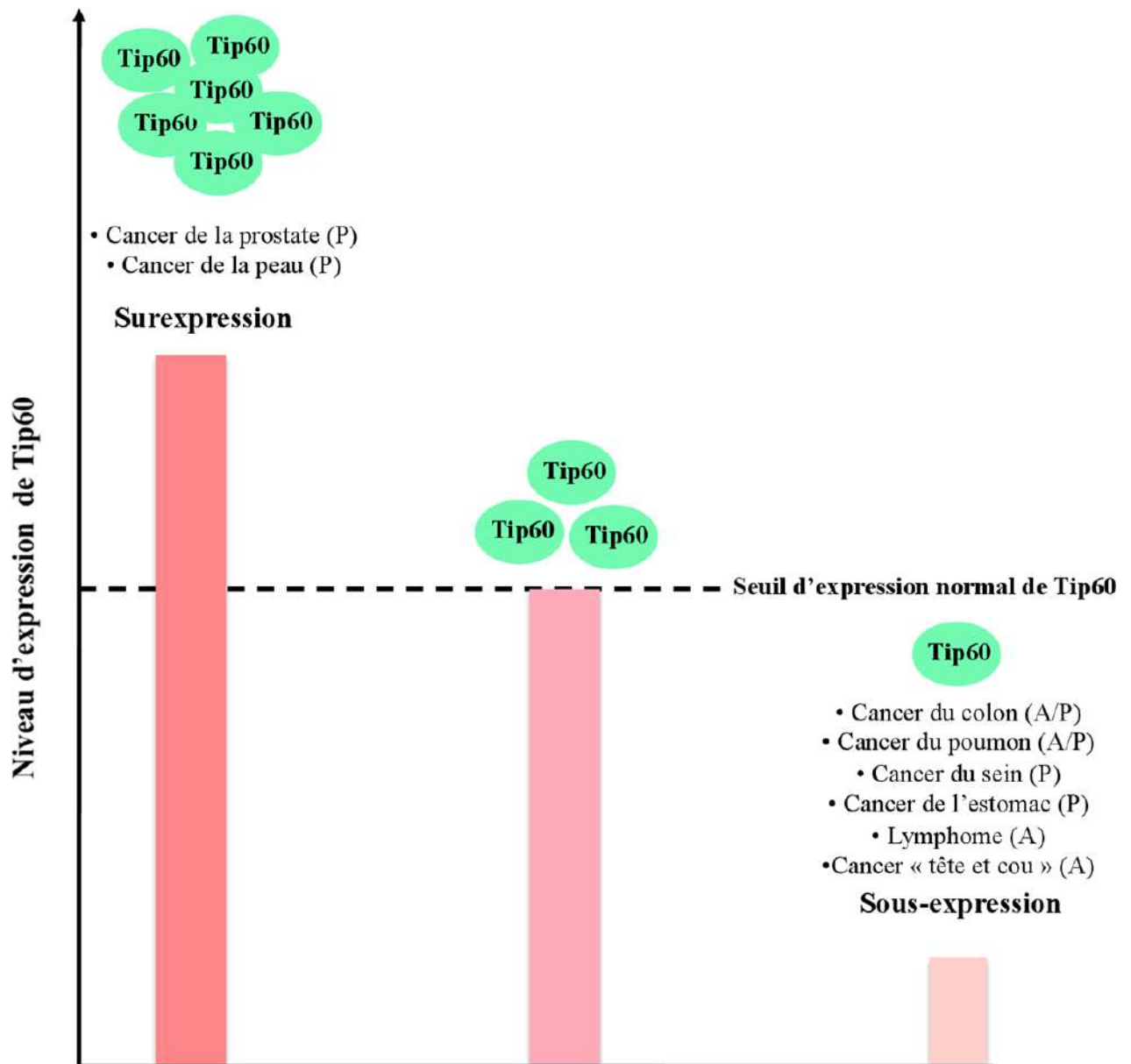


Figure 18 : Illustration de la variation d'expressions protéique et ARN de TIP60 dans les différents cancers. TIP60 est sur-exprimée dans le cancer de la prostate et de la peau et sous-exprimée dans le cancer du côlon, du poumon, du sein, de l'estomac, du lymphome et de la « tête et cou ». Figure tirée de (Mattera et al., 2009).

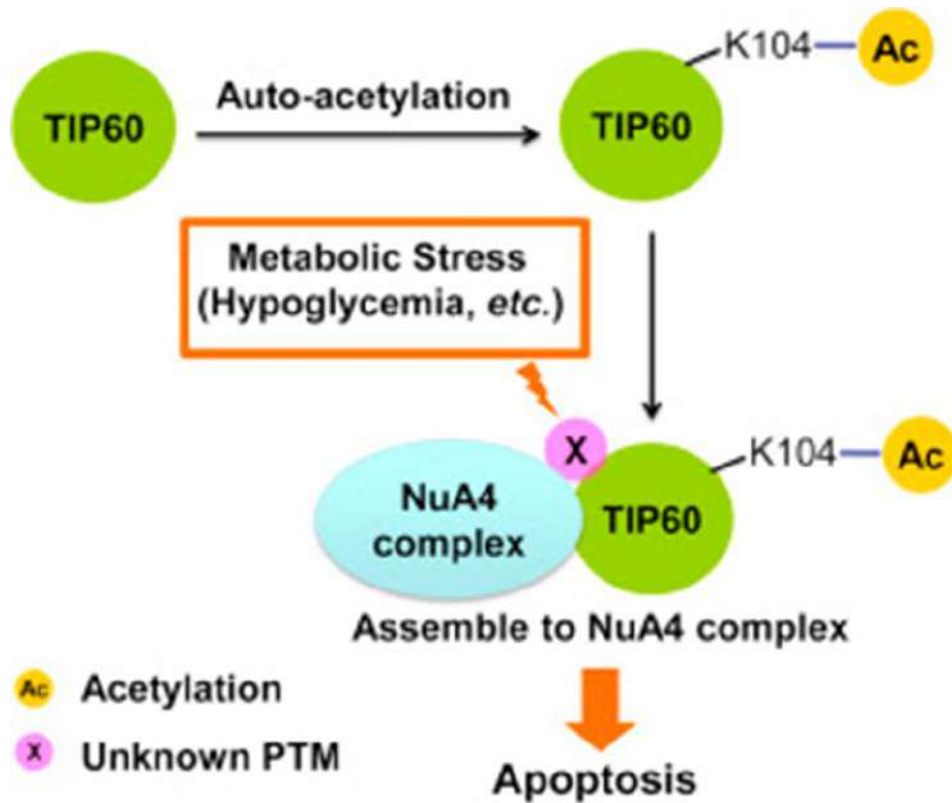


Figure 19 : L'Auto-acétylation de TIP60. L'auto-acétylation de TIP60 sur sa lysine 104 permet son intégration dans le complexe NUA4. Figure tirée de (Fang et al., 2018).

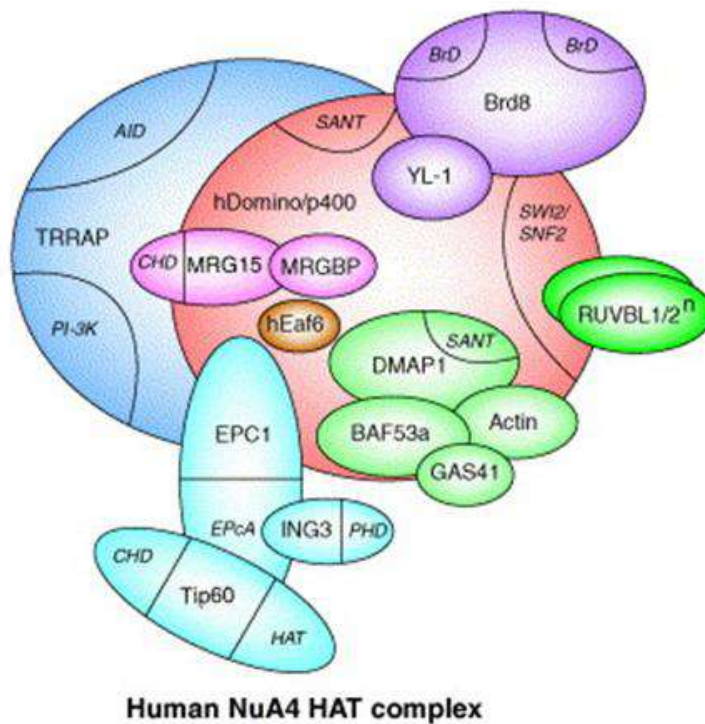


Figure 20 : Structure du complexe NUA4. Le complexe NUA4 est un ensemble de protéines ayant différentes activités telles que l'acétylation par TIP60 et l'ATPase par p400. Image tirée de (Doyon and Cote, 2004).

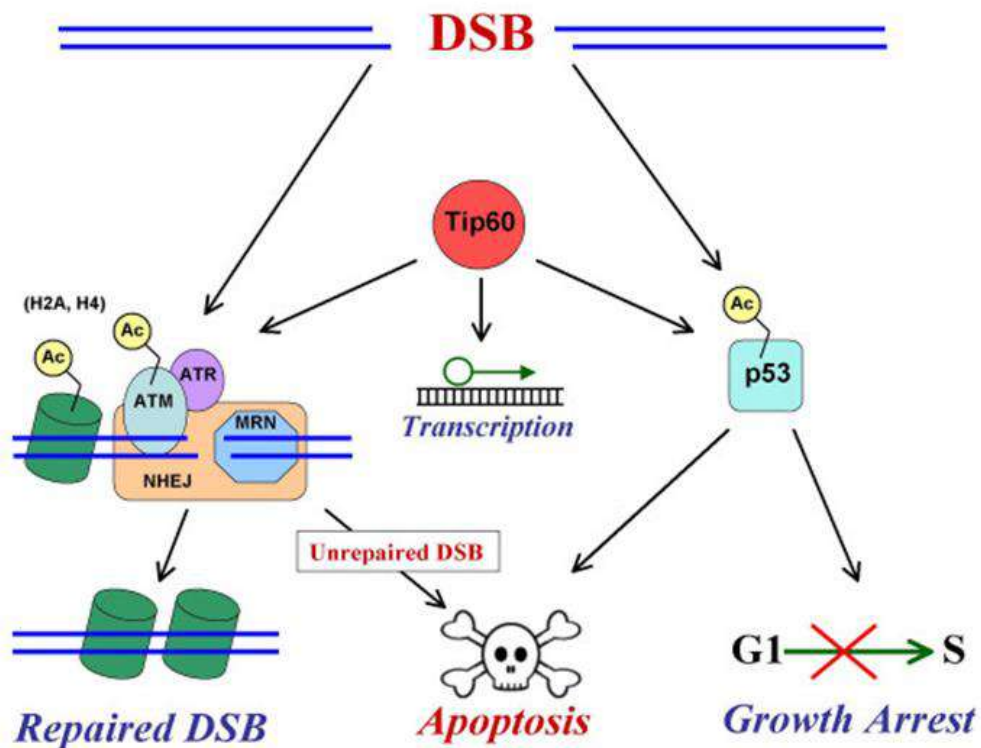
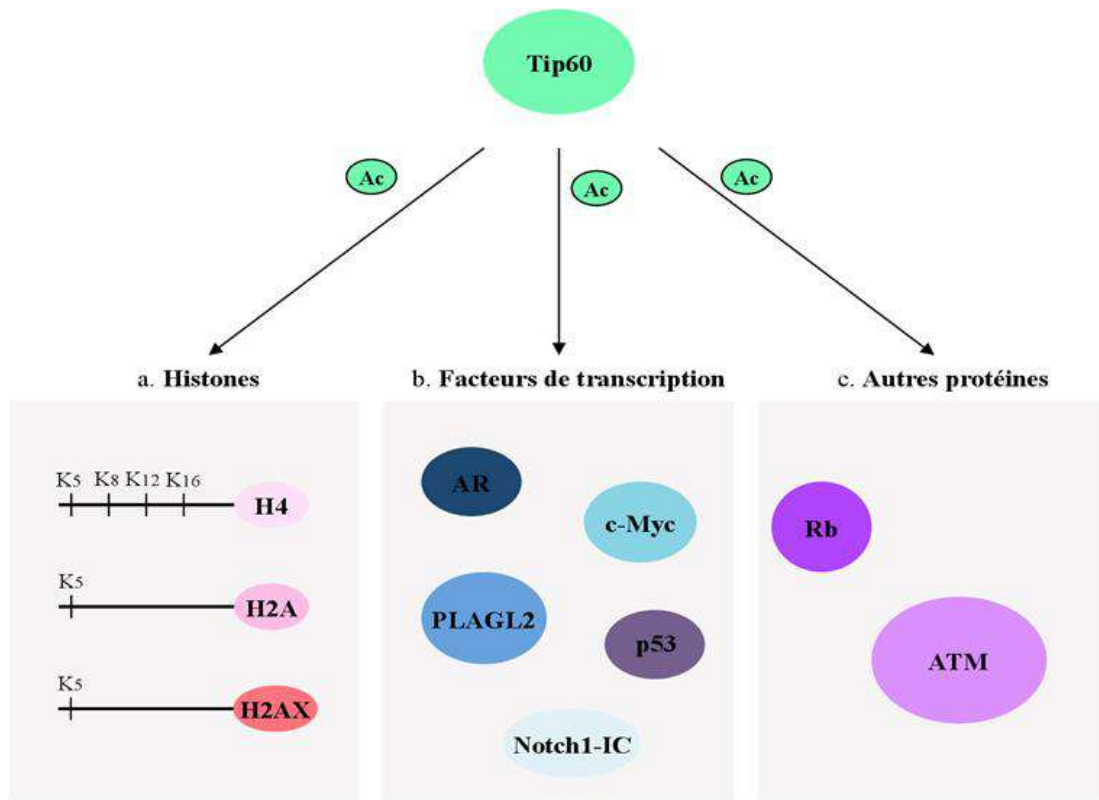


Figure 21 : Représentation des différentes cibles de TIP60 et son implication dans plusieurs processus cellulaires. TIP60 acétyle les histones, les facteurs de transcription et des protéines non-histones. TIP60 est impliquée dans la réparation de l'ADN, l'apoptose, la transcription des gènes et la croissance cellulaire. Image tirée de (Mattera et al., 2009; Nunnari et al., 2008).

- *TIP60 dans le processus de carcinogénèse*

La cancérogenèse peut être définie comme l'acquisition de certaines propriétés par des cellules qui les rendent immortelles et leur confèrent une capacité de prolifération illimitée. La polypléidie et la dérégulation de l'expression des gènes sont deux caractéristiques de la cancérogenèse, ces deux processus sont le plus souvent associés à une dénaturation de la chromatine et surtout de l'hétérochromatine, qui constitue le plus souvent la signature des tumeurs solides. Des protéines telles que les méthyl-transférases, les déméthylases, les histones acétyl-transférases et les désacétylases permettent la stabilité des différentes structures de la chromatine. De nombreuses études ont montré que l'expression de ces protéines est modifiée lors de l'initiation de la carcinogénèse.

De nombreuses études ont montré que lors de l'installation de la carcinogénèse le squelette de maintien de l'hétérochromatine SUV39H1/H3K9me3/HP1 est perdu. SUV39H1 étant une méthyl-transférase tri-méthylant histone H3 sur sa lysine 9 ce qui permet à la protéine HP1 de se fixer et garder compacte l'hétérochromatine. Cette perte serait compensée par la formation d'un nouveau squelette formé de TIP60, P400 et H4K12ac qui empêchera la déstructuration du paysage chromatinien.

Dans cette mini-revue on a montré le rôle de TIP60 dans le maintien de l'intégrité chromatinienne en formant le squelette TIP60/P400/H4K12ac et en faisant intervenir des protéines à bromo-domaines telles que les BRD2/4.

Publication 5:

Exciting History of Tip60 and Its Companions in Carcinogenesis Across the Heterochromatin Landscapes. **Idrissou M**, Rifaï K, Daures M, Penault-Llorca F, Bignon YJ, Bernard-Gallon D. OMICS. 2018 Sep;22(9):626-628.

Exciting History of Tip60 and Its Companions in Carcinogenesis Across the Heterochromatin Landscapes

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To the Editor:

Carcinogenesis can be defined as the acquisition of certain properties by cells that make them immortal and provide them with unlimited proliferation capacity. Polyploidy and deregulation of gene expression are two characteristics of carcinogenesis. These two processes are most often associated with a denaturation of chromatin and especially that of heterochromatin, which constitutes the signature of most solid tumors (Yuen et al., 2005).

Heterochromatin is a tightly packed form of DNA. It is comprised of DNA wrapped around octamers of histones called nucleosomes. These nucleosomes are tightened together and maintained by the action of several proteins and histone marks (Fig. 1A). Proteins such as methyl transferases, demethylases, histone acetyltransferases (HATs), and deacetylases allow the stability of different chromatin structures.

The methyl transferase suppressor of variegated 3–9 homolog 1 (SUV39H1), through the trimethylation of N-terminal tail of histone H3 on lysine 9 (H3K9me3), allows the binding of the heterochromatin protein 1 (HP1) that will preserve the structure of the heterochromatin (Fig. 1A).

In many cancers such as breast and colorectal cancers, it has been shown that there is a loss of SUV39H1 and the H3K9me3 biomarkers. However, it was shown that Tip60 intervened at the level of heterochromatin to compensate for the absence of SUV39H1. Tip60 would acetylate histone H4 on its lysine 12 (H4k12ac) at the heterochromatin, which would allow proteins with bromodomains such as bromodomain-containing protein 2 or 4 (BRD2/4) to settle and thus maintain this structure (Fig. 1A). This TIP60 action could involve the Tip60 complex, with the intervention of ATPase P400 and balance of the Tip60/p400 ratio (Mattera et al., 2009).

Acetyltransferase Tip60 is a protein capable of attaching an acetyl group to other proteins, such as transcription factors and histones. This acetylation activates proteins, and in the case of histones, it has always been associated with the opening of chromatin allowing gene expression (Fig. 1A).

Recent studies have shown the presence of histone acetylation H3 and H4 in the heterochromatin. Acetylation of histone H4 lysine 12 in heterochromatin would maintain a basal transcription level and a plasticity of the heterochromatin by binding bromodomain proteins BRD2 and BRD4 to this mark (Fig. 1B).

Indeed, heterochromatin is the condensed part of the chromosomes, where genes are fewer or not expressed. It is also the genomic location where most of the oncogenes are nested. A skeleton composed of SUV39H1-H3K9me3-HP1 under normal conditions most often maintains this structure (Fig. 1B). Grezy et al. showed that when this normal compaction pathway is defective, HAT Tip60 is recruited on heterochromatin where it mediates histone acetylation H4K12 and BRD2/4 binding. This new pathway is the last step for the denaturation of heterochromatin (Fig. 1B).

In most cancers, there is variation in the transcriptional and/or translational expression of Tip60. For example, in colorectal and breast cancers, there are transcriptions and translations under expression of Tip60, followed by a loss of the Tip60-H4K12ac-BRD2/4 pathway. This leads the cells to a process of carcinogenesis with a loss of the chromosomal architecture and including polyploidy (Fig. 1B).

The presence of Tip60 at the heterochromatin is even more possible, given the presence of P400 in the heterochromatin to incorporate the histone variant H2Az in the G1 phase in the normal cell (Fig. 1A). P400 is an ATPase involved in chromatin remodeling through its SNF2 catalytic domain common to all chromatin remodeling ATPase (Fuchs et al., 2001). The enzymatic activity of this domain allows incorporation of the histone variant H2Az instead of the histone H2A (Fig. 1A).

Indeed, Grezy et al. observed a denaturation of heterochromatin in SUV39H1^{1/2-/-} cells after inhibition of P400 by small interfering RNA similar to the inhibition of TIP60, which suggests that Tip60 is being recruited within its complex by P400. However, other studies conducted on human fibroblasts have shown that the depletion of P400 or histone HA2z variant by short hairpin RNA could induce heterochromatin formation that would be associated with cellular senescence (Gévry et al., 2007). Under normal conditions, P400 may allow chromatin release (Fig. 1A). However, the loss of the SUV39H1-H3K9me3-HP1 skeleton allows the recruitment of Tip60 and the implementation of the new heterochromatin maintenance pathway Tip60-H4K12ac-BRD2/4 (Fig. 1B).

Mattera et al. (2009) have shown another link between Tip60, P400, and carcinogenesis. They demonstrated that the Tip60/

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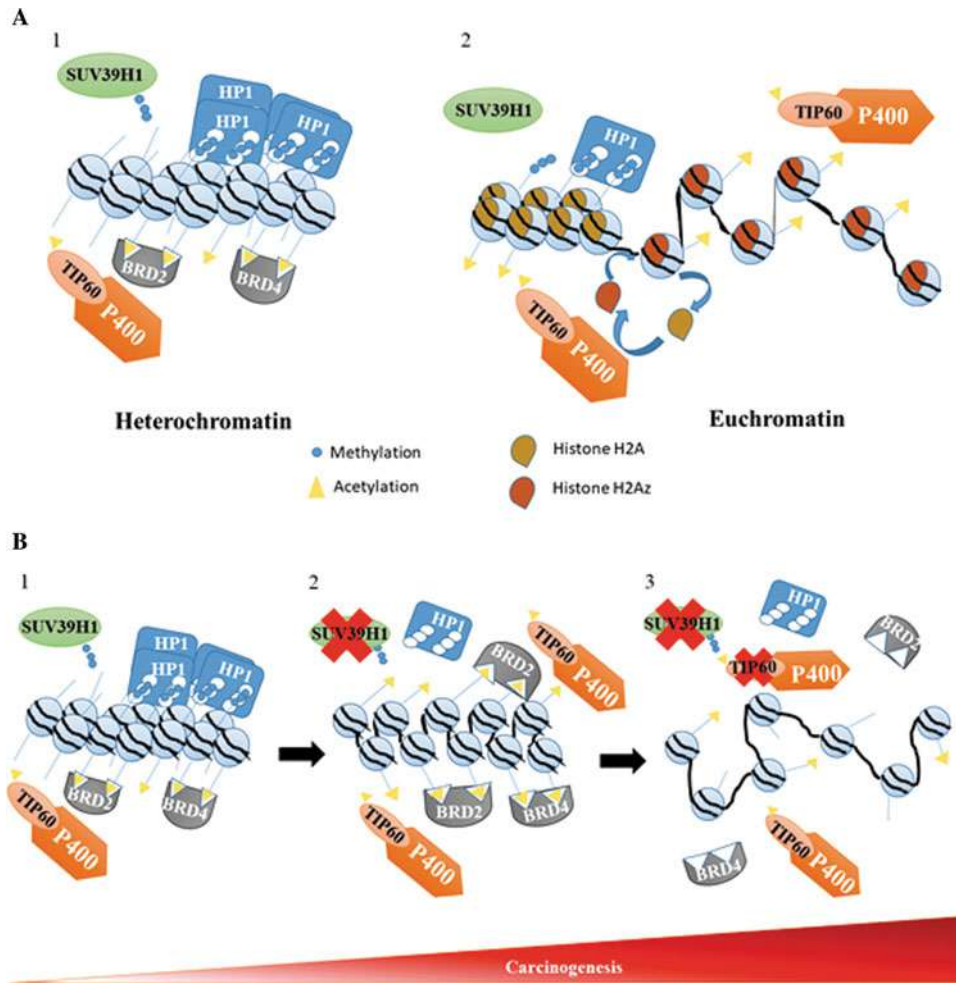


FIG. 1. (A) (1) The heterochromatin representing the condensed chromatin with its skeleton SUV39H1-H3K9me3-HP1 and the presence of the proteins BRD2/4 fixed on the histone acetylation H4 posed by Tip60. (2) The euchromatin representing the chromatin released through acetylation and demethylation of histones and the change of histone H2A by histone variant H2Az through P400. (B) The evolution of heterochromatin in the carcinogenesis process. (1) The heterochromatin in normal conditions. (2) Installation of carcinogenesis with the loss of skeleton SUV39H1-H3K9me3-HP1 through the loss of SUV39H1 but maintenance of structure via new pathway Tip60-H4K12ac-BRD2/4. (3) Advanced cancer stage with heterochromatin denaturation, decrease in Tip60, and loss of the new pathway Tip60-H4K12ac-BRD2/4.

P400 ratio is a key factor toward inducing carcinogenesis. It was shown that the variation in the Tip60/P400 ratio in favor of P400 resulted in a decrease in Tip60 expression and carcinogenesis progression, whereas a variation in this ratio in favor of Tip60 leads to apoptosis of tumor cells. Indeed, it has been shown that P400 is capable of binding its SANT domain to the Tip60 HAT domain, thus P400 inhibits the acetyltransferase activity of Tip60 (Park et al., 2010). This suggests that P400 would be a Tip60 regulator when the latter is underexpressed, as in the case of colorectal or breast cancer (Mattera et al., 2009).

Taken together, we shall also note that Tip60 is a pleiotropic protein involved in several physiological processes, such as transcriptional regulation, DNA repair, cell migration, invasion, alteration of chromatin structure, and genomic instability (Judes et al., 2015). Its involvement in these different processes and the small trip to the land of heterochromatin makes Tip60 an ideal target for a deeper understanding of carcinogenesis and a worthwhile therapeutic target for cancer therapeutics and diagnostic innovation.

Acknowledgment

K.R. is the recipient of a grant from the Hariri Foundation.

Author Disclosure Statement

The authors declare there are no competing financial interests.

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Abbreviations Used

- BRD2/4 = bromodomain-containing protein 2 or 4
H2Az = variant of histone H2A
H3K9me3 = histone H3 lysine 9 trimethylation
H4K12ac = histone H4 lysine 12 acetylation
HA2 = histone A2
HAT = histone acetyl transferase
HP1 = heterochromatin protein 1
P400 = E1A-binding protein p400
SANT = Swi3-Ada2-NCor-TFIIB
SNF2 = sucrose nonfermenting 2
SUV39H1 = suppressor of variegated 3–9 homolog 1
Tip60 = Tat interactive protein 60 kDa

Conclusion de la publication 5:

Dans cette mini-revue nous avons suivis TIP60 dans son voyage au niveau des différentes structures de la chromatine lors de l'installation et de la progression du cancer tel que le cancer colorectal. Ce voyage nous a permis de suivre le rôle de TIP60 dans le remodelage de la chromatine à travers son activité acétyl-transférase sur la lysine 12 de l'histone H4. Cette acétylation qui permet aux protéines BRD2/4 de se fixer sur la chromatine et ainsi maintenir la structure de l'hétérochromatine qui se relâche au fur et à mesure que l'on avance dans le cancer. Ce relâchement est dû à la perte du squelette de l'hétérochromatine SUV39H1/H3K9me3/HP1. La présence de TIP60 au niveau de l'hétérochromatine serait possible grâce au recrutement de P400 qui fait partie du même complexe que TIP60.

- *TIP60 dans le développement du cancer du sein*

Nous avons déjà vu que TIP60 faisait partie d'un mécanisme pléiotropique agissant sur des fonctions cellulaires variées et impliquées dans le développement des cancers et surtout le cancer du sein.

Connaissant l'impact du micro-environnement sur le développement des cancers et la présence des tissus adipeux autour de la plupart des tumeurs. Dans cette mini-revue, on s'est focalisés sur une fonction cellulaire modulée par TIP60 qui est l'adipogenèse et son impact sur le développement du cancer du sein.

Publication 6:

TIP60 Histone Acetyltransferase in Adipose Tissue: Possible Linkages with Breast Cancer Development? Judes G, Dubois L, Rifaï K, Daures M, **Idrissou M**, Bignon YJ, Penault-Llorca F, Bernard-Gallon D. OMICS. 2017 Nov;21(11):684-686.

TIP60 Histone Acetyltransferase in Adipose Tissue: Possible Linkages with Breast Cancer Development?

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To the Editor:

A complex interaction exists between cancer cells and their microenvironment that contributes to cancer progression. Interestingly, several cancers affecting kidney, stomach, ovary, and breast grow near adipose tissues. Although the adipose tissue serves as an energy storage and an endocrine organ that regulates systemic energy and metabolic homeostasis, adipocytes can also modify the tumor microenvironment.

Various studies on breast cancer cell lines have demonstrated that adipocyte factors such as adipokines, cytokines, and growth factors promote survival, adhesion, growth, and migration of tumor cells. Different processes are involved in regulation of adipocyte differentiation and adipogenesis such as the cascade of transcription factor (cytosine-cytosine-adenosine-adenosine-thymidine enhancer-binding proteins and peroxisome proliferator-activated receptor γ [PPAR γ]) or histone modifications. Transcription factors can be subject to post-translational modifications such as phosphorylation, acetylation, sumoylation, and ubiquitination, which regulate different aspects of their function.

Histone acetylation, mediated by histone acetyltransferase (HAT), has a significant role in adipogenesis and particularly with TIP60. The latter is a member of the acetyltransferase MYST (MOZ, Ybf2/Sas3, Sas2, TIP60) family involved in pleiotropic mechanisms and, more specifically, in breast cancer development. TIP60 is implicated in a diverse range of cellular functions, including mRNA synthesis and DNA repair. However, the involvement of TIP60 in adipocyte differentiation and function is unclear. In this letter, we underscore the idea that there might exist a possible connection between TIP60, adipogenesis, and breast cancer.

In vivo, the heterozygous inactivation of TIP60 induces a 50% reduction of TIP60 mRNA, but a compensation by the second allele allows to keep the TIP60 protein level stable. The decrease of TIP60 appears to affect different metabolic parameters. TIP60 can interact with various actors of adipogenesis. The transcription factor PPAR γ plays important roles in differentiation, maintenance, and function of adipocytes. TIP60 interacts with the activation function 1 region of PPAR γ and the acetyltransferase activity of TIP60

contributes to the coactivation of PPAR γ . Its recruitment on PPAR γ target genes in 3T3-L1 adipocytes supports the idea that TIP60 is an important adipogenic factor (van Beekum et al., 2008).

In breast cancer, the level of PPAR γ staining is strongly positively correlated with patients' survival. PPAR γ activity is regulated by post-translational modifications including acetylation. The K154/155 acetylation site of PPAR γ is a major actor of cell lipid synthesis in breast cancer. An alteration of PPAR γ acetylation leads to a decrease of lipogenic differentiation and mRNA expression of lipogenic genes in breast cancer cells (Fig. 1A).

Ubiquitin-specific protease 7 (USP7) is a deubiquitinate enzyme essential in adipogenesis, which regulates the ubiquitination of many proteins such as TIP60. Indeed, USP7 deubiquitinates TIP60 and results in an increase of the TIP60 protein level. Cell cycle genes such as *Cdc20* and *Aurka* are involved in adipogenesis mechanisms, and are coregulated by TIP60 and USP7 (Fig. 1B).

Moreover, TIP60 and USP7 play a role in the early steps in adipogenesis (Gao et al., 2013). In breast cancer, USP7 is upregulated and plays an important role in the tumor development by deubiquitination of two tumor suppressors, PTEN and p53. USP7 expression is associated with lymph node metastasis and a poor prognosis. This USP7 overexpression is positively correlated with the expression of histone demethylase plant homeodomain finger-containing protein 8 (PHF8) and cyclin A2. USP7 physically interacts with PHF8 and allows its stabilization and upregulates cyclin A2. USP7 promotes breast cancer development through its action on these two proteins (Wang et al., 2016). In addition, USP7 participates in the epithelial mesenchymal transition through deubiquitination of transcription factor Snail, increasing breast cancer cell invasion and metastasis development (Fig. 1B).

GPR50 (G protein coupled receptor 50) belongs to the family of G-protein-coupled receptor of melatonin receptors and is involved in energy homeostasis. In fact, in the brain, GPR50 expression is linked to energy status being decreased by both fasting and high-fat diet feeding. Different metabolic alterations, such as reduced fat accumulation and elevated

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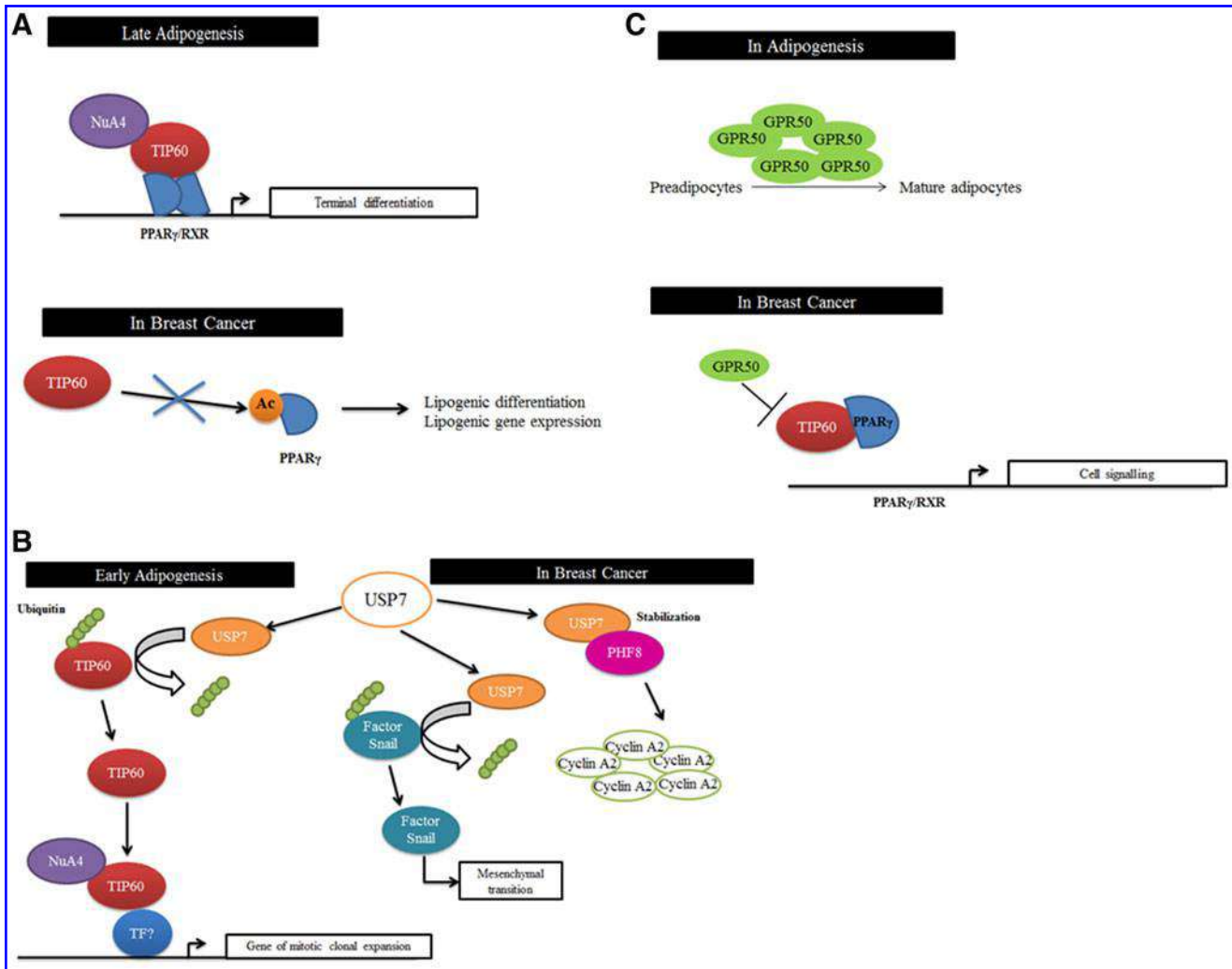


FIG. 1. Link between TIP60 and essential actors in adipogenesis and breast cancer. (A) The TIP60/PPARX complex regulates the expression of adipocyte differentiation genes (van Beekum et al., 2008). In breast cancer, the expression level of TIP60 is decreased and might lead to modification of PPARX acetylation inducing alteration in adipogenesis. (B) The deubiquitinase USP7 prevents the TIP60 degradation and the NuA4/TIP60 complex regulates the expression of mitotic clonal expansion genes such as *Cdc20*, *Aurka* (Gao et al., 2013). In breast cancer, factor Snail is protected from proteasomal degradation by the deubiquitinase USP7 and plays a role in epithelial mesenchymal transition. By interaction, USP7 stabilizes the PHF8 histone demethylase and induces an upregulation of cyclin A2 (Wang et al., 2016). This mechanism promotes tumorigenesis. (C) A progressive expression of GPR50 is necessary to adipogenesis and allows formation or storage of lipids in mature adipocytes. The TIP60/PPARX signaling could be blocked by the binding of GPR50 to TIP60 and altered the TIP60 regulation of nuclear hormone receptor transcriptional activity in breast cancer (Li et al., 2011).

metabolic rate, were shown in *Gpr50*^{-/-} mice. In humans, polymorphisms of *Gpr50* were associated with elevated circulating triglyceride and cholesterol levels. During the differentiation process of 3T3-L1 preadipocyte cell line, an increase of *Gpr50* expression was identified and led to a higher level of its expression in mature adipocytes, needed to the formation or storage of lipid (Fig. 1C).

Besides playing a role in adipogenesis, GPR50 interacts with TIP60 in zinc finger motif, HAT, and LXXLL motif regions. The association between these two proteins could lead to an alteration of TIP60-mediated coactivation of PPAR γ (Li et al., 2011). The role of TIP60 activity for nuclear hormone receptor regulation, such as androgen and estrogen

receptors, is well established. GPR50 action on TIP60-mediated nuclear hormonal receptor transcriptional expression seems to differ according to the type of nuclear hormone receptors. In MDA-MB-231 breast cancer cell line, a stable GPR50 overexpression activates a transforming growth factor beta pathway and leads to a proliferation inhibition and an activation of cell migration. *In vivo*, GPR50 blocks breast tumor development.

Taken together, TIP60 is involved in several adipogenesis mechanisms through its interaction with three important proteins: PPAR γ , USP7, and GPR50. These actors act also in different processes of breast cancer development. These pieces of observations collectively highlight the concept

that TIP60 could be a key component in breast cancer adipose tissue.

Acknowledgments

We thank the ligue contre le cancer-comité du Puy-de-Dôme for research support. K.R. was funded by Hariri Foundation.

Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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Abbreviations Used

AF1 = activation function 1
 GPR50 = G protein coupled receptor 50
 HAT = histone acetyltransferase
 MYST = MOZ, Ybf2/Sas3, Sas2, TIP60
 PHF8 = plant homeodomain finger-containing protein 8
 PPAR γ = peroxisome proliferator-activated receptor γ
 USP7 = ubiquitin-specific protease 7

Conclusion de la publication 6:

Dans cette mini-revue, on a pu mettre en lumière des liens entre TIP60 et l'adipogenèse. En effet, TIP60 interagit avec certains acteurs de l'adipogenèse tels que PPAR γ nécessaire à la différenciation des adipocytes. TIP60 est capable de co-activer PPAR γ en l'acétylant, ce qui fait de TIP60 un facteur adipogène important. Grâce à la protéine USP7, TIP60 est aussi capable d'activer l'expression de gènes tels que *Cdc20* et *Aurka* qui sont impliqués dans l'adipogenèse. Toutefois, les protéines comme GPR50 sont capables d'inhiber cette co-activation de PPAR γ par TIP60. Ces différents mécanismes de l'adipogenèse permettent de suggérer que TIP60 pourrait être un élément clé du tissu adipeux dans le cancer du sein.

- *Regulation de l'acétylation des histones H3 et H4 par TIP60 dans le cancer du sein*

Grâce à la littérature et aux travaux menés dans notre laboratoire sur l'acétylation des histones H3 et H4 par TIP60 dans le cancer du sein, des signatures épigénétiques des différents types moléculaires de cancers du sein ont été mises en évidence. Par exemple, on a pu voir que la marque H3K4ac était exprimée différemment dans les différents types moléculaires de cancer du sein. Cette différence d'expression de la marque H3K4ac n'explique pas son rôle dans le cancer du sein. Le plus souvent associée à l'ouverture de la chromatine et à l'expression des gènes, la marque H3K4ac est aussi observée dans des régions de la chromatine compactée (hétérochromatine) réprimant l'expression des gènes, telles que dans la région péri-centromérique chez la levure *S. pombe* (Xhemalce et al 2009). Cette expression dans les différents compartiments de la chromatine nous a poussé à regarder le rôle de cette marque histone posée par TIP60 dans le cancer du sein.

Dans cette étude, on a voulu clarifier le lien entre TIP60 et la marque H3K4ac dans le cancer du sein, mais aussi voir l'enrichissement de cette marque dans les différents compartiments chromatiniens et son expression dans les différents types moléculaires de cancer du sein et enfin voir l'effet d'une déplétion de TIP60 sur son expression.

Pour cela, une ChIP-qPCR a été réalisé pour mesurer l'enrichissement de la marque H3K4ac sur l'hétérochromatine et l'euchromatine dans 22 tumeurs du sein et leurs tissus sains adjacents. Puis nous avons effectués une ChIP-re-ChIP ciblant à la fois H3K4ac et TIP60 sur des gènes fortement enrichis en H3K4ac dans 54 tumeurs du sein et leurs tissus sains adjacents. Enfin, une déplétion de TIP60 par Sh-RNA dans les différentes lignées continues de cancer du sein TNBC et Luminales (MDA-MB-231 et MCF-7), qui ont ensuite été injectées en suite dans des souris athymique Balb-c pour mimer un état de sous-expression de TIP60 et permettre de confirmer l'acétylation de H3K4 par TIP60 mais aussi de voir le rôle de la marque dans la progression tumorale.

Publication 7:

TIP60: an actor in acetylation of H3K4 and tumor development in breast cancer. Judes G, Dubois L, Rifaï K, **Idrissou M**, Mishellany F, Pajon A, Besse S, Daures M, Degoul F, Bignon YJ, Penault-Llorca F, Bernard-Gallon D. *Epigenomics*. 2018 Nov;10(11):1415-1430.



TIP60: an actor in acetylation of H3K4 and tumor development in breast cancer

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Aim: The acetyltransferase TIP60 is reported to be downregulated in several cancers, in particular breast cancer, but the molecular mechanisms resulting from its alteration are still unclear. **Materials & methods:** In breast tumors, H3K4ac enrichment and its link with TIP60 were evaluated by chromatin immunoprecipitation-qPCR and re-chromatin immunoprecipitation techniques. To assess the biological roles of TIP60 in breast cancer, two cell lines of breast cancer, MDA-MB-231 (ER-) and MCF-7 (ER+) were transfected with shRNA specifically targeting TIP60 and injected to athymic Balb-c mice. **Results:** We identified a potential target of TIP60, H3K4. We show that an underexpression of TIP60 could contribute to a reduction of H3K4 acetylation in breast cancer. An increase in tumor development was noted in sh-TIP60 MDA-MB-231 xenografts and a slowdown of tumor growth in sh-TIP60 MCF-7 xenografts. **Conclusion:** This is evidence that the underexpression of TIP60 observed in breast cancer can promote the tumorigenesis of ER-negative tumors.

First draft submitted: 4 January 2018; Accepted for publication: 13 July 2018; Published online: 16 October 2018

Keywords: acetyltransferase TIP60 • breast cancer • H3K4ac

The histone acetyltransferases (HATs) fall into three main groups, the largest and most diverse of which, namely the MYST family, includes MOZ, YBF2, MOF and TIP60 [1]. TIP60 (also known as KAT5) belongs to a multiprotein complex called human NuA4 complex, composed of up to 16 subunits. The acetyltransferase TIP60 acetylates not only histones H2A, H4 and H3 *in vitro*, but also nonhistone proteins such as p53, c-Myc and ATM [2] and is involved in diverse biological processes, such as DNA damage response signaling, apoptosis, cell cycle progression and transcriptional regulation [3–8]. TIP60 is also a known nuclear receptor coactivator. It binds to the ligand-binding domain of the androgen receptor and enhances hormone-dependent activation of genes by several steroid hormone receptors, including ER α [9]. Because of its pleiotropic functions, the role of TIP60 in tumorigenesis mechanisms is complex and it can act as an oncogene or tumor suppressor according to cancer type [10].

The histone acetyltransferase TIP60 has been shown to be underexpressed in many human cancers of ranging origins, including breast cancer [11–15]. Gorrini *et al.* have shown that TIP60 functions as a haploinsufficient tumor suppressor in a model of tumor induction in mice (E μ -myc transgenic mice), providing a causal link between its underexpression and tumorigenesis [13]. In breast carcinoma, the frequency of TIP60 loss of heterozygosity was higher in the samples with p53 mutations. Thus, downregulation of TIP60 was associated with p53 mutant and TIP60 acted mainly through the p53 pathway. However, the molecular mechanism of TIP60 downregulation and its involvement in breast cancer development remains unclear.

TIP60 and its associated co-factors contribute to transcriptional control by its acetyl-transferase function and activate target gene promoters. However, some studies have shown TIP60 to be involved in the mediation of transcriptional repression [16,17]. Histone modifications are involved in regulating chromatin compaction or chromatin-decondensing. Classically, acetylation has been defined as a modification involved in active transcription and early studies revealed the association of hyperacetylated histones with actively transcribed genes [18]. However, in *Shi*-

zoozoccharomyces pombe, the acetylation of H3K4 by the Mst1, a homolog of the human TIP60, is involved in heterochromatin formation, where it acts as a switch for recruitment between Clr4 and Swi6 [18,19].

In a previous study, we showed a marked variation in the acetylation of H3K4ac in breast tumors compared with normal breast tissues and the proportion of this histone modification differs according to breast cancer subtypes [20–22]. The function of H3K4ac has received little attention compared with other histone modifications and H3K4 is not known in mammals to be a target of TIP60 *in vivo*.

In this study, we analyzed the link between TIP60 and H3K4ac in breast cancer. We found that H3K4 acetylation was dependent on TIP60 expression, suggesting that TIP60 participates in this acetylation in breast cancer and more surprisingly in heterochromatin formation. To analyze the involvement of TIP60 underexpression in breast cancer development *in vivo*, we induced a knockdown of TIP60 in athymic Balb-c mice by shRNA in two breast cancer cell lines (MDA-MB-231 and MCF-7). We identified different effects of TIP60 depletion according to cell lines presenting opposite hormonal status. Sh-TIP60 MDA-MB-231 had a high growth in tumor development compared with sh-TIP60 MCF-7.

Materials & methods

Patients

The chromatin immunoprecipitation (ChIP) and re-ChIP studies included a cohort of 76 breast tumors with 76 matched normal tissues, diagnosed from 1998 to 2014 in the Centre Jean Perrin. Patients gave their informed written consent to give a sample for research. Tissues were stored in nitrogen in the Centre Jean Perrin Biological Resource Center (CRB No. BB-0033-00075). Patients with neoadjuvant chemotherapy, hormone therapy, radiotherapy and with family background of cancer were excluded from the study.

Co-immunoprecipitation

Tumors and matched normal tissues were lysed in digestion buffer containing protease inhibitor mixture (Nuclear complex Co-IP kit, Active Motif, CA, USA). Protein concentration was determined by the Bradford method: 100 µg of proteins was incubated with 5 µg of H3K4ac antibody (pAb-165-050, Diagenode) at 4°C overnight and antibody/bead complexes were then incubated for 1 h at 4°C on a rotator (Protein G Agarose Columns, Active Motif). The immunoprecipitates underwent western blot analysis with TIP60 antibodies (1:100, goat PoAb, sc-5725 X, Santa Cruz, TX, USA).

Chromatin immunoprecipitation, re-chromatin immunoprecipitation assays & qPCR

ChIP was performed using 3 µg of antibodies (Diagenode): H3K4ac (pAb-165-050), H3K9ac (pAb-103-050), H3K9me3 (pAb-056-050), H3K27me3 (pAb-069-050) and also nonimmune rabbit IgG (negative control) (kch-504-250). The ChIP was carried out by SX-8X[®] Automated System (Diagenode, Seraing, Belgium) and performed as described previously [23].

In re-ChIP assays, DNA-containing magnetic beads and H3K4ac antibodies were incubated in elution buffer H (Auto Histone ChIP-seq Kit, Diagenode) with 10 mM dithiothreitol at 37°C for 30 min to elute the immunoprecipitated DNA after the first ChIP assay. The second ChIP assay was performed with the purified DNA by the second antibody (TIP60, goat PoAb, sc-5725 X, Santa Cruz, TX, USA). The ChIP DNA was amplified by qPCR with the ABI 7900 real-time PCR system.

DNA (5 µl) was then analyzed by qPCR (ABI PRISM 7900HT, Applied Biosystems, CA, USA) in triplicate in a 25 µl reaction volume containing 1 × Taqman Universal PCR Master Mix with the forward and reverse primers (400 nM) and probes (250 nM): *ERS1*, *ERS2*, *EZH2*, *BRCA1*, *P300*, *SRC3* as described in Dagdemir *et al.* [24] and *PGR* [23]. The efficiency of ChIP of particular locus was calculated by qPCR and reported as a percentage of starting material: $\%(\text{ChIP}/\text{Total Input}) = 2^{\wedge}9(\text{Ct}[x\% \text{ input}] - \log[x\% / \log 2] - \text{Ct}[\text{ChIP}]) \times 100\%$.

Cell-culture conditions

MCF-7 and MCF10-A cells were grown in RPMI 1640 and DMEM Ham's F12 medium (Gibco, Thermo Fisher Scientific, CA, USA), respectively, supplemented with 10% fetal bovine serum, 1% L-glutamine and specifically to MCF7, 5 µg/ml of insulin was added. The MCF10-A growth medium was completed with hydrocortisone (0.5 µg/ml), cholera toxin (100 ng/ml) and EGF (20 ng/ml; Sigma). MDA-MB-231 cells were grown in L15 Leibovitz's medium supplemented with 15% fetal bovine serum (ATCC, VA, USA) and 1% L-glutamine. All cells

were grown in 500 μ l of gentamicin. Cells were grown at 37°C and 5% CO₂ (MCF-7, MCF-10A) or without CO₂ (MDA-MB-231).

TIP60 silencing

Mission short hairpin RNA (shRNA)-expressing plasmids were purchased from Santa Cruz Biotechnology (TX, USA). TIP60-specific shRNA constructs in a plasmid (ref: sc-37967-SH, shRNA plasmids consist of a mixture of three to five lentiviral vector plasmids. Each individual plasmid encodes shRNA specific target of 19–25 nt) and control shRNA plasmids (ref: sc-108060 C, sc-108066 C) were individually transfected into MDA-MB-231 and MCF-7 cells to a 50–70% confluency. The transfection mixture included 2 μ g shRNA plasmid DNA and 1 μ l of shRNA plasmid transfection reagent. Cells were incubated for 7 h at 37°C. Culture medium was changed after 24 h. For selection of stably transfected cells, a puromycin selection (3.5 μ g/ml) was performed 48 h after transfection. The culture medium was changed and the transduced cells were cultured in fresh medium.

RT-qPCR

RNAs were extracted from tumor and adjacent normal tissues or from breast cell lines and were promptly homogenized using an Ambion extraction kit. Total RNA was extracted according to the manufacturer's instruction (QIAGEN SA, Courtaboeuf, France). The quality of total RNA was assessed with a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Massy, France) and 1–2 μ g of total RNA was reverse-transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, CA, USA). All the genes and control genes studied were amplified in triplicate using the TaqMan Gene expression PCR Master Mix (Applied Biosystems). Data were collected using an ABI PRISM 7900HT sequence detection system. Relative gene expression was determined using the comparative C_T method. Normalization was performed with 18s RNA. The level of each mRNA gene of each tumor was normalized to mRNA of normal tissues. Fold changes were determined using the 2^(- $\Delta\Delta$ CT) method [25].

Immunoblotting

Immunoblotting to detect specific proteins in cell extracts prepared in RIPA buffer (Thermo Fisher Scientific, Illkirch, France) was performed according to standard procedures; 25–50 μ g of proteins was studied on SDS-PAGE gel, electrotransferred onto nitrocellulose membrane and respectively probed with primary antibody. The following antibodies (Abs) were used: TIP60, Santa Cruz, goat PoAb, sc-5725 X, 1: 100; H3K4ac, Diagenode, ref: pAb-165-050, 1:100; Actin, Millipore, ref:CP01, 1:4000), alkaline phosphatase conjugated secondary Abs (antirabbit IgG, 1:2000, S3738 or Donkey anti-Goat IgG, 1:2000, V115A, Promega). Bands were detected using Western Blue[®] Stabilized Substrate for Alkaline Phosphatase (Promega).

Immunofluorescence

Cultured cells were treated with KCl (0.07 M at 37°C). Cells were centrifuged for 20 min at 770 \times g, and fixed with acetic acid and methanol for 20 min at RT; 250 μ l of nuclei per chamber was deposited on the chamber slide system. The slides were irradiated by UV at 30 cm for 3 h. Nuclei were then rinsed in phosphate-buffered saline (PBS) (1 \times) and incubated with 200 μ l of PBS with 5% bovine serum albumin (BSA) for 1 h at RT. The slides were blocked overnight at 4°C with PBS containing 2% BSA and primary antibody recognizing TIP60 (Santa Cruz Biotechnology, TX, USA; ref: 5725, 1:200) or H3K4ac (Diagenode, ref: pAb-165-050, 1:100). After three washes with PBS for 5 min at RT, samples were incubated for 30 min with a 1:500 dilution (in PBS 1 \times /BSA 2%) of antigoat IgG conjugated to the fluorescent dye (Jackson Immunosearch, ref: 711-095-152) or with antirabbit IgG conjugated to the fluorescent dye (Jackson Immunosearch, Cambridgeshire, UK, ref: 705-166-147). Slides were washed three-times with PBS 1 \times and counterstained with Hoechst (1/500; Thermo Fisher Scientific, CA, USA). Images were taken on a Leica SPE confocal microscope (Nanterre, France).

Xenograft studies

Six-week-old female athymic mice (nu/nu genotype, BALB/c background) [26] were purchased from Janvier Laboratories (Le Genest-Saint-Isle, France) and housed under aseptic conditions. All protocols in this study were approved by the ethics committee (No. 20160105184547091). To test the effect of TIP60 shRNA *in vivo*, 5 \times 10⁶ tumor cells transfected with shRNA TIP60 or control shRNA in 50 μ l of cell culture media and 50 μ l of BD Matrigel (BD Biosciences) were injected in the right flank of mice. Tumor growth and response to therapy were determined once a week by measuring with calipers. Mice were sacrificed at 33 days postinoculation for the

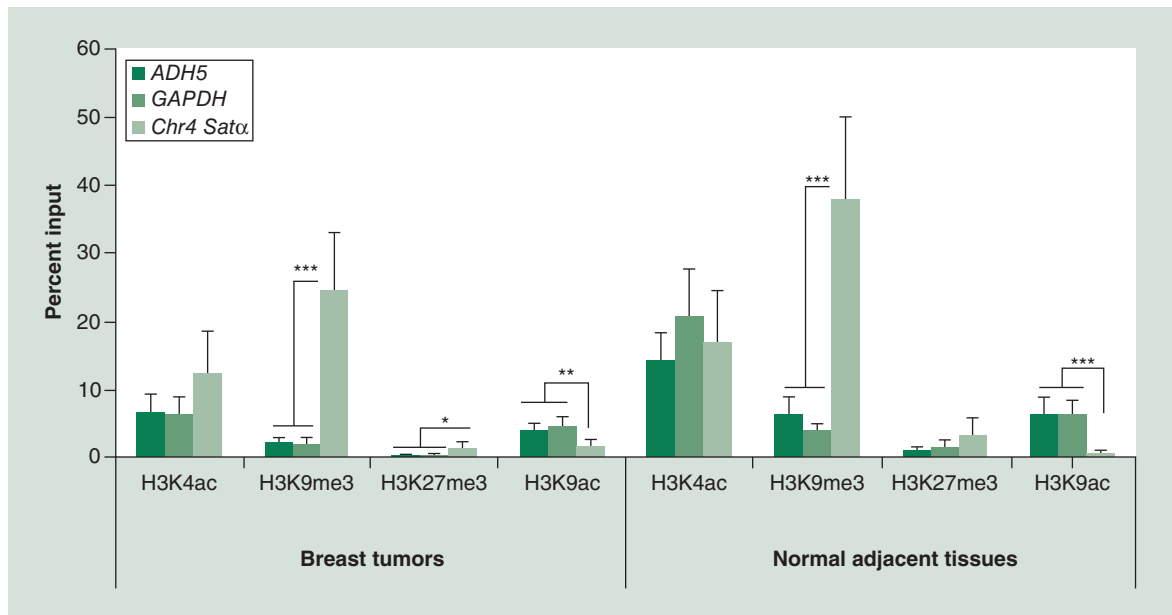


Figure 1. H3K4ac in centromeric heterochromatin and euchromatin in breast cancer. ChIP and qPCR were used to study histone H3 patterns (H3K4ac, H3K9me3, H3K27me3 and H3K9ac) on specific regions of heterochromatin (*Chr4 SATα*) and euchromatin (*ADH5* and *GAPDH*) in breast tumors (n = 22) and their adjacent normal tissues (n = 22); y-axis corresponds to percent input (ChIP/total input) on gene promoters of studied epigenetic marks. Data are expressed as means for each group, and error bars indicate the 95% confidence interval of the mean value. Data were analyzed by Student's t-test.

*p < 0.05; **p < 0.01; ***p < 0.001.

ChIP: Chromatin immunoprecipitation.

MDA-MB-231 cell line and at 48 days postinoculation for the MCF-7 cell line and tumors of these mice were removed and placed in 4% alcohol/formaldehyde/acetic acid. Tumor volumes (mm³) were measured with calipers and calculated using the formula $V = \pi/6(L \times S^2)/2$, where *L* and *S* are the largest and smallest diameters in millimeters.

Immunohistochemistry

Tissues were fixed in alcohol formalin acetic acid and embedded in paraffin; 4 μm sections were mounted on silanized glass slides (Starfrost®, Klinipath, Leuven, B) and dried overnight at 37°C. An Automated Benchmark XT immunohistochemistry instrument (Roche, Bâle, CH) was used to process the slides, which were dewaxed and rehydrated using EZ Prep (Roche). Heat-induced antigen retrieval was performed for 30 min with CC1 (pH 8; Roche). The slides were then incubated at 37°C with primary antibodies: anti-Ki67 (1:25, Dako) for 2 h, an UltraView universal DAB detection kit (Ventana) was used for visualization with a horseradish peroxidase secondary antibody and the signal was amplified using the Ventana amplification kit. Slides were then counterstained with hematoxylin, washed in water and coverslipped with an aqueous Faramount mounting media DAKO (Agilent, CA, USA). For negative control, the primary antibody was replaced by PBS.

Results

Lysine 4 acetylation of histone 3 is found in centromeric heterochromatin in breast cancer

No involvement of H3K4ac in cancer development is reported. In breast cancer, a low enrichment of H3K4ac was found on different gene promoters overexpressed in tumors [20]. This mark could be an actor in heterochromatin formation in breast cancer. To test this hypothesis, we investigated the enrichment of H3K4ac in different regions of heterochromatin (*Chr4 satα*) and euchromatin (*ADH5* and *GAPDH*) by ChIP-qPCR in breast tumors and normal breast tissues (Figure 1). We studied H3K9me3 and H3K27me3 as control marks of heterochromatin and H3K9ac as a control mark of euchromatin. Surprisingly, a similar enrichment of H3K4ac was found in heterochromatin and euchromatin regions. For the control mark enrichments, we achieved the expected results, namely a significant enrichment of H3K9me3 and H3K27me3 in the heterochromatin region compared with

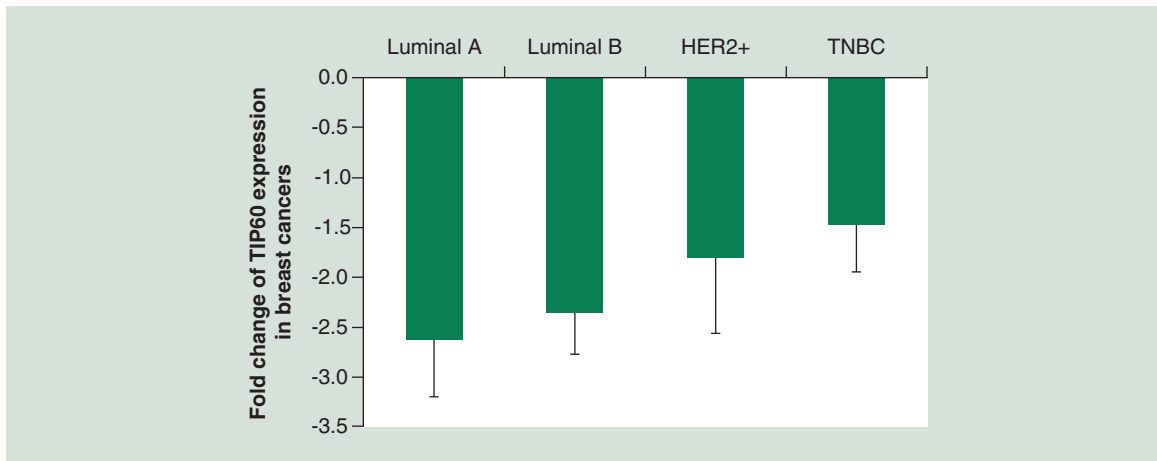


Figure 2. Underexpression of acetyltransferase TIP60 in breast cancer. Graphs showed fold changes ($2^{-\Delta\Delta Ct}$) of gene expression normalized to normal tissue gene expression in breast cancer subtypes (luminal A [n = 7]; luminal B [n = 7]; HER2+ [n = 6]; TNBC [n = 7]); x-axis represents the gene expression fold-change ratios for TIP60 mRNAs. Fold change was used with reference set to 0. Data are expressed as means for each group and error bars indicate the 95% confidence interval of the mean value. TNBC: Triple-negative breast cancer.

euchromatin and conversely for the H3K9ac level, which was increased in euchromatin. In breast tissues, H3K4ac seems to play a direct role not only in the centromeric heterochromatin, but also in the euchromatin.

TIP60 gene expression is downregulated in human breast cancer

The similar enrichment of H3K4ac in heterochromatin and euchromatin raises the question of the role of H3K4ac in regulating gene transcription in breast cancer. These results led us on to study the actor responsible for H3K4 acetylation in breast tissues. In the yeast *S. pombe*, Mst1, a homolog of the human TIP60 acetyltransferase, is responsible for H3K4 acetylation in pericentromeric regions in late S phase and participates in the recompaction of heterochromatin [19]. However, H3K4ac is not known to be a target of TIP60 in humans. Given the importance of TIP60 in cancer, we analyzed the expression of TIP60 in breast carcinoma. By qPCR analysis and $2^{-\Delta\Delta Ct}$ method, we found that *TIP60* mRNA was underexpressed in tumors compared with their adjacent normal tissues (Figure 2). The mRNA of TIP60 presented a 2.36-fold decrease in luminal A, 2.35-fold decrease in luminal B, 1.81-fold decrease in HER2+ and 1.47-fold decrease in triple-negative breast cancer (TNBC) tumors. No significant difference of TIP60 expression was found between breast cancer subtypes. TIP60 underexpression was observed irrespective of breast cancer subtype, suggesting that the decreased TIP60 expression modification occurs early in tumorigenesis.

Co-occupancy of TIP60 & H3K4ac

We opted to focus on luminal breast cancer subtypes because these represent the majority of breast cancers and present a marked underexpression of TIP60. To confirm that TIP60 and H3K4ac can simultaneously co-occupy the same chromatin location, re-ChIP experiments followed by qPCR were performed on regions of a specific gene panel (*BRCA1*, *PGR*, *ESR1*, *ESR2*, *P300* and *EZH2*) involved in breast cancer development. From 54 luminal breast tumors and adjacent normal tissues, a first ChIP was performed with H3K4ac antibody, followed by a second ChIP with TIP60 antibody or IgG as a negative control (Figure 3A). Results confirmed that H3K4ac and TIP60 simultaneously co-occupy the same genomic locations in both tissues. However, this co-occupancy was significantly decreased in breast tumors compared with normal tissues for all the target promoter genes. Using co-immunoprecipitation, we observed a direct interaction between H3K4ac and TIP60 (Figure 3B), suggesting that TIP60 could play a role in acetylation of lysine 4 of histone 3. A physical interaction between TIP60 and H3K4ac exists in breast cancer and seems to be decreased compared with normal tissue.

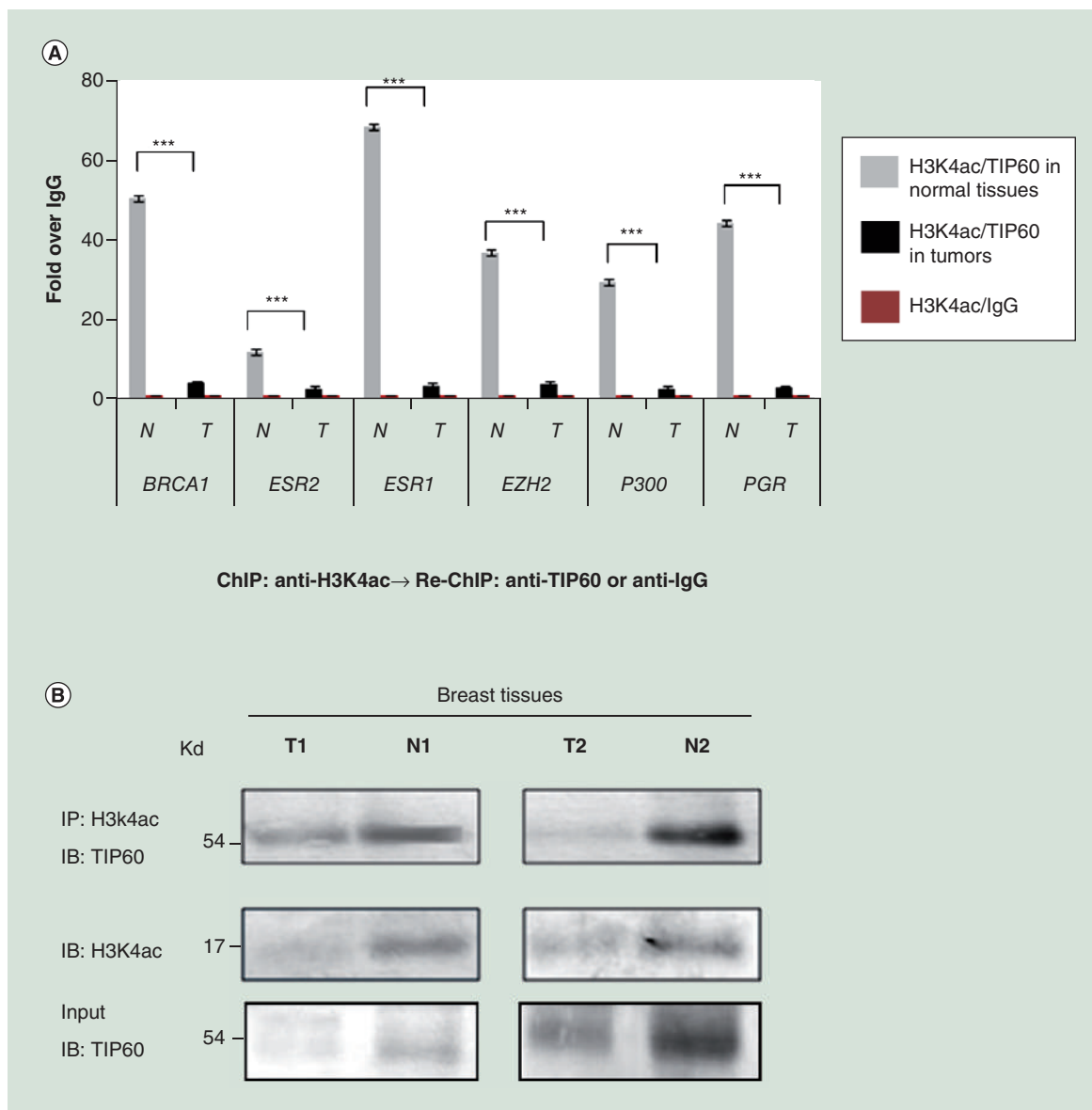


Figure 3. TIP60 shows co-operative binding with H3K4 acetylated in breast cancer. (A) Re-ChIP showed a co-occupancy of TIP60 and H3K4ac in promoter regions of target genes where both proteins bind. Re-ChIP assay was performed with H3K4ac antibody followed by the second immunoprecipitation with TIP60 antibody. The ChIP DNA was amplified by qPCR for the target regions of *BRCA1*, *ESR2*, *ESR1*, *EZH2*, *P300* and *PGR* genes. The fold enrichment of recruitment of H3K4ac and TIP60 at the target regions is relative to respective IgG control. The results are the averages of independent experiments performed on 54 tumors (T) and 54 adjacent normal breast tissues (N). All data are represented as means \pm standard deviation and analyzed by paired Student's t-test. The statistical significance of the difference in the recruitment of H3K4ac and TIP60 between tumors and normal tissues is shown as follows: ***p < 0.001. **(B)** H3K4ac-TIP60 interaction was confirmed by co-immunoprecipitation. Lysates of tissues were immunoprecipitated with H3K4ac antibody on columns (two independent experiments). An immunoprecipitation without antibody was used as negative control. The immunoprecipitates were immunoblotted with TIP60 antibody. ChIP: Chromatin immunoprecipitation.

TIP60 acetylates H3K4 in breast cancer

Because TIP60 interacts physically with H3K4, TIP60 could directly acetylate H3K4. Following this hypothesis, we studied the TIP60 activity in breast cancer *in vitro*. First, we checked two breast cancer cell lines for TIP60 expression by RT-qPCR ($2^{-\Delta\Delta C_t}$ method) and western blot (Figure 4A and B). We opted to focus on MCF-7 and MDA-MB-231 cell lines, because these had different hormonal profiles and represented two subtypes of breast

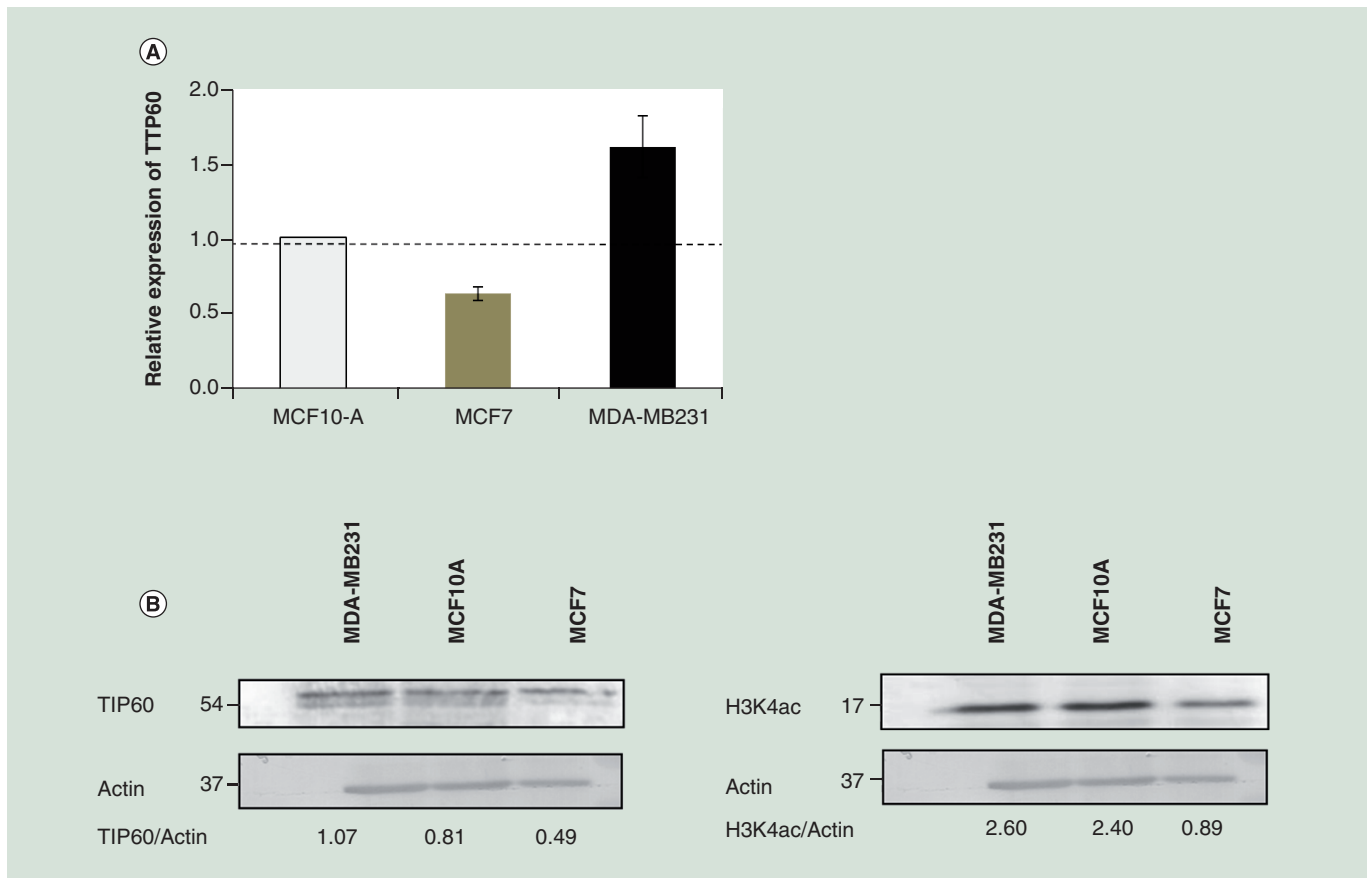


Figure 4. Control of TIP60 expression level in breast cancer cell lines. (A) Analysis of TIP60 mRNA relative quantification ($2^{-\Delta\Delta Ct}$) by RT-qPCR in MDA-MB-231 (TNBC), MCF-7 (luminal) and MCF10-A (control cells) breast cancer cell lines. Graphs showed fold changes of TIP60 expression normalized in relation to TIP60 expression in MCF10-A; x-axis represents the relative fold changes. Fold change was used with reference set to 1. Data were expressed as means for each group, and error bars indicate the 95% confidence interval of the mean value. **(B)** Immunoblots to detect TIP60 and H3K4ac in breast cancer cell lines (MDA-MB-231, MCF10-A and MCF-7). Representative quantification was expressed as relative fold change in protein expression of TIP60 and H3K4ac after normalization to actin density. TNBC: Triple-negative breast cancer.

cancer: luminal (ER+) and TNBC (ER-) respectively. The MCF10-A cell line was taken as a control cell line to study TIP60 expression. We identified two different profiles of mRNA TIP60 expression between MCF-7 and MDA-MB-231 cell lines. A high TIP60 expression was found in MDA-MB-231 compared with MCF10-A, and conversely for MCF7. These results were confirmed by the protein level. These cell lines presented a difference in TIP60 expression, and represent suitable models for investigating the role of TIP60 and consequently its depletion. To investigate the mechanism responsible for the acetylation of H3K4, we transfected MCF-7 and MDA-MB-231 cell lines with scrambled shRNA (sh-control) or shRNA, which specifically targets TIP60 sequences (sh-TIP60) to obtain cells stably underexpressed. TIP60 transfection of cell lines with a sh-TIP60 decreased *TIP60* mRNA level approximately twofold (Figure 5A), and significantly inhibited TIP60 protein expression (Figure 5B) in the two breast cancer cell lines. Importantly, a decrease in acetylation H3K4 staining was found in nuclei after TIP60 inhibition (Figure 6). This result thus showed that the acetylation of H3K4ac is dependent on TIP60 acetyltransferase activity in luminal (MCF-7) and TNBC (MDA-MB-231) breast cancers.

TIP60 contributes to breast tumor development

To investigate effects of TIP60 on breast tumor development *in vivo*, we administered sh-TIP60 transfected to MCF-7 and MDA-MB-231 cells in athymic mice. The two principal subtypes of breast cancer were represented by the MCF-7 cell line for the luminal subtype and the MDA-MB-231 cell line for the TNBC subtype. In TNBC tumors, a TIP60 depletion showed a significant growth-increasing activity compared with cells transfected with

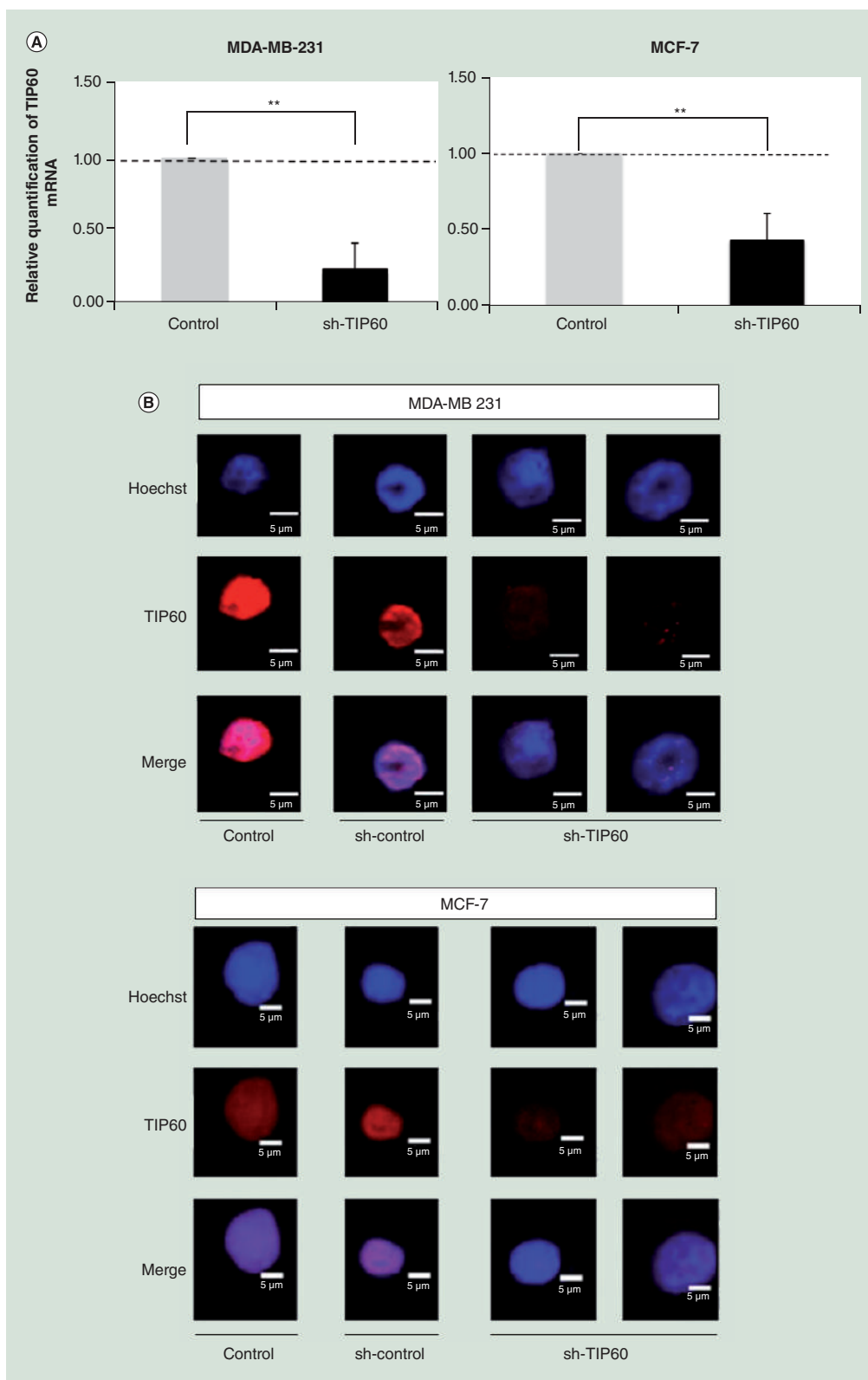


Figure 5. Control of sh-RNA transfection. (A) Analysis of TIP60 mRNA relative quantification ($2^{-\Delta\Delta Ct}$) by RT-qPCR in MDA-MB-231 and MCF-7 cell lines transfected by sh-TIP60 and sh-control. Graphs showed fold changes of TIP60 expression in sh-TIP60 transfected cells normalized TIP60 expression in ShRNA control transfected cells; x-axis represents the relative fold changes in scale. Fold change was used with reference set to 1. Data were expressed as means for each group and error bars indicate the 95% confidence interval of the mean value; data were analyzed by Student's t-test (** $p < 0.01$). **(B)** Representative confocal microscopy images of transfected MDA-MB-231 and MCF-7 cell lines. Endogenous TIP60 (red) was visualized with TIP60-specific antibodies and Hoechst was used to visualize the nuclei (blue). Scale bars indicate 5 μm.

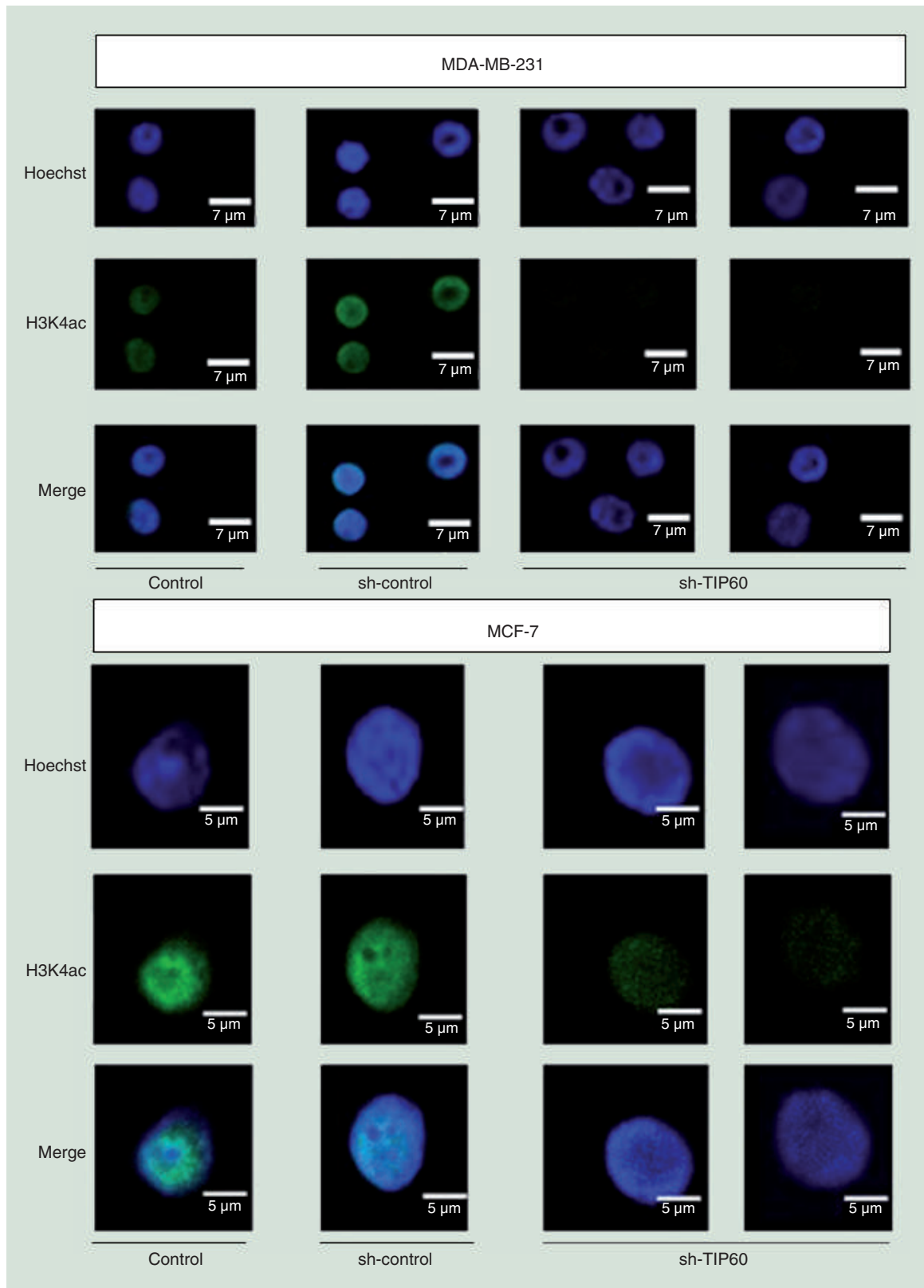


Figure 6. TIP60 inhibition reduces the acetylation of H3K4. Representative confocal microscopy images of transfected MDA-MB-231 and MCF-7 cell lines. H3K4ac modification (green) was visualized using specific antibodies and Hoechst was used to visualize the nuclei (blue). Scale bars indicate 5 and 7 μm.

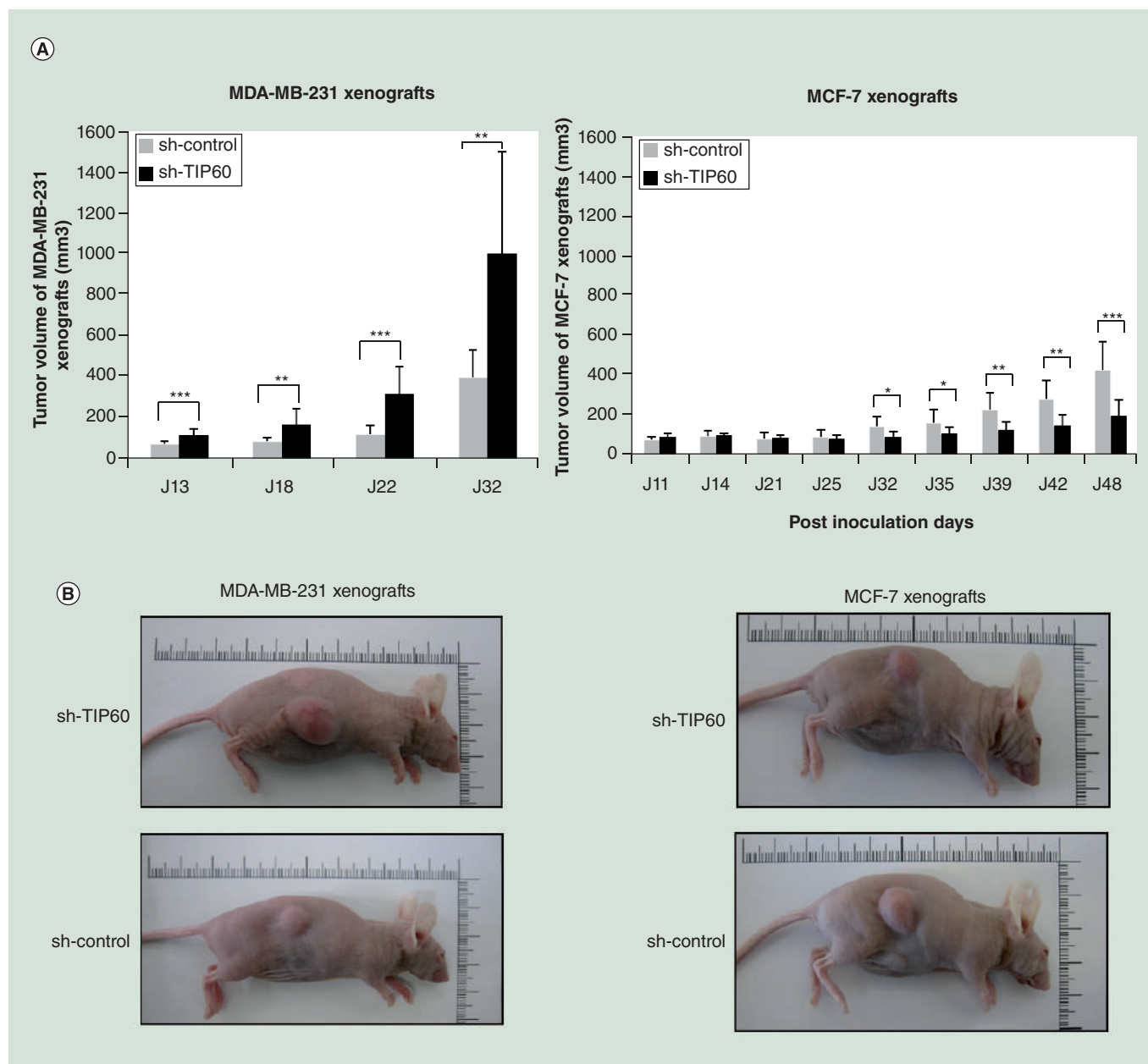


Figure 7. TIP60 depletion in breast cancer xenograft models. (A) Measure of tumor volume in mice after inoculation of MDA-MB-231 cells transfected by sh-TIP60 (n = 11) and sh-control (n = 10), and MCF-7 cells transfected by sh-TIP60 (n = 10) and sh-control (n = 10). Data are expressed as means for each group, and error bars indicate the 95% confidence interval of the mean value. Data were analyzed by paired = TRUE Student's t-test (*p < 0.05; **p < 0.01, ***p < 0.001). TIP60 contributes to development of (MDA-MB-231) triple-negative breast cancer breast xenografts *in vivo*. Conversely, TIP60 depletion reduces development of (MCF-7) luminal breast xenografts *in vivo*. **(B)** Tumor growth in MDA-MB-231 and MCF-7 (sh-TIP60 and sh-control) xenograft models. **(C)** Immunostaining to quantify Ki67 protein and hematoxylin phloxine saffron counterstained showing mitosis (arrows) and cytonuclear atypia (arrowhead) of sh-TIP60 and sh-control MDA-MB-231 and MCF-7 xenografts. Scale bars indicate 100 μ m, and images taken at $\times 20$ magnification. **(D)** hematoxylin phloxine saffron section of sh-TIP60 MCF-7 xenografts showing fibrosis. Scale bars indicate 100 μ m and images taken at $\times 10$ magnification. **(E)** sh-TIP60 and sh-control MDA-MB-231, and MCF-7 xenografts were subjected to ChIP assays using anti-H3K4ac antibodies and IgG antibodies as a control for gene promoters (*BRCA1*, *ERS1*, *ERS2*, *EZH2*, *P300*, *PGR*, *SRC3*). Quantification was performed by qPCR in the immunoprecipitates versus inputs. Values are expressed as fold enrichment over the sh-control samples (standardized relative to 1 for sh-control tumors). Data are expressed as means for each group, and error bars indicate the 95% confidence interval of the mean value. Data were analyzed by paired = TRUE Student's t-test (*p < 0.05; **p < 0.01).

sh-control (Figure 7A and B). Histopathological analysis showed that TNBC tumors arising in sh-TIP60 and sh-

control mice were high-grade mammary adenocarcinomas (grade 3) as determined by the Nottingham histological scoring system and presented a high level of proliferation (Ki-67 = 80%) (Figure 7C). Thus the analysis of cell proliferation in sh-control and sh-TIP60 TNBC tumors showed no significant difference of Ki-67 expression between the two groups. Because TIP60 is required for H3K4 acetylation as previously demonstrated, we examined H3K4ac status on gene panel promoters in sh-TIP60 TNBC tumors (Figure 7E). ChIP studies found a significant decrease in H3K4ac enrichment at the promoter of *ERS1*, *ERS2*, *EZH2*, *P300* and *PGR* in sh-TIP60 TNBC tumors. Thus a depletion of TIP60 *in vivo* had an effect on the H3K4 acetylation patterns of different genes involved in breast cancer development.

Interestingly, we obtained a specific effect of TIP60 depletion in MCF-7 xenografts: the TIP60 depletion showed significant growth delay relative to growth of MCF-7 cells transfected with sh-control (Figure 7A and B), sh-control and sh-TIP60 tumors presented no histological differences. These tumors were high-grade and with 90% of Ki67 expression (Figure 7C). Histopathology nevertheless revealed marked fibrosis in the sh-TIP60 tumors (Figure 7D). Unlike TNBC tumors, the H3K4ac analysis on gene promoters did not show any difference in H3K4ac enrichment between sh-TIP60 and sh-control luminal tumors (Figure 7E).

Discussion

In this study, we identified an important actor, the acetyltransferase TIP60, in breast cancer development and particularly in the acetylation of histone H3. Acetylation of H3K4 had previously been identified by mass spectrometry in mice and humans by Garcia *et al.* [27] and then detected in *S. cerevisiae* [28] and *S. pombe* [19]. This histone modification has been correlated with the activation of transcription in humans by its presence at promoters of active genes [29,30]. In *S. cerevisiae*, Guillemette *et al.* drew the same conclusion; H3K4ac is present on such promoters, and large-scale transcriptional expression analysis in mutated strains for this lysine or different enzymes confirmed this finding [28]. It is noteworthy that the methylation of H3K4 is largely involved in the transcriptional activation in all these models. However, it is surprising that the acetylation and methylation of this same residue both have a transcriptional activator effect. However, in *S. cerevisiae*, as in humans, H3K4ac is found upstream of H3K4me3 at promoters [28]. Thus, the relationship between these two histone marks is unclear. In the yeast *S. pombe*, Xhemalce *et al.* found H3K4ac at the heterochromatin of pericentromere at late S phase and suggested that H3K4ac facilitated eviction of Fld1 and therefore the complex RITS, by affecting its binding to H3K9me2 residues. This eviction would facilitate the recruitment of proteins Swi6 and Fld2 when heterochromatin is reformed [19]. These results suggest that H3K4ac could participate in transcriptional repression in mammals. Few data exist on H3K4ac in humans and particularly in cancer. In a previous study, we analyzed this histone modification on various gene promoters (*ERS1*, *ERS2*, *BRCA1*, *P300*, *PGR* and *EZH2*) in different subtypes of breast cancer and specific H3K4ac enrichments were found in normal tissues and breast tumors [20]. After taking all these factors into consideration, we studied the presence of H3K4ac in constitutive heterochromatin, which formed in centromeric regions. These gene-poor areas are usually made of tandem repetitions, also named satellites, such as Sat- α in Chr4 [31]. We observed an enrichment of H3K4ac not only in the centromeric region, but also in the reference locus of euchromatin (*GAPDH* and *ADH5*). Moreover, the H3K4ac enrichment was more marked in normal breast tissue than in breast tumors. This finding shows that acetylation of H3K4 is decreased in breast cancer. However, no known mechanism explains this depletion of H3K4ac. Accordingly, we focused our research on the acetyltransferase TIP60, whose homologous enzyme MST1 in *S. pombe* is involved in the acetylation of H3K4 [19]. Interestingly, we observed a decrease in TIP60 expression in different subtypes of breast cancer compared with adjacent normal tissues and similar results have already been reported [13,32]. The low enrichment of H3K4ac in breast cancer could be explained by this underexpression of TIP60 acetyltransferase: in breast tumors, a co-localization of TIP60 and H3K4ac was found in the same gene promoters (*ERS1*, *ERS2*, *BRCA1*, *P300*, *PGR* and *EZH2*), which presented a low enrichment of H3K4ac and a physical interaction confirmed the link between these two proteins. To test the involvement of TIP60 in this acetylation, we used a TIP60 shRNA in the breast cancer cell lines and the depletion of TIP60 led to an overall decrease in H3K4 acetylation level. These results suggest that TIP60 participates in the acetylation of H3K4 in breast cancer cells. H3K4ac offers a research approach to explore the mechanism of TIP60 in heterochromatin formation in breast cancer. Interestingly, TIP60 is recruited to pericentric heterochromatin and mediates acetylation of histone H4K12 by the recruitment of BRD2 [16,33]. The underexpression of TIP60 leads to derepression of satellite transcription and decompaction of pericentric heterochromatin. This acetyltransferase TIP60 can play a role in acetylation of histone, such as H4K12, and in heterochromatin formation. We suggest that

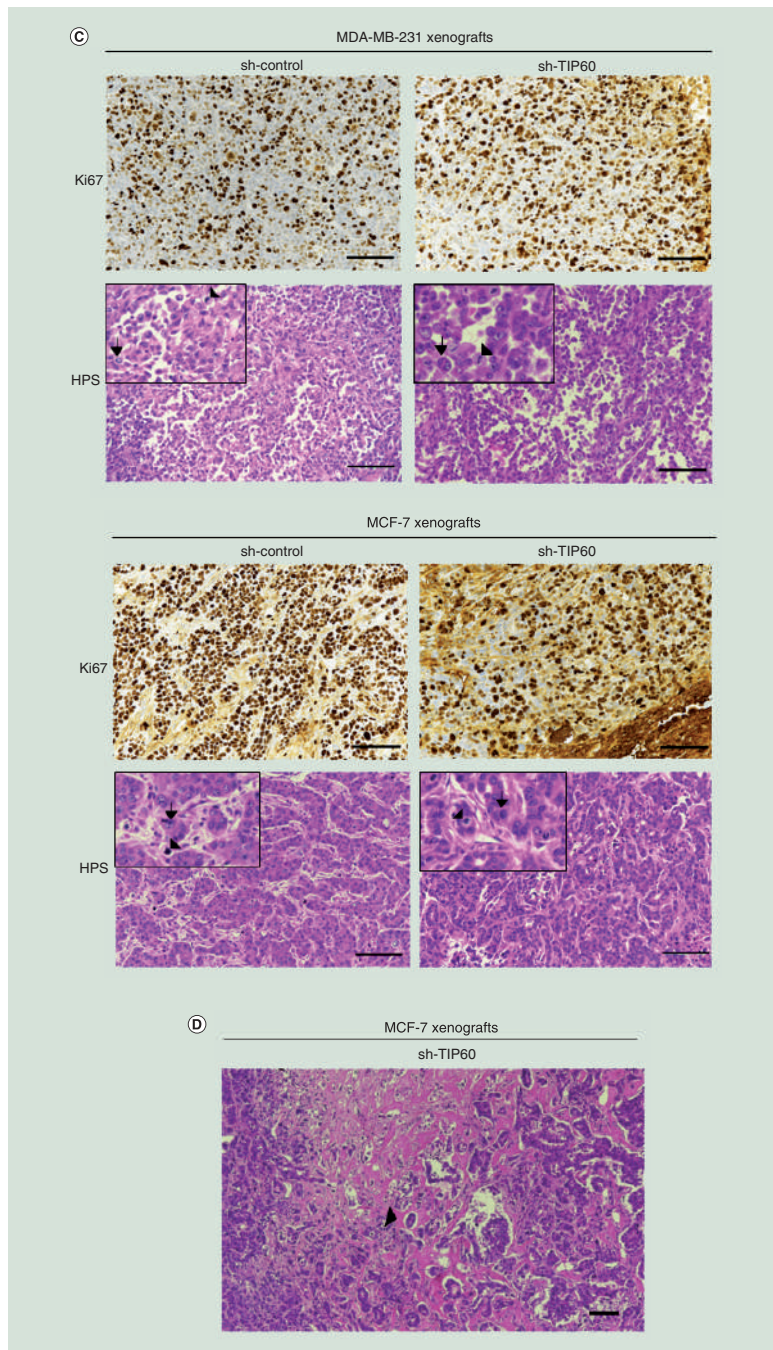


Figure 7. TIP60 depletion in breast cancer xenograft models (cont.). (A) Measure of tumor volume in mice after inoculation of MDA-MB-231 cells transfected by sh-TIP60 (n = 11) and sh-control (n = 10), and MCF-7 cells transfected by sh-TIP60 (n = 10) and sh-control (n = 10). Data are expressed as means for each group, and error bars indicate the 95% confidence interval of the mean value. Data were analyzed by paired = TRUE Student's t-test (*p < 0.05; **p < 0.01, ***p < 0.001). TIP60 contributes to development of (MDA-MB-231) triple-negative breast cancer breast xenografts *in vivo*. Conversely, TIP60 depletion reduces development of (MCF-7) luminal breast xenografts *in vivo*. (B) Tumor growth in MDA-MB-231 and MCF-7 (sh-TIP60 and sh-control) xenograft models. (C) Immunostaining to quantify Ki67 protein and hematoxylin phloxine saffron counterstained showing mitosis (arrows) and cytonuclear atypia (arrowhead) of sh-TIP60 and sh-control MDA-MB-231 and MCF-7 xenografts. Scale bars indicate 100 μ m, and images taken at $\times 20$ magnification. (D) hematoxylin phloxine saffron section of sh-TIP60 MCF-7 xenografts showing fibrosis. Scale bars indicate 100 μ m and images taken at $\times 10$ magnification. (E) sh-TIP60 and sh-control MDA-MB-231, and MCF-7 xenografts were subjected to ChIP assays using anti-H3K4ac antibodies and IgG antibodies as a control for gene promoters (*BRCA1*, *ERS1*, *ERS2*, *EZH2*, *P300*, *PGR*, *SRC3*). Quantification was performed by qPCR in the immunoprecipitates versus inputs. Values are expressed as fold enrichment over the sh-control samples (standardized relative to 1 for sh-control tumors). Data are expressed as means for each group, and error bars indicate the 95% confidence interval of the mean value. Data were analyzed by paired = TRUE Student's t-test (*p < 0.05; **p < 0.01).

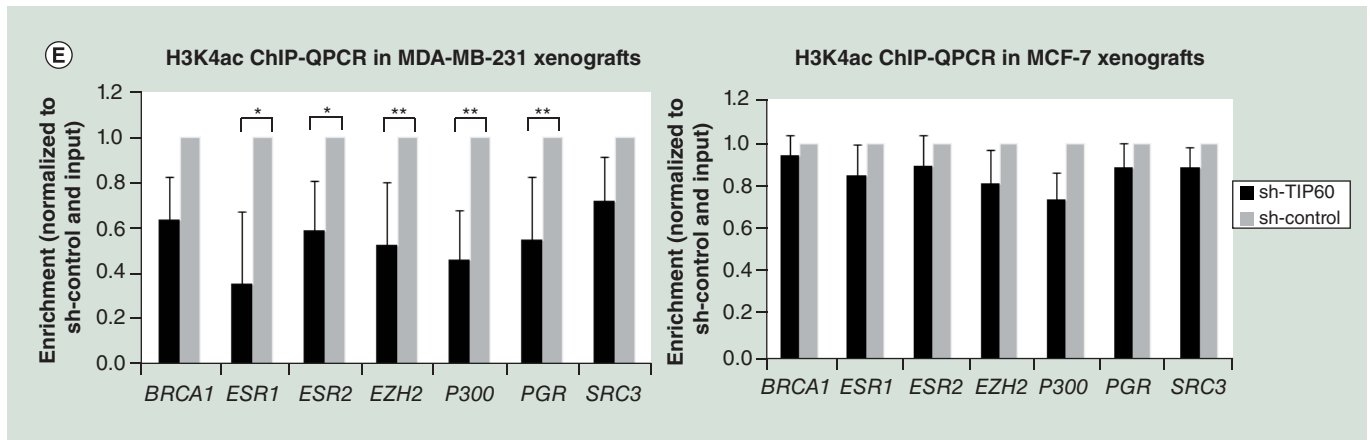


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the same mechanism could explain our results; H3K4ac could be directly involved in heterochromatin formation through TIP60.

Other questions raised by our findings concern the involvement of the TIP60 depletion in breast cancer development. TIP60 is a tumor suppressor often underexpressed in human cancers and is required for an oncogene-induced DNA damage response [13]. A decrease in TIP60 expression blocked tumor suppressor pathways such as the DDR and p53 pathways [34–36]. These results indicate that decreased TIP60 expression correlates with tumor development. However, the molecular mechanism of TIP60 downregulation was not clarified. We therefore investigated the TIP60 depletion effect *in vivo* by transfection of sh-TIP60 to breast cancer cell lines (MDA-MB-231 and MCF-7) then injected to athymic Balb-c mice. Our results showed that MDA-MB-231 being triple negative (ER-, PR- and HER2-), the mesenchymal cell line presented a significant increase in tumor development with metastasis when expression TIP60 was decreased. Moreover, this depletion led to a marked decrease in H3K4ac enrichment on gene promoters and could contribute to dysregulation of gene expression in breast cancer. A recent study showed miR-22 involvement in regulation of TIP60 in breast cancer [37]. The MDA-MB-231 cell line has an elevated level of miR-22 that is required to maintain the metastasis levels by targeting TIP60 and an inhibition of miR-22 expression leads to a reduction of the metastatic phenotype of MDA-MB-231, as well as an elevation of the expression of TIP60. These results confirm the tumor suppressor role of TIP60 in TNBC breast cancer and show that miR-22 could be a potential target to increase the TIP60 level in breast cancer [38].

We found an opposite effect of TIP60 depletion in the MCF-7 cell line, which is ER+ and an epithelial cell line: sh-TIP60 slowed down tumor development. It has been shown that ER is the defining and driving transcription factor in luminal breast cancers and its target genes regulate cell growth and endocrine response [39–41]. Acetyltransferase TIP60 is required for estrogen-induced transcription of a subset of ER- α target genes in human cells. TIP60 is recruited by estrogen and interacts with ER- α and this interaction leads to increased recruitment of histone methyltransferase MLL1 and increased monomethylation of histone H3 at lysine 4 and acetylation of histone H2A at lysine 5 [9]. This modification of histone pattern activates the transcription of target genes involved in the development of breast cancer. In this light, we hypothesize that TIP60 depletion in MCF-7 cell line provides mediation of estrogen-induced transcription of target genes involved in tumorigenesis.

Conclusion & future perspective

We have identified a target of acetyltransferase TIP60 in breast cancer, the lysine 4 of histone H3, which is found in centromeric heterochromatin and euchromatin. TIP60 could thus be recruited to centromeric heterochromatin, where it mediates acetylation of histone H3K4. We conclude that the underexpression of TIP60 observed in breast cancer may promote tumorigenesis in TNBC tumors. However, a comprehensive study is necessary to know mechanisms and pathways involving the acetyltransferase TIP60 in breast cancer development. The acetyltransferase TIP60 could then be used as a new target and a prognostic marker in breast cancer.

Summary points

- TIP60 expression is altered in different breast cancer subtypes. This alteration modifies acetylation status of proteins such as histones.
- A physical interaction exists between TIP60 and H3K4ac and these two proteins are localized on the same promoter region of specific genes in breast cancer. The acetylation of H3K4 seems to be depending to the presence of TIP60.
- In ER-negative tumors (triple-negative breast cancer xenografts), the TIP60 depletion promotes tumor development. An opposite effect is observed in ER-positive tumors (luminal xenografts).
- The reduced expression of TIP60 observed in breast cancer might alter the regulation of steroid hormone dependent-genes and could promote tumor development or slow it down.

Acknowledgements

The authors thank our imaging facility, Imagerie Confocale de Clermont-Ferrand (ICCF) and we gratefully acknowledge the help of C Vachias. We thank M Roche and J Allemand for the immunohistochemical technique.

Financial & competing interests disclosure

We thank the 'Ligue contre le cancer-Comité du Puy-de-Dôme'. K. Rifaï was funded by the Hariri Foundation. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

For *in vivo* xenograft models, all protocols were conducted in accordance with the 2010/63/UE Directive after approval by the institutional review board C2E2A from Auvergne Region (approval number: APAFIS#33399-201601 1 109302187 v5). The samples were obtained from a Biological Resources Center (BB-0033-00075) and a prior signed informed consent was obtained from each patient. It was done in accordance with the Council of Europe's Recommendation on Research on Biological Materials of Human Origin (Rec [2006]) from 2006.

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Conclusion de la publication 7:

Cette étude nous a tout d'abord permis de confirmer la présence de H3K4ac dans l'hétérochromatine et l'euchromatine mais aussi dans les tissus tumoraux et sains du sein. La présence de cette marque dans les différents compartiments de la chromatine et dans les tumeurs et tissus sains pourrait suggérer un rôle dans la formation et le maintien de la chromatine.

On a pu confirmer que TIP60 et H3K4ac étaient Co-localisées et qu'il y avait une interaction physique entre eux sur le promoteur des gènes enrichis en H3K4ac dans le cancer du sein. Ce qui confirme le dépôt de cette marque par TIP60 dans les différents compartiments de la chromatine.

L'inhibition de TIP60 par Sh-RNA nous a permis de mettre en évidence une diminution de la marque H3K4ac dans le noyau cellulaire suggérant ainsi l'importance de TIP60 pour cette marque. Cette inhibition nous a permis aussi de voir une diminution de la croissance tumorale dans les xénogreffes effectuées avec la lignée continue de cancer du sein luminal (MCF-7 Sh-TIP60). Tandis que dans les tumeurs des souris réalisées avec la lignée continue de cancer du sein TNBC (MDA-MB-231 Sh-TIP60), on observe une augmentation de la croissance tumorale. Ces résultats suggèrent un rôle différent de TIP60 dans les deux types moléculaires de cancer du sein. Dans le type moléculaire Luminal (MCF-7) exprimant *ER+*, *PR+* et *HER2+*, TIP60 agit comme un oncogène car sa délétion entraîne une diminution de la tumeur. Alors que dans le type moléculaire TNBC (MDA-MB-231) n'exprimant pas les récepteurs *ER-*, *PR-* et *HER2-* TIP60 agit comme un suppresseur de tumeurs car sa déplétion entraîne une augmentation de la croissance tumorale.

2.3 Epi-drogues dans le traitement des cancers du sein

Les cancers sont des pathologies à multiples dysfonctionnements cellulaires, ce qui les rend difficiles à suivre et à traiter. De nos jours de plus en plus d'études ont permis de développer de nouveaux traitements contre les cancers. Cependant la plupart de ces traitements sont invasifs et néfastes pour les cellules saines d'où la nécessité de traitements plus ciblés. Ces vingt dernières années, les avancées sur la compréhension des mécanismes épigénétiques et l'élaboration des profils épigénétiques des cancers a permis l'utilisation de molécules chimiques ou naturelle appelées « épi-drugs » pour lutter contre le cancer.

Dans cette revue, nous avons recensé différentes molécules chimiques ou naturelles pouvant moduler le profil épigénétique dans une cellule cancéreuse et être utilisées comme traitement contre le cancer du sein. En effet, nous avons observé que les sous-types de cancer du sein ont des profils épigénétiques différents par rapport aux cellules saines. La mise en place de bases de données OMICS répertoriant les différentes modifications de l'ADN, les micro-ARNs, les protéines et les modifications post-traductionnelles dans les différents types de cancer du sein, nous a amené à recenser les principales épi-drogues pouvant être utilisées comme thérapies ciblant les sous-types au pronostic défavorable.

Publication 8:

Epi-drugs as triple-negative breast cancer treatment. Idrissou M, Sanchez A, Penault-Llorca F, Bignon YJ, Bernard-Gallon D. Epigenomics. 2020 Apr;12(8):725-742. doi: 10.2217/epi-2019-0312. Epub 2020 May 12.



Epi-drugs as triple-negative breast cancer treatment

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Triple-negative breast cancer (TNBC) types with poor prognosis are due to the absence of estrogen receptors, progesterone receptors and HEGFR-2. The lack of suitable therapy for TNBC has led the research community to turn toward epigenetic regulation and its protagonists that can modulate certain oncogenes and tumor suppressors. This has opened an important new field of therapy using epi-drugs, in preclinical and clinical trials. The epi-drugs are natural or synthetic molecules capable of inhibiting or modulating the activity of epigenetic proteins such as DNA methyltransferases, modulating the expression of interferon microRNAs, as well as histone methyltransferases, demethylases, acetyltransferases and deacetylases. This review investigated the epi-drugs used in the treatment of TNBC.

First draft submitted: 17 October 2019; Accepted for publication: 26 February 2020; Published online: 12 May 2020

Keywords: DNMT inhibitors • epi-drugs • HAT inhibitors • histone deacetylase inhibitors • histone demethylase inhibitors • histone methyltransferase inhibitors • microRNA • therapy • triple-negative breast cancer

Breast cancer is the most diagnosed cancers in women worldwide, with nearly 2.1 million new cases diagnosed in 2018, which amounts to approximately one in four of all female cancers. It is also the leading cause of cancer-related deaths in women worldwide, with 626,679 deaths in 2018 accounting for 15% of all cancer deaths in 2018 [1].

Generally, this cancer is classified by molecular subtypes based on expression of the estrogen receptor (ER), progesterone receptor (PR) and HER2 receptors for better patient management and treatment. The luminal subtype is composed of luminal A expressing (ER, PR and HER2) receptors and luminal B which are expressed (ER, PR) but HER2 may or may not be expressed. Due to expression of these receptors, these subtypes are easily targeted for therapies inducing a better prognosis. In addition, the HER2-enriched subtype expresses HER2 receptors and does not express the ER and PR receptors. Then, triple-negative breast cancer (TNBC) subtype is the most aggressive with the worst prognosis, frequently found in younger patients. It represents 15–20% of all breast cancers, and is characterized by the absence of ER, PR and HER2 receptors, making them a subtype with poor prognosis. The TNBC subtype has attracted a lot of research and remains a major challenge in the fight against breast cancer [2,3]. This research has recently focused on the epigenetic mechanisms involved in tumorigenesis, which are DNA methylation, post-translational histone modifications and microRNAs [4–7]. These are transmissible and reversible mechanisms which modify gene expression without changing gene sequences. In research advances, it has been observed that the subtypes of breast cancer have different epigenetic profiles in comparison to healthy cells. The establishment of omics databases listing the different modifications in mRNA, microRNA, proteins and post-translational modifications in the different types of breast cancer will allow the development of numerous therapies targeting subtypes with poor prognosis [8,9].

Today's therapies increasingly use natural and synthetic molecules called 'epi-drugs', which activate or inhibit proteins that modulate epigenetic mechanisms. These proteins with the ability to modulate epigenetic mechanisms can be 'writers' (DNA methyltransferases [DNMTs], histone methyltransferases [HMTs] and histone acetyltransferase [HATs]), 'erasers' (histone demethylases [HDMs] and histone deacetylases [HDACs]) or 'readers' (bromodomains, tudor domains, chromodomains and PHD finger domain proteins) [10], and they play various roles in cellular pro-

cesses impacted by tumorigenesis, such as DNA repair, cell growth, apoptosis, cell differentiation and chromatin remodeling [5,6,10].

Epi-drugs & DNA methylation

DNA methylation is the addition of a methyl group to the carbon at position 5 of the cytosine of the CpG dinucleotides, this addition is done through DNMTs. It is the most extensively studied epigenetic process in human cancer. Cancer cells generally show hypomethylations of DNA and hypermethylations of tumor suppressor gene promoters that create genomic instability resulting in overexpression of oncogenes and underexpression of tumor suppressor genes [11–13]. In breast cancer, as in most cancers, there is a hypermethylation of some of the genes that promote the development of carcinogenesis, such as ER promoter (*ESR*, *PR*), pro-apoptotic genes, cell cycle inhibitor genes, DNA repair genes (*BRCA1*) and many others [11,14]. In TNBC where there is no expression of *ER* and *PR* and *HER2*, there is always hypermethylation of gene promoters such as the promoter of the *BRCA1* gene [15].

The DNA methyltransferase

The DNA methylation is conducted by DNMTs that are able to modify the carbon in the 5-position of the cytosine of CpG dinucleotides by adding a methyl group. In mammals, three of these DNMTs (DNMT1, DNMT3a and DNMT3b) are active on DNA, while DNMT2, or TRDMT1, does not methylate DNA but tRNA [12]. Each of these DNMTs have very specific roles in the DNA methylation process that occurs during certain cellular processes such as gametogenesis, embryogenesis or somatic tissue development [16]. These DNMTs can be combined with other methyltransferases or can recruit HDACs from the gene promoter to play their role in maintaining transcriptional repression [14].

DNMT1

DNMT1, also known as maintenance methyltransferase, is the main enzyme responsible for maintenance of DNA methylation. It has been shown to copy methylation motifs on the newly formed DNA strand when DNA is replicated [12]. Like most DNMTs, it consists of two regions: a catalytic C-terminal region acting as a methyltransferase and a multidomain N-terminal region that has a regulatory and recognition function for hemimethylated or nonmethylated DNA [12]. In breast cancer, there is generally an overexpression of DNMTs, but DNMT1 is not significantly overexpressed in tumors compared with healthy tissues [12]. Nevertheless, its wide-reaching role in DNA methylation makes it a potential target for treatment against TNBC.

DNMT2

DNMT2 is the smallest DNMT in mammals, without the N-terminal regulatory region. It is composed exclusively of C-terminal regions [12]. DNMT2 has been shown to be implicated in DNA damage recognition, DNA recombination and mutation repair [12]. It has been demonstrated that human DNMT2 does not add methyl groups on DNA but to cytosine 38 of aspartic acid tRNA [12]. Its role in cancer is still unknown, but DNMT2 expression decreases in hepatocellular carcinomas.

DNMT3

DNMT3, or *de novo* methyltransferase, introduces a new methylation profile that is not inherited through DNA. This methylation differs from maintenance methylation, which replaces the existing methylation profiles on the new DNA strands during replication [12]. There are three DNMT3s: DNMT3a, DNMT3b and DNMT3L. DNMT3L is a regulatory factor for *de novo* methylation. Very similar to DNMT3A and DNMT3B, DNMT3L does not have the amino acid residues necessary for methyltransferase activity in the C-terminal domain. Its role is unclear and further studies are needed. DNMT3A and DNMT3B are the active DNA methyltransferases because they have methyltransferase activity in the C-terminal domain. They cannot distinguish between nonmethylated CpG and hemimethylated CpG sites, and therefore cannot copy inherited methylation profiles or contribute to maintaining existing methylation profiles [12]. The DNMT3B gene showed the highest expression range compared with DNMT1 and DNMT3A in breast cancer tumor tissue.

DNMT inhibitors as therapy for TNBC

DNMT inhibitors are natural or synthetic molecules able to interact with DNMTs and thus inhibit them. These molecules include nucleoside analogs, antisense oligonucleotides, low molecular weight molecules or natural compounds, some of which have shown their ability to inhibit DNMTs in breast cancer.

Nucleoside analogs

Nucleoside analogs are DNMT inhibitors that are phosphorylated by cellular kinases. These inhibitors trap DNMTs by binding to DNA and forming a covalent bond with DNMTs, making them unavailable for methylation and inducing demethylation of DNA replication. A number of agents are in clinical development and numerous clinical have tested 5-aza-2'-deoxycytidine (Decitabine; CAS No. 2353-33-5, Tocris, Bristol, UK), Zebularine (CAS No. 3690-10-6, Tocris) and others [17].

5-aza-2'-deoxycytidine or decitabine, which has been accepted by many international regulatory agencies, including the US FDA and the European Commission, as a demethylating agent and has been used for solid tumors such as breast cancer [18]. Decitabine transforms into active phosphorylated nucleotides and incorporates into DNA as a substitute for cytosine, where it irreversibly binds to DNMT, trapping enzymes in the DNA. Subsequent depletion of DNMT stock and inhibition of DNMT function by decitabine result in silencing DNA methylation of tumor suppressors, which are most often hypermethylated on their promoters in cancers. There is no consensus over the relationship between DNMT and decitabine response in solid tumor. Research has shown that decitabine therapy significantly inhibited the growth of tumors with high levels of DNMT protein, suggesting that DNMT protein levels could be a biological marker to guide clinical drug selection for TNBC [18]. Preclinical studies in breast cancer models have shown high ER re-expression and greater restoration of tamoxifen sensitivity when we combined decitabine and HDAC inhibitors [19].

Zebularine is a primary inhibitor of DNMT, acting like an analog of cytidine during the incorporation into DNA. This incorporation prevents the future methylation of DNA [20]. Nakamura *et al.* reported that zebularine exhibits antitumor effects in hepatocarcinoma cell line (HepG2) [21]. Then recently, the same authors demonstrated that zebularine is able to induce apoptosis in human cholangiocarcinoma [22]. Recent research has shown that zebularin is highly effective when combined with trichostatin and tumor necrosis factor-related apoptosis inducing ligand in TNBC cells [23]. To date, no major clinical trial has been conducted with zebularine in breast cancer.

Antisense oligonucleotides

Antisense oligonucleotides are small sequences, able to hybridize to specific RNAs acting consequently as inhibitors of translation of mRNAs. This strategy has been used in cancer research and treatment by blocking translation of DNMT1.

ASO98 also known as MG98 is an antisense oligonucleotide inhibitor of DNMT1, used in breast cancer cell lines. This study was conducted by Yan *et al.*, where in TNBC cell lines (MDA-MB231 and Hs578t), a re-expression of ER α was found [24]. In view of these early promising results, a Phase I clinical trial was conducted in solid cancers by Davis *et al.* [25], but MG98 given as a 21 day continuous infusion every 4 weeks was not tolerated. Likewise Stewart *et al.* realized a Phase I trial with MG98 administration twice weekly for 3 weeks in a renal cancer patient and MG98 seemed to be well tolerated [26].

Low molecular weight molecules

Inhibiting proliferation of tumor cell lines by low molecular weight molecules RG108, which is a DNMT inhibitor that reactivates the expression of tumor suppressor genes in tumor cells by DNA demethylation [27,28]. In TNBC cells, RG108 allows re-expression and activation of PKD1 when combined with Suramin [29].

Procainamide (PCA) (CAS No. 614-39-1; Sigma-Aldrich, MI, USA) is an approved drug, commonly used in cardiac arrhythmias but PCA is also able to prevent DNA methylation inducing re-expression of genes and specifically *GSTP1*, a detoxifier gene, in prostate cancer [30,31]. Different authors have shown that PCA is also used as an inhibitor of methylation in breast cancer cell lines leading to restoration of tumor suppressor expression [32]. This demonstrates that PCA can be used as an epigenetic drug in cancer and clearly in TNBC.

Disulfiram (CAS No. 97-77-8; Tocris) is an aldehyde dehydrogenase and non-nucleoside DNMT inhibitor. Although the mode of action is still unknown, disulfiram would be able to interact with the thiol group of the catalytic site of DNMTs as it would act on aldehyde dehydrogenase [33,34]. Disulfiram is able to inhibit the invasion of tumor and endothelial cells and show its ability to modulate reactive oxygen species accumulation and

overcome synergistic resistance to cisplatin in breast cancer cell lines [35]. It shows a capacity to suppress cancer stem-like properties and STAT3 signaling in TNBC cells and able to inhibit the epithelial–mesenchymal transition induced by TGF- β and stem-like characteristics in breast cancer via ERK/NF- κ B/Snail pathway. Furthermore, the autophagic apoptosis in cancer cells induced by the activation of ER stress [36].

Hydralazine (CAS No. 304-20-1; Sigma-Aldrich), an FDA-approved antihypertensive, is an DNMT inhibitor able to bind CpG island sequences and interferes with the translocation of DNMTs along the DNA sequence [14,37]. The combination of hydralazine and thiazolidinedione show apoptosis and arrest of proliferation in TNBC cells [38]. Phase II clinical trials conducted with hydralazine in combination with magnesium valproate have been shown to be effective in solid tumors such as breast cancer [39].

Natural compounds ('epi-nutrients')

They are molecules already present in nature and food such as tea, cashew nut, marine sponge and others.

These are usually antioxidants and exhibit inhibition of carcinogenesis. A main component of green tea has been extensively studied, the epigallocatechin-3-gallate (EGCG), which is an inhibitor of catechol-O-methyltransferase. The structural similarity between DNMT and catechol-O-methyltransferase suggests EGCG-driven inhibition of DNMT that results in the re-expression of hypermethylated genes such as *CDKN2A*, *RAR-B* and *MGMT*. In TNBC cells, EGCG leads to a decrease in cell viability and proliferation [40]. The combination of EGCG with topotecan shows the same effects on the proliferation and viability of TNBC cells but increases the apoptosis of these cells [41]. Tests on preclinical models have shown a loss of tumor masses dependent on dose of EGCG [42]. The clinical trials conducted with EGCG were Phase I and were conducted to prevent dermatitis radiation in adjuvant radiotherapy on patients with breast cancer [43].

Curcumin or diferuloylmethane derives from the rhizomes of *Curcuma longa*, commonly used in medicine in India for over 6000 years. Curcumin is a polyphenol with an anticancer potential, able to suppress initiation, promotion and metastasis in different tumors, to downregulate different factors of transcription, the chemokines, the kinases, the cell factors of adhesion and the growth factors such as EGFR and HER2 [44]. In spite of its low absorption and rapid elimination, curcumin is commonly used in clinical trials in association with different agents to affect its metabolic elimination [45]. Clinical trials conducted on postmenopausal breast cancer patients show that, the combination of curcumin with hydroxytyrosol and omega-3 fatty acid leads to a decrease in inflammation and pain in patients with aromatase-induced musculoskeletal symptoms [46].

Epi-drugs & post-translational histone modifications

Post-translational modifications of histones are mechanisms that consist of a modification of the histone after its translation. They most often consist of the addition or removal of methyls and acetyls groups on the N-terminal histone tails. HMTs or HATs make additions, while HDMs or HDACs remove these groups. Given the role of histones on the condition of chromatin, these modifications allow the compaction or relaxation of chromatin, thus allowing the transcription or not of the genes involved in cancers.

Histone methylation

Histone methylation is the epigenetic process in which one or more methyl groups are added to the histone tails of the amino residues. The process is controlled by histone HMTs and HDMs. HMTs can add methyls to the lysine or arginine of the histone tails, while HDMs can remove them. In cancer, these two enzymes are seen to vary, and this variation is used as a diagnostic factor or to follow tumor progression in some cancers including breast cancer [47–50].

Histone methyltransferases

Histone methyltransferases are enzymes that use SAM as co-substrate for the transfer of methyl groups to amino residues (lysine and arginine) on N-terminal histone tails. This methylation of the residues subdivided methyltransferases into two main groups: KMTs and arginine methyltransferases [48,51].

The SUV39H is the sole enzyme responsible for laying down the trimethylation of histone H3 on the H3K9me3 on the chromatin. At first, SUV39H was exhibited as necessary in the heterochromatin for the silencing of gene expression [52], but we still have no clear picture of how SUV39H contributes to gene regulation in the euchromatin region and, more importantly, to pathogenesis [53]. It has been shown that in many cancers,

including breast and colorectal cancers, there is a loss of SUV39H1 and the H3K9me3 mark, which leads the cells into a process of carcinogenesis with loss of the chromosomal architecture including polyploidy [52,54].

EZH2 is a protein belonging to the polycomb group which is described as having a repressive action by methylating histone H3 lysine 27 and inducing silencing of tumor suppressors in cancer [55]. In tumor cells, EZH2 inhibits antimetastatic genes such as E-cadherin and inhibitors of metalloproteinases, by promoting cell propagation and independence of base growth [56]. Inactivation of the EZH2 gene altered cell adhesion and migration in some diseases, suggesting that H3K27me3 participates in the regulation of angiogenesis. Furthermore, EZH2 is upregulated in prostate cancer to normal prostate tissues [57,58]. This overexpression of EZH2 has also been reported in breast cancer [59].

The KMT G9a is a growth hormone that also plays the role of methylate histone H3 lysine residues 9 and 27, resulting in transcriptional repression. Several studies suggest that G9a promotes the proliferation and survival of cancer cells [60,61]. Its role in cancer is related to its post-transcriptional upregulation in response to hypoxia [62] and its role in silent tumor suppressors [63,64]. Other studies demonstrated the key role of G9a HMT in cell proliferation and neovascularization in cancer, proving that further research will be indicated by targeting G9a HMT with inhibition to induce antitumorigenic actions [65].

DOT1L, an HMT of H3K79, is also required for angiogenesis. Investigations in human umbilical vein endothelial cells by silencing DOT1L demonstrated a decrease in the formation of functional vascular networks. DOT1L acts with the transcription factor ETS-1. This complex supports the expression of VEGFR2 implicated in angiogenesis by establishing ERK1/2 and AKT signaling pathways. A knockdown with DOT1L was also realized in mice and exhibited the pro-angiogenic part of DOT1L by reduction in vascular density [66].

SET-7 is another KMT recently found to be implicated in angiogenesis. SET-7 was described as acting with GATA1, also known to regulate the angiogenesis. The complex SET7–GATA1 leads to upregulation of VEGF transcription and promotes angiogenesis. The inactivation of SET-7 resulted in an inhibition of cell growth in breast cancer and also *in vitro*, in addition to a decrease in VEGF secretion resulting in reduced human umbilical vein endothelial cell tube formation [67].

Histone methyltransferase inhibitors as a therapy for TNBC

Depending on the methyltransferase activity and their involvement in hypermethylation of oncosuppressor genes like BRCA1 in breast cancers [68–70], the inhibitors on HMTs could reduce the methylation in tissues preventing carcinogenesis. Following below are different HMTs listed.

S-adenosylmethionine analogs were the first inhibitors used as anticancer drugs. However, these compounds target not only HMT but also other enzyme classes using AdoMet as a methyl donor, such as DNMT [71], and so this low specificity is a bottleneck to their use. Investigations were conducted with SAM on cell metabolism and showed its implication in apoptosis of tumor cells contrary to normal cells and specifically in colon, liver, stomach, breast or prostate cancer. It has been shown at molecular level that SAM induces *uPA* gene silencing, thus inhibiting tumor cell invasion *in vitro* and metastatic and cancer growth *in vivo*.

DZNEP (3-deazaneplanocin A) (CAS No.120964-45-6; Tocris) is an inhibitor of KMTs and more particularly of EZH2. It acts at the level of S-adenosyl-L-methionine, the endogenous substrate that binds to the SET domain of EZH2 to allow the transfer of a methyl group to the amino group of the lysine 27 side chain of histone H3, thus inducing the suppression of transcription [72]. The overall protein levels of H3K27me3 and EZH2 vary according to the different subtypes of breast tumors. A high proportion of EZH2 is often associated with triple-negative and basal-like tumors and *vice versa* for luminous tumors [73]. Lately, in our laboratory, we studied three modifications of histone H3 (H3K4ac, H3K9ac and H3K27me3) between two cell lines representing two types of breast cancer: luminal subtype (MCF-7) and mesenchymal-like (MDA-MB-231). We conducted a comparative study of the effect of different ‘epi-drugs’ including DZNEP, SAHA and NaBu on histone changes in the *BRCA1*, *P300* and *SRC3* genes involved in breast cancer. We found that DZNEP had no effect in MCF7, whereas in MDA-MB 231 treated with DZNEP that H3K27me3 significantly decreased on *SRC3* gene [74].

Different arginine methyltransferase inhibitors (AMI) 1–6 are well documented [5]. AMI is a strong specific inhibitor for arginine N-methyltransferases and less effective on KMTs. Among these AMIs, P2015666 is effective on TNBC cells by reducing cell proliferation and mammosphere formation [75]. Few clinical trials are underway, due to the toxicity reported by investigations in different cell lines.

Histone demethylases

Histone demethylases or lysine demethylases (KDMs) are proteins able to remove a methyl group from the lysines in the histone tails. Classified into six families according of their catalytic activities and their target histone lysine residues, KDMs 1–6 can be distinguished.

First family of KDMs, KDM 1 is the family composed of the KDM 1A and 1B proteins both exhibiting the SWIRM domain. SWIRM is able to demethylate and more specifically the mono- or dimethyl group on the lysines 4 and 9 of the histone H3, thus leading to transcriptional activation or inactivation [76–79]. Besides, SWIRM allows also to demethylate nonhistone proteins such as p53 and DNMTs by KDM 1A and certain proteins of the NF- κ B pathway by KDM 2A [80,81]. However, there are structural differences between KDM 1A and 2A, KDM 1B do not have any tower domain, which allows interaction with the CoREST protein [82].

Specific to the H3K36me2 mark, the KDM2 family is composed of KDM 2A and 2B [83]. These two proteins are able to induce gene repression by regulating p15INK4B by demethylation of the H3K36me2 mark [83,84]. Indeed, KDM 2A might be able to cause the NF- κ B pathway arrest, while KDM 2B might act against oxidative stress and induction of apoptosis via its action on the proteins p53 and RB [85].

The KDM3 family in humans includes KDM 3A, KDM 3B and JMJD 1C. KDM 3A, also known as JMJD 2A, is the most studied protein of the family and the only one with a demethylase activity. So, KDM 3A is a demethylase specific for the mark H3K9 mono- and dimethylated, KDM 3A is also able to bind to the androgen receptor and acts in spermatogenesis [86,87].

KDM 4A–4D form the KDM4 family and are specific demethylases for H3K9 and H3K36 di- or trimethylated marks [88,89]. KDM 4A seems to act on protein synthesis while KDM 4B and 4C are involved in tumorigenesis through their signalization actions on NOTCH1. KDM 4B is also able to work together with N-MYC [90]. The role of KDM 4D is still poorly understood and not really known.

The KDM5 family is mainly associated with transcriptional repression via its interaction with the polycomb repressive complex 2 that owns the EZH2 methyltransferase. This family is composed of KDM 5A, 5B, 5C and 5D, which are able to suppress the di- and trimethylated groups on the H3K4 marks. KDM 5A is also able to bind to RBP-J blocking the interaction between RBP-J and NOTCH [91]. KDM 5B is generally localized in the testis and binds to the androgen receptor implicated in the regulation of the transcription [92,93].

The KDM6 family includes UTX and JMJD3 demethylases, which are able to suppress repressive marks in the transcription, like the di- and trimethylation of H3K27.

JMJD3 and UTX exhibits characteristics of a tumor suppressor, for UTX via its implication in cell cycle arrest [94,95] and for JMJD3, its interaction with p53 in cyclin-dependent kinase inhibitor 2A expression [96,97].

Demethylase inhibitors as a therapy against TNBC

Despite the fact the HDMs are antagonist to HMTs, inhibition of HDMs could prevent in cancer, the expression of oncogenes which are hypomethylated leading to transcription [98]. Some of these inhibitors such as GSK-J4, Bizine or Trans-2-PhenylCycloPropylAmine are used in cancer and specifically in breast cancer.

GSK-J4 (CAS No. 1373423-53-0; Tocris) an ester derivative of GSK-J1, a JMJD3 inhibitor that modulates the pro-inflammatory response of macrophages [99]. GSK-J4 shows significant antitumor activity, Hashizume *et al.* demonstrated that GSK-J4 treatment on brainstem gliomas inhibited cell growth and prevented colony formation [100]. This study also showed a decrease in tumor growth in mouse xenografts in the GSK-J4 group compared with the control group. These same observations were observed in a study of luminal and triple-negative breast tumor cells treated with GSK-J4: a decrease in cell proliferation, mammospheric and colony number and size and a tumorigenicity inhibitory effect in xenografts [101]. Decreased cell growth is also observed in acute lymphoblastic leukemia T cells [102]. GSK-J4 also induces cell death in ovarian cancer stem cells at a nontoxic concentration for normal fibroblasts. The xenograft study also shows inhibition of the ability to initiate the tumor [103]. In addition to cancer, GSK-J4 also acts on the various pathologies related to a disruption of the expression of JMJD3. For example, this drug reduces the inflammatory response induced by CD4⁺ T cells as well as inflammation in autoimmune encephalomyelitis [104]. The blocking of JMJD3 by GSK-J4 protects insulin-producing pancreatic cells from cytokine induced apoptosis, showing an interest in reducing Type I diabetes [105]. GSK-J4 also shows an interest in blocking the reactivation of Herpes Simplex 1 virus induced by the demethylation of H3K27me3 [106]. These different observations support the hypothesis that GSK-J4 has a strong potential as a new agent to fight different types of cancer.

2-PCPA and Bizine are two HMD inhibitors. 2-PCPA causes the suppression of invasion and metastasis of TNBC cells by inhibiting the interaction between LSD1 and Slug [107,108]. However, Bizine, which is similar to phenelzine, reduces proliferation and cell growth in prostate cancer cell lines [109].

Histone acetylation

Histone acetylation is a post-translational modification controlled by the balance between HAT and HDAC activity. To lead the chromatin to a euchromatin structure, HATs catalyze the transfer of acetyl groups from acetyl-CoA to the amino group of lysine residues and form ϵ -N-acetyl lysine, thus causing the chromatin to relax [110]. HDACs, on the other hand, can catalyze deacetylation and induce chromatin compaction to form heterochromatin; this process is done by hydrolysis of acetyl groups from lysine residues and often leads to transcriptional repression of genes. The balance between the histone acetylation and deacetylation profile regulated transcription. Several diseases, including cancer such as breast cancer, are often thought to be associated with dysfunctions in enzymes involved in these events [111].

Histone acetyltransferases

HATs are the enzymes that add acetyl groups to the tails of histones and other proteins. In humans, there are three main subfamilies: MYST family (Moz, Ybf2, Sas [2–3] and Tip60), Gcn5-related N-acetyltransferase (GNAT) family and p300/CBP family [112,113]. The proteins forming these subfamilies act as transcription factors and as steroid receptor co-activators with catalytic activity.

MYST family is named after its founding members MOZ, Ybf2/Sas3, Sas2 and TIP60. These acetyltransferases are considered to be transcriptional co-activators that play an important role in several processes such as regulating transcription and the process of repairing DNA damage, particularly in double-strand breaks. TIP60 ‘KAT5’ is the most studied protein in the MYST family [114–116]. There is ample evidence to suggest that MYST complex proteins (ESA1, SAS2, MOF, TIP60, MOZ and MORF) have various roles in different nuclear processes.

P300/CBP family is composed of the acetyltransferases p300 and CBP, which share a sequence similarity of 75%. P300 and CBP interact with more than 400 different cellular proteins, such as p270 and other proteins involved in apoptosis, differentiation and cell growth [117,118]. These interactions also allow them to play the role of transcriptional co-activators and are involved in multiple signal-dependent transcriptional events. Recent data indicate that *p300/CBP* genes are altered in various human tumors, suggesting that CBP has tumor suppressing activity [119].

The superfamily of GNATs, which includes KAT2A and KAT2B are two highly homologous acetyltransferases. KAT2A, also known as GCN5, is one of the two major complexes involved in chromatin modification [120], KAT2B, called PCAF also plays a role in chromatin modification. GNAT inhibition plays a crucial function in the tumor growth of breast cancer *in vivo* and *in vitro* [121].

HAT inhibitors as therapy for TNBC

Chemical or natural, HAT inhibitors are molecules able to block the binding of acetyl groups to the histone tails.

Chemical inhibitors

TH1834 is a specific inhibitor of acetyltransferase TIP60 that belongs to the MYST family. TH1834 would deprive TIP60 acetyl CoA, thus stopping its ability to perform its acetyltransferase function. This inhibition of acetylation by TH1834 has been demonstrated *in vivo* and *in vitro* [122,123]. Association of TH1834 with ionizing radiation strongly influences genome instability, DNA damage and apoptosis in breast cancer cell and other cancers [122,124]. Knowing the essential role of tip60 in the tumorigenesis process, inhibition of its activity on certain genes such as oncogenes could be a therapeutic approach to fight cancer [123,125–127].

NU9056 specifically inhibits Tip60 activity. In prostate cancer cells, NU9056 treatment induced apoptosis through caspase-3. Using this method, other small molecule inhibitors of Tip60 have also been reported, Lys-CoA and disulfide inhibitor A [128]. Actually, NU9056 is rather developed, but in our laboratory the effects of this inhibitor on TNBC cell lines are underway.

Natural inhibitors

Anacardic acid is a substance of cashew nut shell that is capable of specifically inhibiting histone acetyltransferase P300 and PCAF. Anacardic acid induced inhibition of cell proliferation, induced cell cycle arrest in the G₀/G₁ phase, suppressed cell invasion and migration and induced apoptosis in breast cancer cells [129–131].

Garcinol is a natural histone acetyltransferase inhibitor (HATi). Isolated from *Garcinia morella* fruit extracts, garcinol is able to inhibit chronic inflammation, proliferation and induces apoptosis of TNBC cells and murine models of metastatic breast cancer [132].

Curcumin is a pleiotropic molecule, in (Natural compounds ("epi-nutrients") of DNMT inhibitors as therapy for TNBC) section and is able to act on DNMT as well as on HAT [124]. Curcumin has been identified as the first HATi permeable to p300/CBP cells. The activity-dependent transcription of p300 HAT chromatin is effectively suppressed by curcumin [121]. Furthermore, curcumin could also inhibit histone acetylation *in vivo*. Curcumin is one of the most promising HATi in clinical trials, where it emerges with great promise as a therapeutic agent [122].

Histone deacetylases

There are currently 18 different known iso-enzymes of HDACs, divided into four subclasses.

Members 1, 2, 3 and 8 constitute the class I of HDACs which are found expressed in different cell compartments. HDACs 1, 2, 3 and 8 are exhibited specifically in the nucleus but HDAC 3 is also present in the cytoplasm due to its nuclear export signal [133]. These HDACs have domains that allow them to perform their deacetylation and transcription repression activities [133,134].

On the other side, HDACs 4, 5, 7 and 9 (class IIa HDACs) and HDACs 6 and 10 (class IIb HDACs) form the family of class II HDACs. Due to the presence of histidine instead of lysine in its catalytic site, its deacetylase activity is less important than other HDACs [135,136]. Class IIb HDACs including HDAC 6, which are not only found in the cytoplasm, but also in the nucleus in complex with HDAC 11. HDAC 10 is found both in the cytoplasm and the nucleus. HDAC 6 works as a tubulin deacetylase, regulating the motility of microtubule-dependent cells [137]. HDAC 11, mainly expressed in the nucleus, constitutes the class IV of HDACs with a strong homology with the classes I and II of HDACs [138].

The HDACs of class III, also called sirtuins, classified into seven proteins (SIRT1–7) homologous to the yeast protein family SIR2. SIRT1 is the most similar to SIR2, having the most robust HDAC activity. Like HDAC classes I, II and IV, sirtuins also have nonhistone substrates. However, they have several particular characteristics compared with other conventional HDACs. Indeed, they have two different enzymatic activities: lysine deacetylase and mono-ADP-ribosyltransferase. Sirtuins can be located in the cytoplasm (Sirt 1 and 2), nucleus (Sirt 1, 2, 3, 6 and 7) or mitochondria (Sirt 4 and 5). Sirtuins are omnipresent in the cell and play an essential role in maintaining genomic integrity. They target different histone marks, especially the activator marks H3K9ac and H4K16ac, but also target transcription factors, structural proteins, nuclear receptors, HATs, as well as nonhistone chromatin components [139,140]. Due to their deacetylase activity, sirtuins are involved in many pathways such as apoptosis, cell proliferation, cell differentiation, gene expression regulation, chromatin remodeling, DNA repair, metabolism regulation, stress response in many diseases such as cancer [141–146]. However, the nature of their functions in cancer remains highly controversial [147–150], particularly the functional roles of SIRT1 [151–154].

Histone deacetylase inhibitors as therapy for TNBC

Panobinostat, also known as LBH589, is well known as an inhibitor of overexpressed HDACs in cancer, which has led to its use in epigenetics. Many clinical trials have shown that panobinostat is effective alone or in combination with other treatments, and also that it has low toxicity. A study on TNBC cell lines showed its specificity for them, which was evaluated by cytotoxicity and allowed to characterize the effects of panobinostat on breast cancer gene regulation, associated signaling pathways and morphology [155].

Vorinostat is an HDAC inhibitor (HDACi) at the most advanced stage of clinical development and the first to be approved by the FDA [150,156]. It is able to bind the HDAC catalytic site, allowing the hydroxamic fraction to chelate the zinc ion located in the HDAC catalytic regions, resulting in an accumulation of both hyperacetylated histones and transcription factors due to the inhibition of deacetylation. This process has been shown in breast cancer [157,158].

Entinostat (MS-27-275) one of the first oral HDACis, with antiproliferation effect in *in vivo* and *in vitro* breast cancer. In many clinical studies, it has shown its efficacy in ER⁺ breast cancer when combined with aromatase inhibitors [159], but also in ER⁺ and TNBC breast cancer when combined with AZA. Its effect on TNBC breast cancers allows the re-expression of CD44/CD24 surface receptors, thus inducing inhibition of the migration capacity of tumor cells [19]. Other studies have shown its ability to induce apoptosis and cell growth arrest when combined with trastuzumab in HER2-breast cancer [160].

Table 1. List of epi-drugs and their target proteins in the different epigenetic processes and their biological effects in cancer.

Epigenetic processes		Epigenetic proteins	Epi-drugs	Biological effects of epi-drugs	Ref.
DNA methylation		DNMTs (DNMT1, DNMT3a and DNMT3b)	- Decitabine - Zebularine - MG98 - RG108 - Procainamide - Disulfiram - Hydralazine - Tea polyphenols - Curcumin	Re-expression of ER receptor; restoration of the expression of the anti-angiogenic genes <i>TSP1</i> , <i>JUNB</i> and <i>IGFBP5</i> ; inhibition of tumor vascularization; initiation of damage response; reactivate expression of tumor suppressor gene; inhibit tumor proliferation; inhibition of invasiveness and collagenase; induce cell death and apoptosis in cancer; inhibition of endothelial cell migration and invasion; inhibit tumor cell growth	[19,22,27,28,30–32,37,38,41,42,45]
Post-translational histone modifications	Histone methylation	Histone methyltransferase: - SUV39H - EZH2 - G9a - DOT1L - SET7	- S-adenosylmethionine - DZnep - AMI-1 - Gsk2807	Inhibition of <i>in vitro</i> tumor cell invasion and growth of metastases; induce cell cycle arrest and apoptosis of tumor cells; inhibit activation of the nuclear receptor reporter gene in MCF-7 cells and HIV-1 RT polymerase	[5,74,75]
		Histone demethylase: - LSD1 - JMJD1C - JMJD3 - UTX	- GSKJ4 - JIB-04 - 2-PCPA - Bizine	Inhibit transcription of oncogenes; induce cell death and apoptosis in cancer; reducing Type I diabetes; inhibit tumor proliferation	[101–103,105,132]
	Histone acetylation	Histone acetyltransferase: - Tip60 - p300/CBP - GCN5 - ATF-2	- TH1834 - NU9056 - Anacardic acid - Garcinol - Curcumin	Induce apoptosis through caspase-3; inhibit the transcription of oncogenes; inhibition of tumor cell invasion and growth	[124,129–132]
		Histone deacetylase: -HDAC (1–11) -Sirtuin (SIRT 1–7)	- Panobinostat - Vorinostat - Entinostat - Valproic acid - Trichostatin A - Sirtinol - Resveratrol	Act against TNBC cell growth, survival and tumorigenesis; reactivate the transcription of tumor suppressor genes; inhibit cell proliferation	[157,158,161,162,169,172]
MicroRNA	Tumor suppressor	Oncosuppressor miRNA: - Let-7 family - miR-200 family - miR-335	Synthetic miRNA mimic oncosuppressor miRNA	Inhibit translation of oncoproteins; induce cell arrest and apoptosis; inhibit tumor proliferation; inhibit cell migration and invasion	[176–180,184]
	Oncogenic	OncomiRNA (Oncomir): - miR-10b - miR-21 - miR-155 - miR-221/222	Antisense oligonucleotides targeting oncomirs	Reactivate expression of tumor suppressor gene; induce cell arrest and apoptosis; inhibit cell migration and invasion; inhibit tumor proliferation	[176–178,180,182,184]

2-PCPA: Trans-2-PhenylCycloPropylAmine hydrochloride; AMI: Arginine methyltransferase inhibitor; DNMT: DNA methyltransferase; DZNEP: 3-deazaneplanocin A; HDAC: Histone deacetylase; SUV39H: Suppressor of variegation 3 (9) homolog; TNBC: Triple-negative breast cancer.

HDACs classes I and II trichostatin A has shown its efficacy in inhibiting the proliferation of most breast cancer cells, especially those expressing ER and has little or no effect on normal cells (MCF10A) [161,162]. This inhibition of proliferation requires the reactivation of the cell cycle and apoptosis of these cells, making it a potential candidate for the treatment of TNBC [163,164].

Valproic acid (VPA) is an HDACi capable of inducing cell failure, apoptosis, cell migration and ER re-expression in breast cancer cells. The use of VPA in the different ER⁺ or ER-breast cancer cell lines leads to an increase in the RNA and protein level of the CDK inhibitor of P21 and a decrease in the protein level of Cyclin B, thus causing the cell cycle to stop. VPA is able to induce apoptosis, stop the migration and adhesion of ER cells by inhibiting survivin expression and decreasing CD44 expression. VPA is even able to modulate immune response and inflammation in breast cancer [165–167].

Discovered in 2001 by Grozinger *et al.*, sirtinol is a specific inhibitor of the *in vitro* activity of Sir2 from yeast and human Sirt1/Sirt2 [168]. Sirtinol has been studied in the treatment of breast cancer and has shown that its use can stop tumor growth *in vivo* and induce apoptosis, autophagy, stopping the cell cycle, growth, viability and colony

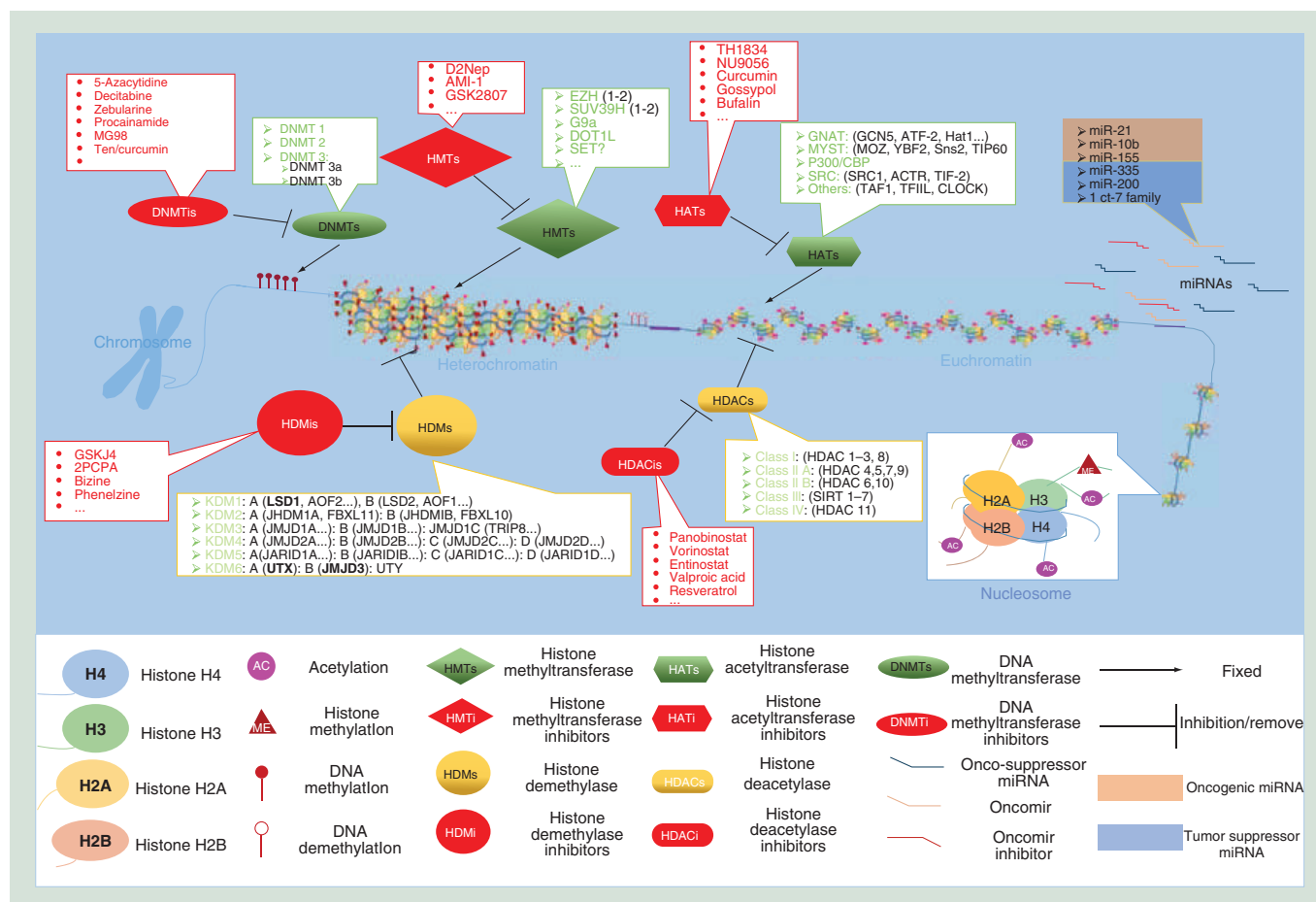


Figure 1. Epigenetic antagonists, actors and their inhibitors on the chromatin.

DNMT: DNA methyltransferase; HAT: Histone acetyltransferase; HATI: Histone acetyltransferase inhibitor; HDAC: Histone deacetylase; HDACi: Histone deacetylase inhibitor; HDM: Histone demethylase; HDMi: Histone demethylase inhibitor; HMT: Histone methyltransferase; HMTi: Histone methyltransferase inhibitor; ME: Histone methylation.

formation in breast cancer cells *in vitro*. In some types of breast cancer cells, sirtinol can induce an increase in their sensitivity to chemotherapeutic treatment such as camptothecin or cisplatin [169].

Resveratrol is an HDACi with a broad spectrum of target classes including cyclooxygenases, lipoxygenases, kinases and sirtuins. It has anticancer [170,171], anti-inflammatory, hypoglycemic and other beneficial cardiovascular effects [172]. Resveratrol is able to modulate epigenetic regulators, methylation negatively and acetylation of histones positively, which restores expression of the *BRCA1*, *p53*, *p21* genes in human breast cancer cell lines. Resveratrol is also able to induce premature senescence and inhibit the epithelial–mesenchymal transition of cancer cells by induction of the tumor suppressor Rad9 [173].

Therapies targeting microRNA

MicroRNAs are small, noncoding RNAs with 19–22 nucleotides that regulate gene expression by inhibiting mRNA translations or degradations. Many biological mechanisms such as proliferation, survival, differentiation, invasiveness and cell death are impacted by miRNAs, which are able to regulate gene expression involved in these processes. In humans, there are more than 460 miRNAs that play oncogenic or tumor suppressor roles in cancers [174,175]. The aberrant variation in the expression of these miRNAs in different types of cancers has been associated with different phases of tumor development such as tumor invasion, metastasis or resistance to antitumor treatment [176,177].

Tumor suppressor microRNA

As its name suggests, tumor suppressor miRNAs are small RNAs that bind to the mRNA of oncogenic proteins. In tumor progression, tumor suppressor miRNAs have their expression rate significantly decreased. Among these miRNAs we can cite the miR-127, miR-335, the miR-200 family or the LET-7 family which attacks the expression of oncoproteins [178–180]. The use of prodrug mimics of miR-127 reduces the viability and motility of TNBC cells and makes them more sensitive to chemotherapy [181]. This ability to inhibit the expression of oncoproteins has made them one of the major areas of research in the development of cancer therapy in recent years [182].

Oncogenic microRNA

On the other hand, miRNAs can act as oncogenes (oncogenic microRNA [oncomiRNA] or oncomiR). These oncomiRs have shown an important role in the development and progression of human cancer. Recent research has identified miRNAs whose expression is strongly linked to tumor progression, among these oncomiRs are miR-21, miR-10b, miR-155 and miR-221/222 which are both tumor-forming and metastasis promoting activities or tumor-suppression inhibitory activities [180,182,183]. Ma *et al.*, have shown that the use of oligonucleotides such as antisense oligonucleotides or antagomiRs which are modified oligonucleotides able to inhibit these oncomiRs in a stable and specific manner [171,184]. AntagomiR use has shown effective inhibition of oncomiRs in mammary carcinoma metastasis in mice and primates [182,184].

Conclusion

This review highlights the enormous impact of synthetic or natural molecules such as DNMTi, HMTi, HDMTi, HATi, HDACi, antagomiRNAs and miRNA mimics which are listed in Table 1 and illustrated in Figure 1. All these epigenetic actors play an important role in new therapies for cancer. The increasingly advanced research in the field of epigenetics has led to a better understanding of this process in the formation of tumor onset, growth and invasion. This deeper understanding has served as a platform for using epi-drugs or epi-nutrients in treatment and therapy for many cancers, including breast cancer. As we have seen, epi-drugs can induce the re-expression of ERs, activation of apoptosis pathways or suppression of survival pathways in TNBC (Table 1), all of which point to a bright future for using epi-drugs in TNBC.

Future perspective

Since most epi-drugs act on the global epigenome and in all cells, targeting the cancer cells becomes a major challenge for research. However, specific epi-drugs, such as antagomiRs inhibiting oncomiRs or synthetic microRNAs mimicking tumor suppressor microRNAs, make possible to target cancer cells more specifically. These advances in epi-drugs and their ability to discriminate between normal and cancer cells will be possible through the creation and updating of omics databases that bring epigenomic profiles of different cancer cells at different stages. These omics databases had been developed to standardize clinical trials. Recently, Raju *et al.* [9] created an omics platform providing genomic, transcriptomic and proteomic data for TNBCs. This might lead to better therapeutic targeting to prevent TNBC.

Executive summary

Epi-drugs & DNA methylation

- DNA methyltransferases inhibitors are able to interact with DNA methyltransferases resulting in the restoration of a normal DNA methylation. These molecules include nucleoside analogs, antisense oligonucleotides, low molecular weight molecules or natural compounds such as curcumin which is able to block DNA methylation.

Epi-drugs & post-translational histone modifications

- Histone methyltransferase inhibitors (HMTi) and demethylase inhibitors (HDMi) act on two antagonistic mechanisms. The deregulation of the balance between these two mechanisms (methylation and demethylation) led to a tumor progression. Inhibitors such as DZNEP or GSKJ-4 respectively HMTi and HDMi will maintain the balance and return to a normal status.
- Histone acetyltransferase inhibitors (HATi) and histone deacetylases inhibitors (HDACi) are chemical or natural molecules. HATi is able to block the binding of acetyl groups to the histone tails. On the other hand, HDACi is able to block the deacetylation. Vorinostat (CAS No. 149647-78-9; Sigma-Aldrich, MI, USA) and Entinostat (CAS No. 209783-80-2; Sigma-Aldrich) are the first HDACi to be approved for development in clinical studies. Curcumin seems to be a promising HATi in clinical trials.

Therapies targeting microRNA

- MicroRNAs are small, non-coding RNAs with 19–22 nucleotides that regulate gene expression by inhibiting mRNA translations or degradations that play oncogenic (oncomiRNA) or tumor suppressor roles in cancer.

Financial & competing interest disclosure

This work was supported by a grant from the French Ligue Régionale Contre Le Cancer – Comités du Puy-de-Dôme et de l'Allier. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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Conclusion de la publication 8:

Cette revue nous a permis de mettre en évidence les épi-drogues chimique ou naturelles agissant comme des inhibiteurs des protéines épigénétiques telles que :

-Les DNMTis comme la curcumine ou la zebularine qui inhibent l'activité des DNMTs modulant ainsi le profil épigénétique aux niveaux de L'ADN.

-Les HMTis tels que le DZNEP et des HDMis comme le GSK-J4 qui eux jouent sur la méthylation des histones permettant l'activation ou l'inhibition des gènes impliqués dans le cancer du sein.

-Les HATis comme TH1834 et NU9056 ou les HDACis tels que l'entinostat et le vorinostat qui interviennent au niveau de l'acétylation des histones jouant sur le remodelage de la chromatine et l'activation des protéines impliquées dans certains mécanismes cellulaires dérégulés dans le cancer du sein.

-Et enfin, Antagomir inhibant les oncomir ou les micro-ARNs synthétiques mimant les micro-ARNs suppresseurs de tumeurs.

Plusieurs de ces épi-drogues sont en étude clinique et ont un avenir prometteur pour le ciblage et la personnalisation des traitements contre le cancer du sein.

III. OBJECTIFS DU PROJET DE RECHERCHE

Avec l'avancée des recherches sur le cancer du sein et la compréhension de l'implication de la régulation épigénétique dans le processus de carcinogénèse, de nombreuses protéines épigénétiques se sont vues mettre en lumière pour lutter contre cette pathologie.

Dans notre laboratoire de nombreuses études menées sur ces protéines ont été réalisées dans le but de mieux caractériser et stratifier le cancer du sein mais aussi de comprendre leur rôle dans cette maladie.

Nos études précédentes sur SIRT1, une histone déacétylase (HDAC) nous a permis de stratifier les sous types moléculaires de cancer du sein en fonction de l'expression de SIRT1 mais aussi d'envisager l'utilisation de SIRT1 comme cible thérapeutique. D'autres études sur une protéine antagoniste à SIRT1 qui est TIP60 une histone acétyl-transférase (HAT) nous a permis de valider les cibles de ce dernier qui sont les histones H3 et H4. Ces histones H3 et H4 sont aussi les cibles de SIRT1 et sont impliquées dans l'expression de certains gènes impliqués dans la carcinogénèse mammaire.

Dans l'optique d'approfondir nos connaissances du rôle de TIP60 dans le cancer du sein, cette thèse a pour but de décortiquer l'activité de TIP60 dans le cancer du sein et de mieux comprendre les mécanismes mis en place dans l'initiation et le développement du cancer du sein impliquant TIP60. Pour mener à bien cet objectif, il a été subdivisé en deux axes.

Dans le premier axe, connaissant les cibles de TIP60 et leurs variations dans le cancer du sein, on s'est posé la question : « Quel est l'effet de l'inhibition de l'activité de TIP60 dans les différents sous types moléculaires de cancer du sein ? ».

Pour répondre à cette question, deux inhibiteurs chimiques spécifiques de TIP60 ont été utilisés à savoir : TH1834 qui inhibe l'activité de TIP60 en le privant d'acétyl-CoA et NU9056 qui se fixe sur le site actif de TIP60 l'empêchant de jouer son rôle acétyl-transférase.

Dans un premier temps ces inhibitions se font *in vitro* sur différentes lignées cellulaires continues de cancer du sein luminales exprimant *ER*, *PR* et n'exprimant pas *HER2* (MCF-7 et T47D), les lignées TNBC n'exprimant aucun des récepteurs suivant *ER*, *PR* et *HER2* (MDA-MB-231, MDA-MB-453 et MDA-MB-468) et une lignée non cancéreuse (MCF-10A). Dans ce cas nous avons regardé la variation de la marque cible de TIP60, H3K4ac après l'inhibition de son activité et nous avons regardé la variation de l'ensemble des récepteurs nucléaires qui existe après l'inhibition par TH1834 ou NU9056. Le but est de vérifier l'efficacité des inhibiteurs sur l'activité de TIP60 mais aussi de voir le rôle de cette activité résiduelle sur l'expression des récepteurs nucléaires connus pour être dérégulés dans le cancer du sein.

Dans un deuxième temps, cette inhibition est faite *in vivo* avec l'inhibiteur TH1834 avec des souris nudes BALBc qui ont reçu une injection de lignée cellulaire continue de cancer du sein luminal (MCF-7) ou TNBC (MDA-MB-231). Dans ce cas nous avons regardé la croissance tumorale des xénogreffes dans les souris. Cela dans le but de voir l'effet de l'inhibition de l'activité de TIP60 sur la croissance des tumeurs du sein.

Dans le second axe, connaissant le rôle de TIP60 dans d'autres cancers tels que le cancer colorectal où dans la littérature, TIP60 est impliquée dans la formation d'une structure de maintien de l'intégrité et la plasticité de l'hétérochromatine lors de l'installation de la carcinogénèse. On s'est alors posé la question : « Quel est le rôle de TIP60 dans la plasticité et l'intégrité chromatinienne lors de la carcinogénèse du sein ? ».

Pour répondre à cette question, on s'est focalisé sur les variations des supposés acteurs de ce maintien de l'hétérochromatine à savoir TIP60, H4K12ac et P400 dans 77 tumeurs du sein et leurs tissus sains associés, mais aussi dans les différents types de lignées continues de cellules du cancer du sein.

Tout d'abord, on a regardé la variation de l'enrichissement de H4K12ac sur les différents compartiments de la chromatine et dans les différents types moléculaires de tumeurs et lignées continues du cancer du sein. Cela dans le but de caractériser ou de stratifier les types moléculaires du cancer du sein en fonction de H4K12ac mais aussi de voir le lieu d'expression de la marque dans la chromatine.

Ensuite, nous avons regardé la co-localisation entre TIP60 et la marque H4K12ac sur les différents compartiments de la chromatine. Cela dans le but de valider l'interaction entre TIP60 et H4K12ac dans l'hétérochromatine et l'euchromatine.

Enfin, nous avons regardé la variation d'expression de TIP60, P400 et H4K12ac dans 77 tumeurs du sein et leurs tissus sains associés, mais aussi dans les différents types de lignées continues de cancer du sein. Cela dans le but de corréler l'expression de ces protéines et marques avec la formation du squelette de maintien de l'hétérochromatine dans la carcinogénèse.

Ces objectifs nous permettront sans doute de mieux comprendre le rôle de TIP60 dans la carcinogénèse du sein mais aussi de valider la formation du squelette TIP60/P400/H4K12ac dans le cancer du sein.

IV. RESULTATS DE L'ETUDE

1 Les effets de l'inhibition de l'activité de Tip60 dans la progression tumorale du cancer du sein

Comme nous l'avons annoncé dans la partie objectif, le but de cette thèse est de répondre à deux questions qui permettront de mieux comprendre le rôle de TIP60 dans le cancer du sein.

La première question est de savoir « Quel est l'effet de l'inhibition de l'activité de TIP60 dans les différents sous types moléculaires de cancer du sein ? ».

Pour répondre à cette question nous avons opté pour une stratégie d'inhibition de l'activité de TIP60. Une étude précédemment effectuée dans notre laboratoire avait été réalisée. Une déplétion de TIP60 par Sh-RNA et xénogreffes entraînant une inhibition stable de sa transcription. Cette inhibition montrait une amplification de la croissance tumorale dans les souris injectées avec la lignée continue de cancer du sein luminal (MCF-7 Sh-RNA-TIP60), alors qu'au contraire dans les souris injectées avec la lignée continue de cancer du sein TNBC (MDA-MB-231 Sh-RNA-TIP60), on observait un ralentissement de la croissance tumorale. Ces observations nous indiquent que TIP60 joue un rôle clé dans la croissance tumorale du sein. Or connaissant son importance structurale dans le complexe NUA4 et la fonction pléiotropique de TIP60, nous avons voulu inhiber juste son activité pour voir son effet dans la croissance tumorale et mieux comprendre son mode action dans le cancer du sein.

1.1 Aperçu de l'inhibition chimique de l'histone acétyl-transférase TIP60 in vitro.

Dans cette première étude, pour mener à bien notre stratégie et répondre à la question de l'effet de l'inhibition de l'activité de TIP60 dans les différents sous types moléculaires de cancer du sein *in vitro*, nous avons utilisé 6 lignées continues: 3 lignées continues de cancer du sein TNBCs (MDA-MB-231, MDA-MB-453 et MDA-MB-468) et 2 lignées continues de cancer du sein luminales (MCF-7 et T47D) et une lignée continue non cancéreuse (MCF-10A). Ces lignées continues sont traitées avec deux inhibiteurs chimiques de TIP60 qui sont : TH1834 qui prive TIP60 d'acétyl-CoA et NU9056 qui bloque le site actif de TIP60.

Premièrement, la cytotoxicité des deux inhibiteurs est vérifiée par la technique de viabilité cellulaire dite XTT qui consiste à mesurer l'activité mitochondriale de la cellule. Cette technique permettra d'obtenir la dose à laquelle les traitements feront 50% de cellules mortes et vivantes, appelé IC50. Cela permettra d'utiliser au mieux les deux inhibiteurs et de rendre plus efficace leurs inhibitions en étant le moins toxique pour les cellules.

Ensuite, après avoir traité les cellules avec les deux inhibiteurs, nous avons regardé l'expression protéique de TIP60 et l'une de ses cibles qui est la marque H3K4ac par Western blot. Dans le but de vérifier si les deux traitements TH1834 et NU9056 agissent effectivement sur l'activité de TIP60 et non sur son expression.

Puis pour être sûr que la diminution de l'accumulation de la marque H3K4ac est bien dû à l'inhibition de l'activité de TIP60, nous avons effectué une ChIP-qPCR pour observer les variations de l'enrichissement en H3K4ac au niveau des promoteurs des gènes de l'hétérochromatine (Myoglobine) et de l'euchromatine (GAPDH).

Enfin, connaissant le lien étroit qu'il existe entre le cancer du sein, les récepteurs hormonaux et que la classification des différents types moléculaires de cancer du sein est basée sur l'expression de ces récepteurs. Nous avons voulu voir l'effet de l'inhibition de l'activité de TIP60 sur les différents récepteurs. Pour cela, nous avons utilisé la technique dite Taqman Low Density Array (TLDA) qui permet de déterminer le profil d'expression d'un grand nombre de gènes. Dans notre étude, des plaques 384 puits contenant tous les 48 différents récepteurs nucléaires et 16 gènes endogènes utilisés comme contrôles. La plaque est scindée en 2 parties contenant chacune les 64 gènes en triplicatas et chaque partie sera utilisée pour déposer les extraits de cellules traitées ou non traitées. L'expression relative de ces gènes est déterminée par qPCR ce qui nous permettra de voir la variation d'expression des gènes impactés par l'inhibition de l'activité de TIP60.

Publication 9:

Digging deeper into breast cancer epigenetics: Insights from chemical inhibition of histone acetyl-transferase TIP60 *in vitro*. **Idrissou M**, Lebert A, Boisnier T, Sanchez A, Houfak Khoufak FZ, Penault-Llorca F, Bignon YJ and Bernard-Gallon D. OMICS. 2020 Sep 22. doi: 10.1089/omi.2020.0104. PMID: 32960142

AU1 ▶

Digging Deeper into Breast Cancer Epigenetics: Insights from Chemical Inhibition of Histone Acetyltransferase TIP60 *In Vitro*

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Abstract

Breast cancer is often sporadic due to several factors. Among them, the deregulation of epigenetic proteins may be involved. TIP60 or KAT5 is an acetyltransferase that regulates gene transcription through the chromatin structure. This pleiotropic protein acts in several cellular pathways by acetylating proteins. RNA and protein expressions of TIP60 were shown to decrease in some breast cancer subtypes, particularly in triple-negative breast cancer (TNBC), where a low expression of TIP60 was exhibited compared with luminal subtypes. In this study, the inhibition of the residual activity of TIP60 in breast cancer cell lines was investigated by using two chemical inhibitors, TH1834 and NU9056, first on the acetylation of the specific target, lysine 4 of histone 3 (H3K4) by immunoblotting, and second, by chromatin immunoprecipitation (ChIP)-qPCR. Subsequently, significant decreases or a trend toward decrease of H3K4ac in the different chromatin compartments were observed. In addition, the expression of 48 human nuclear receptors was studied with TaqMan Low-Density Array in these breast cancer cell lines treated with TIP60 inhibitors. The statistical analysis allowed us to comprehensively characterize the androgen receptor and NR3C2 receptors in TNBC cell lines after TH1834 or NU9056 treatment. The understanding of the residual activity of TIP60 in the evolution of breast cancer might be a major asset in the fight against this disease, and could allow TIP60 to be used as a biomarker or therapeutic target for breast cancer progression in the future.

Keywords: breast cancer, epigenetics, TIP60, drug development, precision medicine, biomarkers

Introduction

POST-TRANSLATIONAL MODIFICATIONS OF HISTONE are mechanisms of chromatin regulation and are characterized by a set of markers (acetylation, methylation, phosphorylation, among others), which are deposited on the N-terminal tails of the histones. This set of markers constitutes the histone code that determines the structure and activity of the different chromatin regions (Bannister and Kouzarides, 2011; Izzo and Schneider, 2010; Santos-Rosa and Caldas, 2005; Turner, 2000).

One of the major mechanisms of post-translational histone modification is histone acetylation that participates with two types of proteins: histone acetyltransferase (HATs) and histone deacetylase (HDACs). They take part in cellular and

metabolic pathways, gene regulation, organization, and chromatin maintenance (Idrissou et al., 2018; Judes et al., 2018). Their dysfunctions are implicated in many diseases such as cancer (Yuen et al., 2005).

HATs are able to acetylate histone or nonhistone proteins. This reversible capacity makes them essential tools for the activation or deactivation of different cellular pathways (Choudhary et al., 2009; Eberharter and Becker, 2002; Turner, 2000). This protein family is subdivided into three subfamilies in humans: GNAT, P300/CBP, and MYST family consisting: Moz, Ybfz, Sas(2–3), and TIP60. The dysfunction of HATs is associated with the development of cancers such as breast cancer (Arif et al., 2010; Avvakumov and Cote, 2007; Di Martile et al., 2016; Doyon et al., 2004; Utley and Cote, 2003).

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TIP60, one of the most studied HATs in the MYST family, has been found in the development of some cancers. Given its pleiotropic nature, it allows to act as a transcription factor or coactivator of some nuclear receptors such the steroid receptors (Halkidou et al., 2003). In many cancers, there is a decrease in RNA and protein levels of TIP60 (Gorrini et al., 2007) and are now used to stratify the different breast cancers from less to more aggressive (McGuire et al., 2019). Inhibition of residual TIP60 activity in breast cancer could result in the death of tumor cells by apoptosis (Brown et al., 2016).

After numerous studies on HDAC inhibitors as a therapeutic treatment for cancer, the research community has recently launched the development and the use of HAT inhibitors as a clinical treatment for cancer (Brown et al., 2016). TH1834 is a TIP60-specific inhibitor similar to its substrate acetyl-CoA, acting as a competitive acetyl-CoA inhibitor, thereby inhibiting the acetyl transferase activity of TIP60 (Gao et al., 2014). NU9056 is a small compound able to bind specifically to the thiol active site of TIP60, blocking it to play its acetyltransferase activity (Coffey et al., 2012). The first studies of these inhibitors showed a decrease in breast tumor growth *in vivo* (Idrissou et al., 2019) and *in vitro*, in cell death of breast and prostate cancer cell lines when coupled with ionizing radiation (Coffey et al., 2012; Gao et al., 2014).

In light of the involvement of TIP60 in different cell pathways and coactivation of nuclear receptors, it is of broad interest and timely to follow on the effects of the chemical inhibitors of residual TIP60 activity on their expressions. These nuclear receptors had already been used to classify breast cancer into molecular subtypes. The use of chemical inhibitors would lend novel epigenetic insights toward therapeutics innovation and precision medicine in breast cancer.

AU5 ► Materials and Methods

Cell culture

All six human cell lines in this study were purchased from the ATCC (American Type Culture Collection, Manassas, VA, USA) and conserved in the Biological Resource Center of Jean Perrin Comprehensive Cancer Center, identified under No. BB-0033-00075 (Clermont-Ferrand, France).

(ER+, PR+) breast cancer cell lines, MCF-7 and T-47D, were used as representative of the luminal subtype. (ER-, PR-, and HER2+) breast cancer cell line, MDA-MB 453, was used as HER2+ subtype. (ER-, PR-, and HER2-) breast cancer cell lines, MDA-MB 231 and MDA-MB 468, were used as representative of the triple-negative subtype. MCF10A, a fibrocystic dystrophy breast cell line considered as nontumoral, was included as a control.

MCF-7 and T-47D cells were cultured in RPMI-1640 medium (Gibco Grand Island, New York, NY) with insulin (1–4 mg/mL; Novo Nordisk, Denmark), MDA-MB 453, MDA-MB 231, and MDA-MB 468 cells were cultured in Leibovitz's L-15 medium (Gibco). Both media were supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 1% L-glutamine (Invitrogen), 0.1% gentamicin (Panpharma, Luitré, France), and incubated in a humidified atmosphere at 37°C containing 5% CO₂ for MCF-7 and T-47D and without CO₂ for MDA-MB 453, MDA-MB 231, and MDA-MB 468. MCF10A cells were grown in DMEM/F-

12 medium (Gibco) containing 10% horse serum, 1% L-glutamine, 0.1% gentamicin, and completed with insulin (10 µg/mL), cholera toxin (100 ng/mL), epidermal growth factor (20 ng/mL), and hydrocortisone (500 ng/mL) (Sigma) in a humidified atmosphere at 37°C containing 5% CO₂.

Cell viability assay

Viability assays were performed with the XTT Cell Viability Kit (Biotium, Hayward, CA) according to the manufacturer's protocol and using the setting made by the laboratory (Daures et al., 2018). Cells were treated with increased doses of TH1834 (Axon Medchem) (MDA-MB-231 [56.71 µM], MDA-MB-453 [48.68 µM], MDA-MB-468 [33.34 µM], MCF7 [66.92 µM] and MCF-10A [111.9 µM] for 48 h and T47D [40.63 µM] for 24 h) or NU9056 (Tocris) (MDA-MB-231 [29.40 µM], MDA-MB-453 [21.14 µM], MDA-MB-468 [31.30 µM], and T47D [11 µM] for 72 h and MCF7 [11.38 µM] MCF-10A [6.828 µM] for 48 h).

Protein extraction and immunoblot analysis

Whole protein extracts from cultured cells were obtained using RIPA buffer (Thermo Fisher Scientific), containing protease and phosphatase inhibitor cocktail (Sigma-Aldrich). Around 25–40 µg of extracted proteins were resolved by electrophoresis on 8–15% sodium dodecyl-sulfate polyacrylamide gel (SDS-PAGE) (Bio-Rad, Hercules, CA), then electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P, PVDF, 0.45 µm; Merck Millipore) in transfer buffer (25 mM Tris-HCL [pH 7.6], 192 mM glycine, 10% methanol). The membranes were blocked with 5% nonfat milk in 0.1% TBS-tween and later immunoblotted with the following primary Abs: anti-TIP60 Ab (1/500, GTX112197) and anti-H3k4me3 Ab (1/500, GTX55482) were purchased from GenTex, anti-H3k4ac Ab (1/750, C15410322; Diagenode, Seraing, Belgium), and anti-GAPDH Ab (1/5000, sc-47724; Santa Cruz Biotechnology). Membranes were then washed and incubated with alkaline phosphatase-conjugated secondary Abs: anti-mouse IgG (1/2000, S3721) and anti-rabbit IgG (1/2000, S3738) (Promega, Madison, WI). Immunolabeling was detected using Western Blue® Stabilized substrate for Alkaline Phosphatase (Promega) at room temperature.

Chromatin immunoprecipitation assays and quantitative real-time PCR

Chromatin immunoprecipitation (ChIP) assays were performed on chromatin extracted from breast cancer cell lines and a fibrocystic cell line using the Auto iDeal ChIP-seq Kit for Histones (C01010171; Diagenode) according to the manufacturer's instructions. The extracted chromatin was later sonicated for 15 min (30 cycles, 30 sec ON/30 sec OFF) at 4°C with Bioruptor™ sonicator (Diagenode). Three micrograms of the following polyclonal antibodies (Abs) were used: anti-H3k4ac po-Ab (C15410322), anti-H3k4me3 po-Ab (C15410003) (Diagenode), and 1 µg of nonimmune rabbit IgG (Kch-504-250; Diagenode) serving as a negative control. The ChIP was carried out by SX-8X® IP-Star® Compact Automated System (Diagenode). The samples were incubated for 3 h with antibodies coating on protein A-coated magnetic beads, then for 10 h at 4°C for immunoprecipitation

reaction. Later on, 4 μL of elution buffer iE2 was added to the samples and the input was prepared with 2 μL of extracted chromatin in 100 μL of elution buffer iE1/iE2. The reverse crosslinking was performed for 45 min at 65°C. At the end, immunoprecipitated DNA (IP DNA) and total DNA (input) from both ChIP assays was purified by MicroChIP DiaPure Columns (C03040001; Diagenode) and analyzed by real-time qPCR.

AU6▶

Real-time qPCR was performed in triplicate at 25 μL final reaction volume 5 μL of IP or input, 1 \times de SYBR[®] Green PCR Master Mix (Applied Biosystems), 200 nM for each GAPDH (C17011047; Diagenode), and Myoglobin Exon 2 primers (C17011006; Diagenode), on a 7900 HT Fast Real-Time PCR System (Applied Biosystems). The recovery level of proteins was disclosed by the rate of IP relative to Input. The efficiency of ChIP of a particular genomic locus can be calculated from qPCR data and reported as a percentage of starting material: $\%(\text{ChIP}/\text{Total Input}) = 2^{[(\text{Ct}(\text{x}\% \text{input}) - \log(\text{x}\%)/\log 2) - \text{Ct}(\text{ChIP})]} \times 100\%$. Ct (input) and Ct (ChIP) are threshold values obtained from the exponential phase of qPCR for the immunoprecipitated DNA sample and input sample, respectively. Log (x%)/log2 accounted for the dilution 1/x of the input.

RNA extraction, reverse transcription, and quantitative real-time PCR

TH1834 and NU9056 treatment cells were plated in 6-well plates at 5000 cells with IC₅₀ concentration (Table 1). After incubation time of TH1834 and NU9056 treatment, RNA extraction, reverse transcription, and quantitative real-time PCR analysis (TaqMan Low-Density Array [TLDA]) were performed according to the manufacturer’s protocol and using the setting made previously in the laboratory (Daures et al., 2018).

T1▶

For qPCR using TLDA, 48 gene expressions and 16 RNA controls (Table 2) were quantified using a TaqMan[®] Human Nuclear Receptor Array, which was a 384-well microfluid card (Applied Biosystems).

T2▶

Statistical analyses

Relative expression levels of TIP60, H3K4ac, and H3K4me3 proteins assayed by immunoblotting were assessed numerically using ImageJ software. Data analyses were performed using Prism 6 software (GraphPad Software, Inc., La Jolla, CA). Statistical significance was calculated with a one-way ANOVA test with a Multiple comparison test to assess statistical significance between condition groups of ChIP assay. A *p*-value <0.05 (*) was considered significant, *p*<0.01 (**) very significant, and a *p*<0.001 (***) highly significant, as stated in the figure legends.

Results

Effects of inhibitors (TH1834/NU9056) on cell viability

To determine the cytotoxicity of TH1834 or NU9056, chemical inhibitors of acetyltransferase TIP60 (KAT5), five breast cancer cell lines (MDA-MB-231, MDA-MB-453, MDA-MB-468, MCF-7, and T47D) and a fibrocystic dystrophy cell line (MCF-10A), were treated with increasing concentrations for 24, 48, or 72 h. An IC₅₀ study of TH1834 and NU9056, exhibited a wide disparity in their concentration and incubation times according to the cell line: the IC₅₀ values of MDA-MB-231 (TH1834: 56.71 μM at 48 h/NU9056: 29.40 μM at 72 h), MDA-MB-453 (TH1834: 48.68 μM at 48 h/NU9056: 21.14 μM at 72 h), MDA-MB-468 (TH1834: 33.34 μM at 48 h/NU9056: 31.30 μM at 72 h), T47D (TH1834: 40.53 μM at 24 h/NU9056: 11 μM at 72 h), MCF-7 (TH1834: 66.92 μM at 48 h/NU9056: 11.38 μM at 48 h), and MCF-10A (TH1834: 111.9 μM at 48 h/NU9056: 6.83 μM at 48 h) (Table 1).

Effects of inhibitors (TH1834/NU9056) on acetyltransferase activity of TIP60

In this study, we performed western blotting to investigate the effects of TH1834 and NU9056 inhibitors on the activity of the acetyltransferase TIP60 in breast cancer or fibrocystic cell lines. For that, the epigenetic mark H3k4ac, which is acetylated by TIP60 (Judes et al., 2016), was assessed by comparison with H3k4me3, used as a control (Figs. 1 and 2).

◀F1 ◀F2

The blots showed that there is no variation of H3K4me3 mark in the studied cell lines after inhibitor treatment (TH1834/NU9056), except for T47D cell line after NU9056 treatment (Figs. 1 and 2). The absence of H3K4me3 variation effectively shows that TIP60 would not have any effect on this mark. However, the expression of TIP60 is found without any variation, except for cell lines, MDA-MB-453 and 468, where there are significant increases after treatment with NU9056 and TH1834 respectively, but also significant decreases in cell lines T47D and MCF-10A after TH1834 treatment and cell line MDA-MB-231 after NU9056 treatment (Fig. 2). Furthermore, depending on the expression of TIP60, we can observe more or less significant decrease of H3K4ac mark in the different cell lines, except for the two luminal cell lines (T47D and MCF-7) after treatment with TH1834, where no variation of H3K4ac mark is observed (Fig. 1). This more or less significant decrease in H3K4ac could suggest that these inhibitors TH1834 and NU9056 would act on the acetyltransferase activity of TIP60 in breast cancer cell lines.

In addition, to test the activity of TIP60 after chemical inhibition of TH1834 or NU9056 on the promoter of genes, which are known to be enriched for H3K4ac in breast cancer

TABLE 1. DOSES OF THE INHIBITORS AND THE INCUBATION TIME REQUIRED IN THE DIFFERENT BREAST CELL LINES AS DETERMINED BY IC₅₀

TIP60 inhibitors	IC ₅₀ values (μM) of TIP60 inhibitors in breast cell lines/Incubation time (h)					
	MDA-MB-231	MDA-MB-453	MDA-MB-468	T47D	MCF-7	MCF-10A
TH1834	56.71 μM /48 h	48.68 μM /48 h	33.34 μM /48 h	40.53 μM /24 h	66.92 μM /48 h	111.9 μM /48 h
NU9056	29.40 μM /72 h	21.14 μM /72 h	31.30 μM /72 h	11 μM /72 h	11.38 μM /48 h	6.828 μM /48 h

TABLE 2. GENE LIST DESIGNED ON TAQMAN LOW-DENSITY ARRAY

<i>Genes symbol</i>	<i>Assay references</i>	<i>Gene names</i>
AR	Hs00171172_m1	Androgen receptor
ESR1	Hs00174860_m1	Estrogen receptor- α
ESR2	Hs00230957_m1	Estrogen receptor- β
ESRRA	Hs01067166_g1	Estrogen-related receptor- α
ESRRB	Hs01584024_m1	Estrogen-related receptor- β
ESRRG	Hs00155006_m1	Estrogen-related receptor- γ
NR3C1	Hs00230818_m1	Glucocorticoid receptor
NR3C2	Hs00230906_m1	Mineralocorticoid receptor
PGR	Hs00172183_m1	Progesterone receptor
NR1D1	Hs00253876_m1	Rev-ErbA α
NR1D2	Hs00233309_m1	Rev-ErbA α
NR1H2	Hs01027215_g1	Liver X receptor- β
NR1H3	Hs00172885_m1	Liver X receptor- α
NR1H4	Hs00231968_m1	Farnesoid X receptor
NR1I2	Hs01114267_m1	Pregnane X receptor
NR1I3	Hs00231959_m1	Constitutive androstane receptor
PPARA	Hs00231882_m1	Peroxisome proliferator-activated receptor- α
PPARD	Hs00602622_m1	Peroxisome proliferator-activated receptor- β/δ
PPARG	Hs00234592_m1	Peroxisome proliferator-activated receptor- γ
RARA	Hs00940446_m1	Retinoic acid receptor- α
RARB	Hs00233407_m1	Retinoic acid receptor- β
RARG	Hs00171273_m1	Retinoic acid receptor- γ
RORA	Hs00536545_m1	RAR-related orphan receptor- α
RORB	Hs00199445_m1	RAR-related orphan receptor- β
RORC	Hs01076112_m1	RAR-related orphan receptor- γ
THRA	Hs00268470_m1	Thyroid hormone receptor- α
THRB	Hs00230861_m1	Thyroid hormone receptor- β
VDR	Hs01045840_m1	Vitamin D receptor
HNF4A	Hs00230853_m1	Hepatocyte nuclear factor-4- α
HNF4G	Hs01071345_m1	Hepatocyte nuclear factor-4- γ
NR2C1	Hs00915961_m1	Testicular receptor 2
NR2C2	Hs00231489_m1	Testicular receptor 4
NR2E1	Hs01128417_m1	Homolog of the Drosophila tailless gene
NR2E3	Hs00183915_m1	Photoreceptor cell-specific nuclear receptor
NR2F1	Hs00818842_m1	Chicken ovalbumin upstream promoter-transcription factor I
NR2F2	Hs00819630_m1	Chicken ovalbumin upstream promoter-transcription factor II
NR2F6	Hs00172870_m1	V-erbA-related
RXRA	Hs00172565_m1	Retinoid X receptor- α
RXRB	Hs00232774_m1	Retinoid X receptor- β
RXRG	Hs00199455_m1	Retinoid X receptor- γ
NR0B1	Hs00230864_m1	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
NR0B2	Hs00222677_m1	Small heterodimer partner
NR5A1	Hs00610436_m1	Steroidogenic factor 1
NR5A2	Hs00187067_m1	Liver receptor homolog-1
NR4A1	Hs00374230_m1	Nerve growth factor IB
NR4A2	Hs00428691_m1	Nuclear receptor related 1
NR4A3	Hs00545009_g1	Neuron-derived orphan receptor 1
NR6A1	Hs00364256_m1	Germ cell nuclear factor
18S	Hs99999901_s1	18S ribosomal RNA
ACTB	Hs99999903_m1	Actin beta
B2M	Hs99999907_m1	Beta-2-microglobulin
GAPDH	Hs99999905_m1	Glyceraldehyde-3-phosphate dehydrogenase
GUSB	Hs99999908_m1	Glucuronidase beta
HMBS	Hs00609297_m1	Hydroxymethylbilane synthase
HPRT1	Hs99999909_m1	Hypoxanthine phosphoribosyltransferase 1
IPO8	Hs00183533_m1	Importin 8
PGK1	Hs99999906_m1	Phosphoglycerate kinase 1
POLR2A	Hs00172187_m1	RNA polymerase II subunit A
PPIA	Hs99999904_m1	Peptidylprolyl isomerase A
RPLP0	Hs99999902_m1	Ribosomal protein lateral stalk subunit P0
TBP	Hs99999910_m1	TATA-box-binding protein
TFRC	Hs99999911_m1	Transferrin receptor
UBC	Hs00824723_m1	Ubiquitin C
YWHAZ	Hs00237047_m1	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

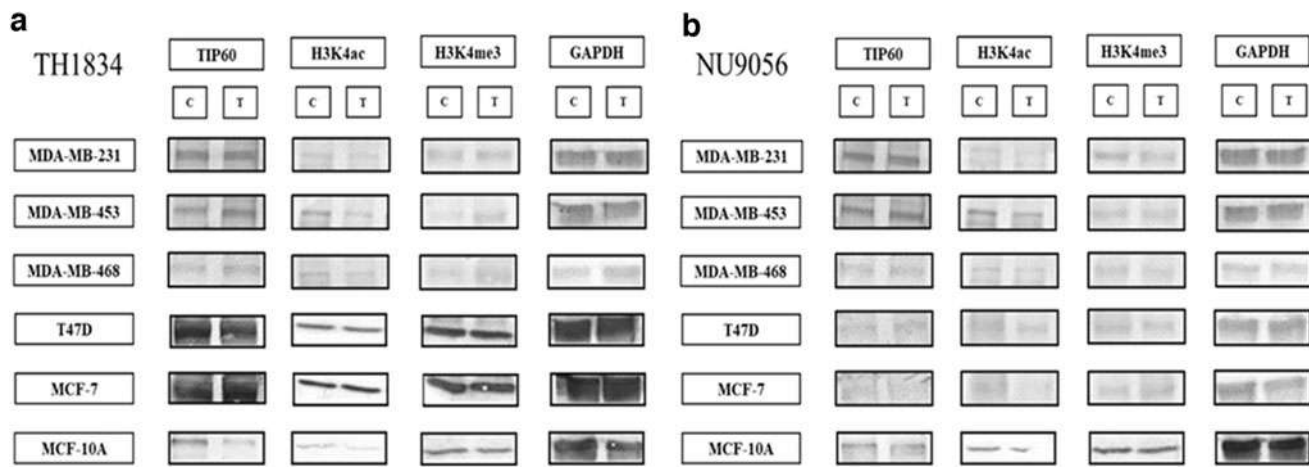


FIG. 1. TIP60, H3K4ac, H3K4me3, and GAPDH protein expressions in the different breast cancer cell lines with TH1834 or NU9056 treatment compared with control cells without any treatment. **(a)** Immunoblot of cells treated with TH1834. Slight decreases of H3K4ac were observed in the different treated cell lines; on the other hand, there is no variation in the other quantified proteins. **(b)** Immunoblot of cells treated with NU9056. Slight decreases of H3K4ac in the different treated cell lines were obtained and no variation was exhibited with the other quantified proteins. C = Control cells; T = treated cells.

development and heterochromatin formation (Judes et al., 2016, 2018), we investigated the enrichment of H3K4ac in heterochromatin (Myoglobin) and euchromatin (GAPDH) regions by ChIP-qPCR in breast cancer cell lines and a fibrocystic dystrophy cell line (Figs. 3 and 4). H3K4me3 was used as a negative control mark for TIP60 activity.

An important enrichment of H3K4me3 mark was observed specifically in the heterochromatin; this mark is known to be repressive, so more expressed on myoglobin gene (heterochromatin). In addition, no variation after NU9056 treatment was found and few variations after TH1834 treatment, except for the MDA-MB-453 and 468 breast cancer cell lines were significant for the H3K4me3 mark after treatment (Figs. 3 and 4). This absence of variation of H3K4me3 after the different treatments (TH1834 and NU9056) shows that TIP60 would not act on this mark.

On the other hand, a decrease of H3K4ac enrichment in all genes (GAPDH and Myoglobin) and in all breast cancer cell lines studied was observed for both TH1834 and NU9056 treatments (Figs. 3 and 4). This decrease in H3K4ac is significant not only on GAPDH in the cell lines MDA-MB-453 and MCF-10A for the TH1834 treatment (Fig. 3), but also on the Myoglobin gene in the MDA-MB-453 cell line for the NU9056 treatment (Fig. 4). This decrease of H3K4ac in the different chromatin compartments, namely heterochromatin (Myoglobin) and euchromatin (GAPDH) shows the role that TIP60 has on this mark and the efficacy of the two chemical inhibitors TH1834 and NU9056 on the acetyltransferase activity of TIP60.

Effects of inhibitors (TH1834/NU9056) on nuclear receptor expression

To investigate the impact of TIP60 inhibition on nuclear receptor gene expression, and identify potential new actors in breast cancer, we performed transcriptomic analysis using TLDA technology on 64 selected genes (Table 2). Gene expression was explored in breast cancer cell lines representing luminal (ER+, PR+) MCF-7 and T-47D, triple-negative

breast cancer (TNBC) (ER-, PR-, and HER2-) MDA-MB 231 and MDA-MB 468, luminal androgen receptor (LAR) (ER-, PR-, AR+ and HER2+) MDA-MB 453, and a fibrocystic dystrophy cell line MCF-10A considered as nontumor cells. RNA expression of nuclear receptors of the different cell lines treated or untreated by (TH1834/NU9056) are represented in heat map.

The nuclear receptor heat map shows as expected an underexpression of receptors such as (ER and PR) in the TNBC and LAR cell lines and an overexpression of these receptors in the luminal cell lines, the LAR cell line (MDA-MB-453) shows an overexpression of AR receptors as predicted also. The inhibition of tip60 by (TH1834/NU9056) shows a variation of certain nuclear receptors, which may be over- or underexpressed after treatment without any significance. The AR and NR3C2 nuclear receptors are found more representative for TH1834 and NU9056 treatments in TNBC cell lines.

The heat map of the mineralocorticoid receptor NR3C2 of the family of Estrogen Receptor Like shows overexpression in the TNBC cell lines (MDA-MB-231 and 468) and underexpression in the MDA-MB-453 cell line only after treatment with TH1834 (Fig. 5a). For the luminal cell lines (T47-D and MCF-7) and the nontumorigenic MCF-10A cell line we observe underexpression of the NR3C2 receptor only after treatment with NU9056 (Fig. 6b and Supplementary Fig. S7a). The Nuclear receptor subfamily 3 group C member 2 (NR3C2) is a gene involved in the hypothalamic/pituitary/adrenal axis inducing a negative feedback of the cortisol in the hypothalamus and the pituitary gland, and increasing the risk of breast cancer (Nan et al., 2015).

The nuclear AR shows underexpression in the TNBC cell lines (MDA-MB-231 and 468) and overexpression in the MDA-MB-453 cell line after TH1834 treatment (Fig. 5a). Usually in TNBC, the expression of androgen receptors is between 20% and 40%, but in the LAR cell line (MDA-MB-453) the AR expression is more important. AR is believed to be involved in tumor proliferation through EGFR (Mandel et al., 2018; Traish and Morgentaler, 2009). TIP60 is capable

F4 ► F3 ►

◄ AU7

◄ F5

◄ F6 ◄ AU8

◄ SF7

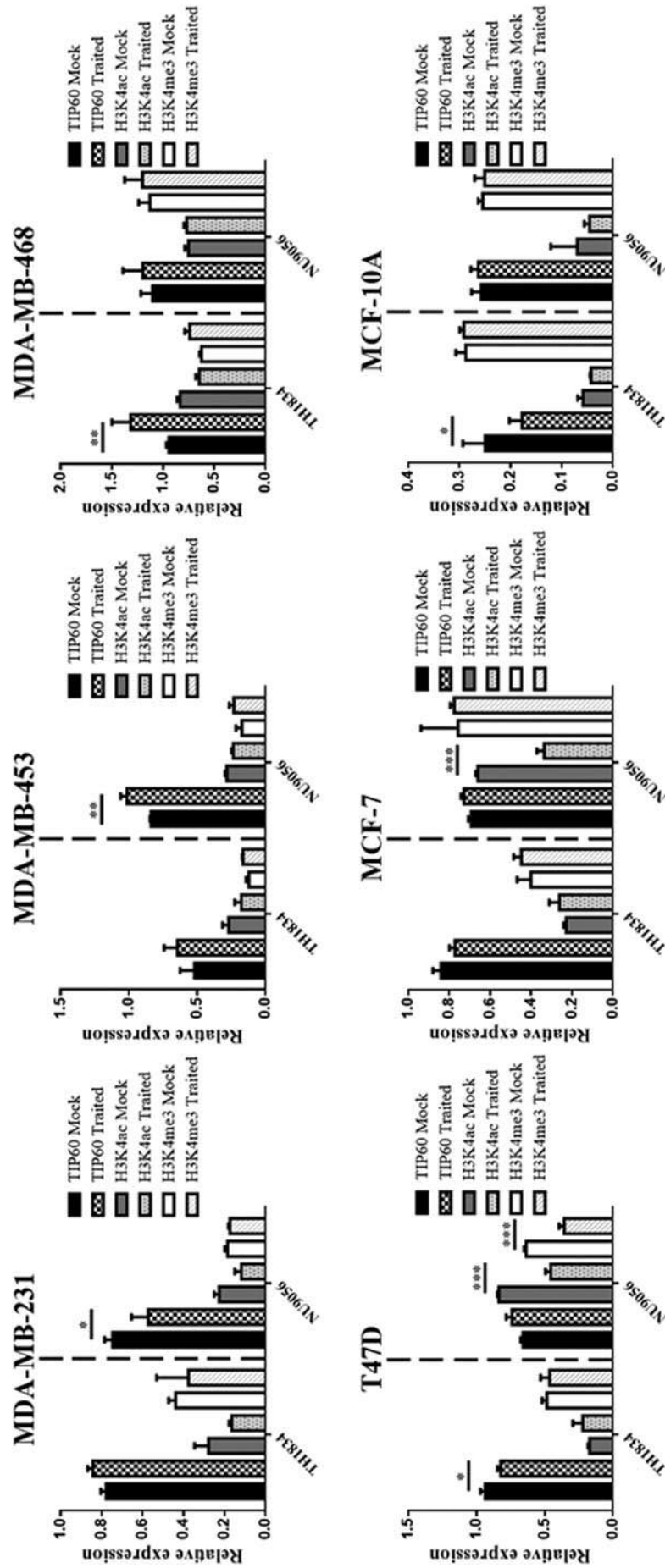


FIG. 2. TIP60, H3K4ac, H3K4me3, and GAPDH semiquantitative protein expressions corresponding to immunoblotting of the different breast cancer cell lines treated with TH1834 and NU9056. Errors are SD of triplicates (quantification was done by ImageJ). Error bars are SD of triplicates, and *p*-values according to two-tailed student's *t*-test (**p* < 0.05, ***p* < 0.01, and ****p* < 0.001). (Black bar = TIP60 without treatment, black checkerboard bar = TIP60 with treatment, gray bar = H3K4ac without treatment, gray checkerboard bar = H3K4ac with treatment, white bar = H3K4me3 without treatment, white checkerboard bar = H3K4me3 with treatment).

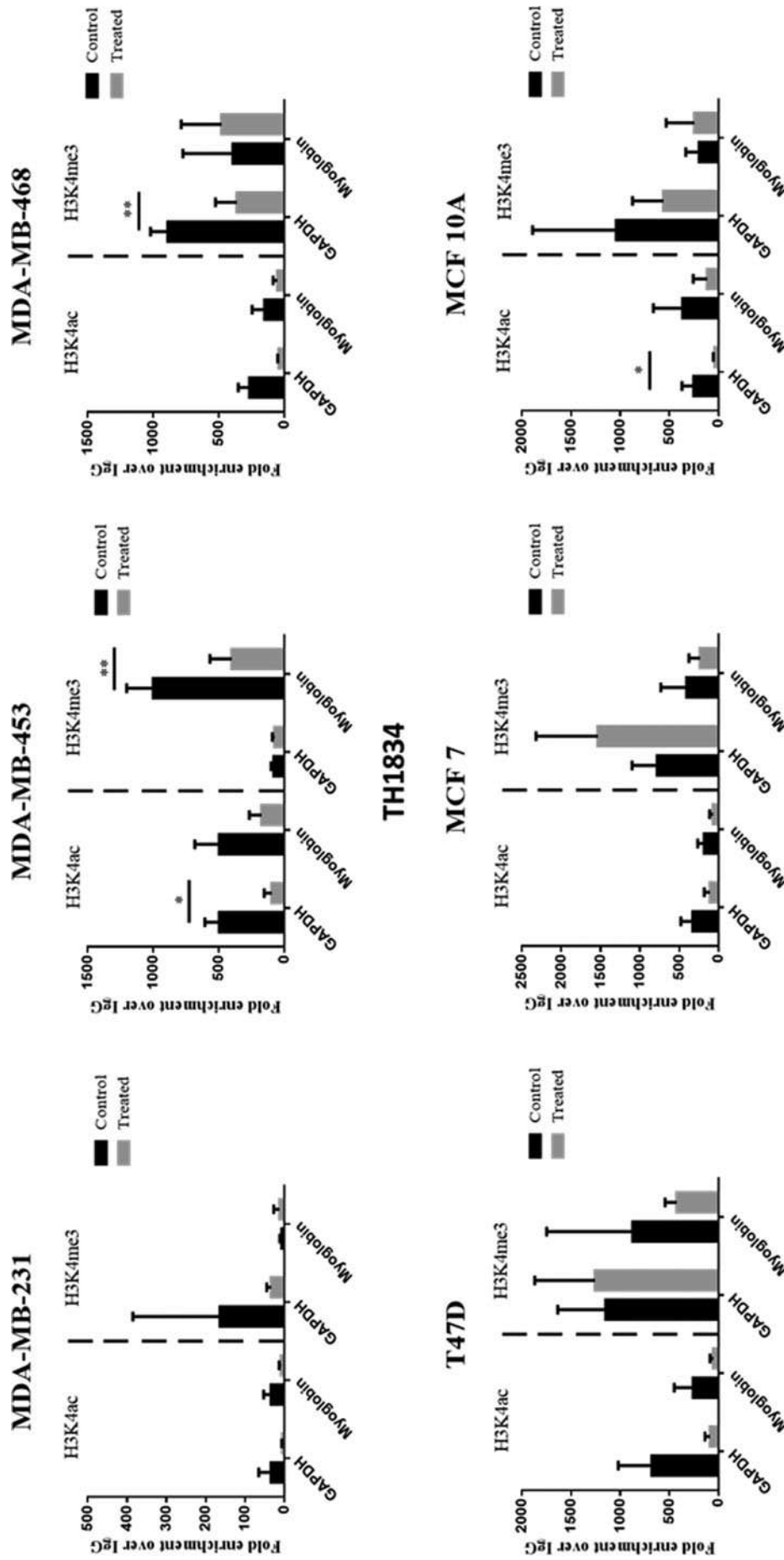


FIG. 3. Enrichment on H3K4ac in fold enrichment over IgG (illustrating the activity of TIP60) and H3K4me3 (negative control) on euchromatin (GAPDH) and heterochromatin (Myoglobin) genes in breast cancer and fibrocytic dystrophy cell lines with or without TH1834 treatment. Error bars are SD of triplicates, and *p*-values according to two-tailed student's *t*-test (*indicating $p < 0.05$ and ** $p < 0.01$). (Black bar = Control cells and gray bar = treated cells).

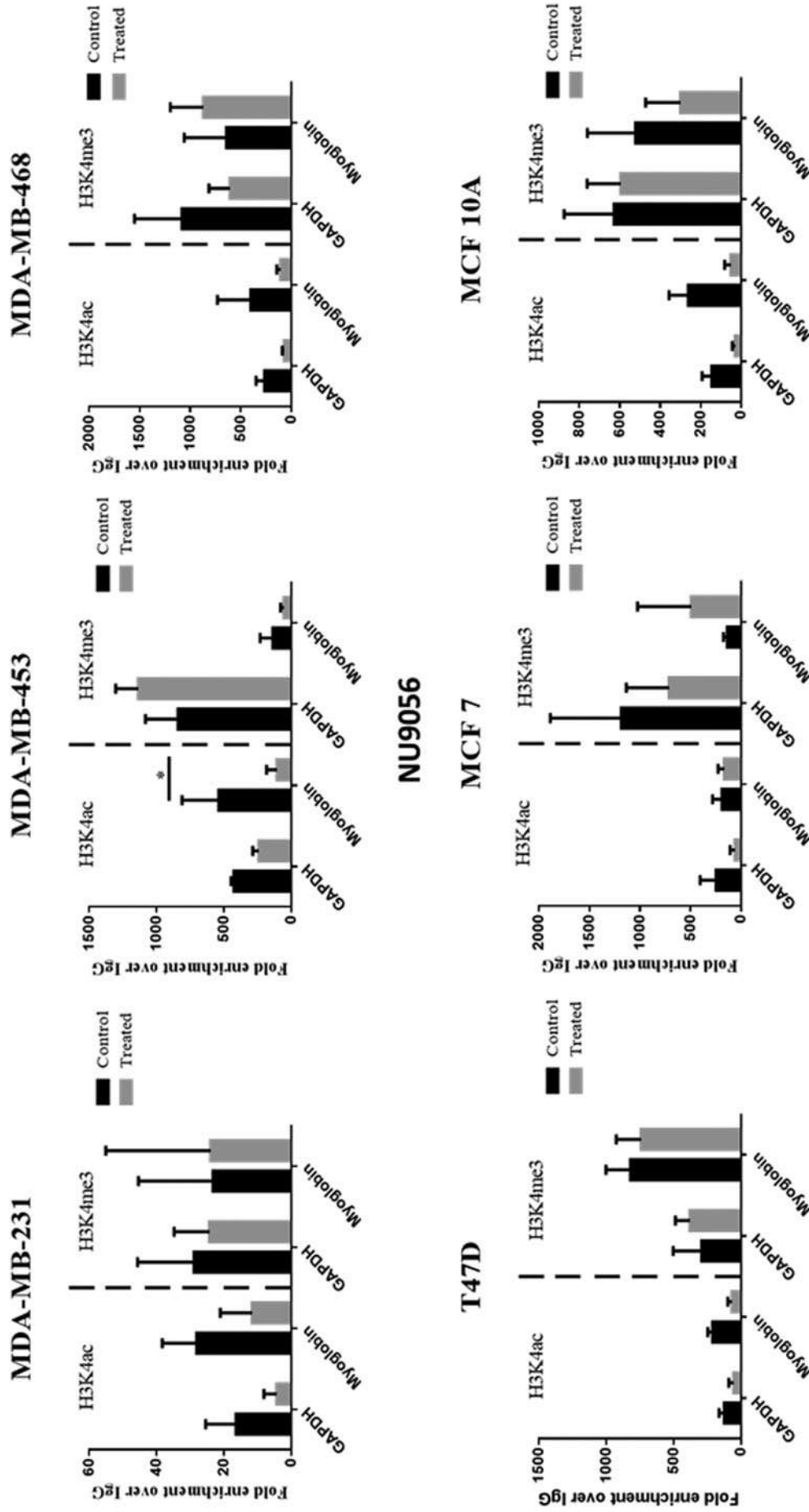
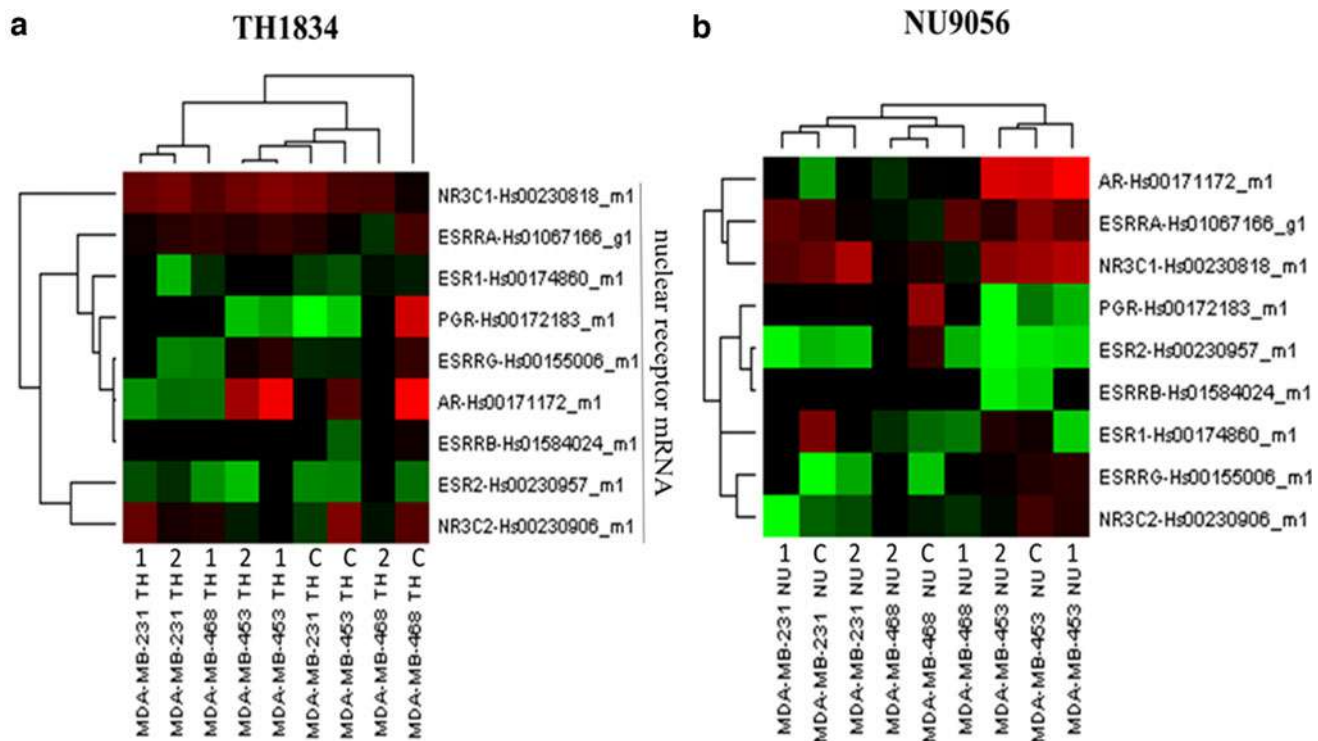


FIG. 4. Enrichment on H3K4ac in fold enrichment over IgG (illustrating the activity of TIP60) and H3K4me3 (negative control) on euchromatin (GAPDH) and heterochromatin (Myoglobin) genes in breast cancer and fibrocystic dystrophy cell lines with or without NU9056 treatment. Error bars are SD of triplicates, and *p*-values according to two-tailed student's *t*-test (*indicating $p < 0.05$). (Black bar=Control cells and gray bar=treated cells).



AU10 ▶ **FIG. 5.** The heat map of estrogen receptors with or without TH1834 or NU9056 treatment in different TNBC cell lines. **(a)** Shows expression of estrogen receptors with or without TH1834 in TNBC cell lines by Δ ct plus. **(b)** Shows expression of estrogen receptors with or without NU9056 in TNBC cell lines by Δ ct plus. (Red) overexpression and (green) underexpression. (C=Control cells; TH 1-2=treated cells with TH1834 and NU 1-2=treated cells with NU9056). TNBC, triple-negative breast cancer.

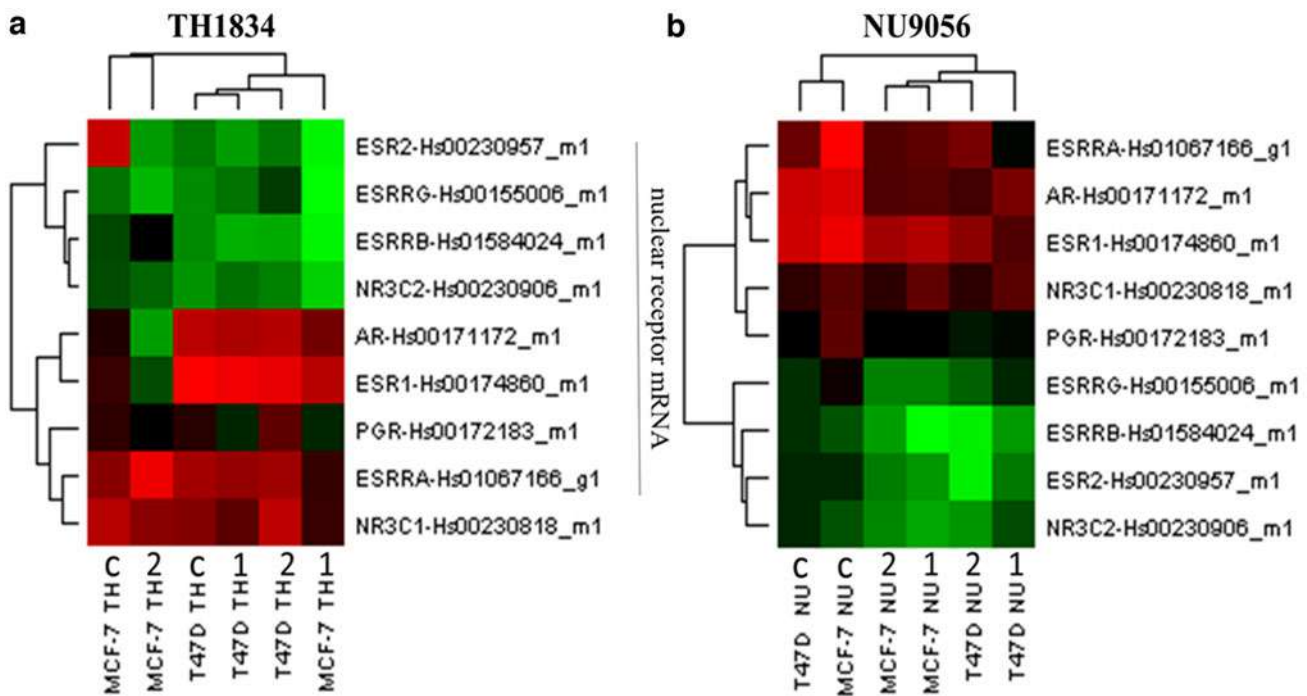


FIG. 6. The heat map of estrogen receptors with or without TH1834 or NU9056 treatment in different luminal breast cancer cell lines. **(a)** Shows expression of estrogen receptors with or without TH1834 in luminal cell lines by Δ ct plus. **(b)** Shows expression of estrogen receptors with or without NU9056 in luminal cell lines by Δ ct plus. (Red) overexpression and (green) underexpression. (C=Control cells; TH 1-2=treated cells with TH1834 and NU 1-2=treated cells with NU9056).

of acetylating AR, thus enabling it to activate the transcription of its target genes (Fu et al., 2004).

The other classified nuclear receptors represented on the heat map in different families such as Estrogen Receptor Like (SF4 ▶), Retinoid X Receptor Like (Supplementary Fig. S4), Thyroid Hormone Receptor Like (Supplementary Fig. S5), and a group consisting of the remaining families (Miscellaneous, Never Growth Factor IB-Like, Steroidogenic, and Germ Cell Nuclear Factor-Like) (Supplementary Fig. S6). These nuclear receptors show little or no variation of the expression in the different breast cancer subtypes after treatment with TH1834 and NU9056.

Discussion

In both TNBC and luminal breast cancers, there is always a slight expression of TIP60. This expression of TIP60 helps to maintain residual TIP60 activity in breast cancers. TIP60 has pleiotropic activity in several mechanisms involved in tumor growth and development, such as apoptosis, DNA repair, cell cycle, or epithelial/mesenchymal transition. For example, the diacetylation of TWIST by TIP60 allows BRD4 to bind to it and activate the recruitment of P-TEFb, which will phosphorylate the polymerase at the WNT5A promoter causing its transcription (Shi et al., 2014). WNT5A is known to be strongly linked to metastasis in several cancers (Asem et al., 2016).

Histone acetylation is the most studied and known activity of TIP60. It has been shown that the acetylation of H3 on its lysine 4 is specific to TIP60 (Judes et al., 2018); hence the interest in using this marker to follow the inhibition of TIP60 activity. The transcriptional inhibition of TIP60 by siRNAs has been extensively studied and its effects in the different mechanisms involved in breast cancer are known. On the other hand, the inhibition of its activity by chemical inhibitors such as TH1834 and NU9056 are not well known and studied. A first study in our laboratory had shown the effect of TH1834 on tumor growth in breast cancer, where we could see a decrease in tumor growth in TNBC and a decrease in tumor growth in luminal breast cancer (Idrissou et al., 2019). On the other hand, for the NU9056 inhibitor, no study has yet been done on the effects of the NU9056 inhibitor on the activity of TIP60 in breast cancer. Only one study was done on NU9056 and TIP60 but in prostate cancer (Coffey et al., 2012).

In this study, first, we verified the effects of these inhibitors on the activity of TIP60 by measuring the level of acetylation of the H3K4 marker in the different types of breast cancer. We found a decrease in this acetylation on H3K4 after treatment with TH1834 and NU9056 in the studied cell lines. This decrease in acetylation, which was more observed in ChIP-qPCR on the euchromatin GAPDH and heterochromatin Myoglobin genes, represents the decrease in TIP60 activity at the level of these genes in the different breast cancer cell lines. This is evident because TH1834 is known to be an inhibitor that binds to the acetyl-CoA-binding site. On the other hand, NU9056 binds to the active thiol site of TIP60. Then, they prevent the use of acetyl-CoA by TIP60.

Considering the link between nuclear receptor expression and breast cancer but also with TIP60, it was interesting to investigate the effects of TIP60 inhibition on the expression of the 48 existing nuclear receptors. Indeed, it is known that

TIP60 is able to play the role of coactivator or activator by acetylating some of these nuclear receptors, such as ER, AR, and NR1I2 (PXR) (Bakshi et al., 2017; Fu et al., 2004; Lee et al., 2013; Xie et al., 2009).

In this study, we were able to demonstrate by reverse transcription/qPCR (TLDA) followed by statistical analysis that most of these receptors vary shortly after treatment with TH1834 and NU9056. On the other hand, the nuclear AR receptor showed underexpression in the MDA-MB-213 and 468 cell lines and overexpression in the MDA-MB-453 cell line after treatment with TH1834, while the opposite was observed for the NR3C2 receptor. The relationship between AR and TIP60 is well known because TIP60 is capable of acetylating AR. On the other hand, the impact of these TIP60 inhibitors on AR expression would mean that TIP60 could act on AR transcription. For the NR3C2 receptor, its relationship between it and TIP60 is not well established, and we just know that it is one of the nuclear receptors involved in the occurrence of breast cancer (Nan et al., 2015).

Conclusions

This study offers new insights *in vitro* on the effects of the inhibition of TIP60 activity on nuclear receptors with an eye to deciphering the epigenetics of breast cancer further. It would be possible to follow the level of acetylation of the AR receptors by TIP60 after inhibition of its activation. This will allow a better understanding of inhibition on processes such as cell growth and survival involving the target AR genes. We can also study whether TIP60 is capable of acetylating NR3C2 or acting as a complex coactivator of this receptor. A better understanding of the acetyltransferase activity in breast cancer could allow TIP60 to be used as a biomarker or therapeutic target for breast cancer advancement.

Author Disclosure Statement

The authors declare they have no competing financial interests.

Funding Information

This work was supported by a grant from the French Ligue Régionale Contre Le Cancer–Comités du Puy-de-Dôme et de l'Allier.

Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4
Supplementary Figure S5
Supplementary Figure S6
Supplementary Figure S7

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Abbreviations Used

AR	= androgen receptor
ATCC	= American Type Culture Collection
ChIP	= chromatin immunoprecipitation
HATs	= histone acetyltransferase
HDACs	= histone deacetylase
HPA	= hypothalamic/pituitary/adrenal axis
LAR	= luminal androgen receptor
TLDA	= TaqMan Low-Density Array
TNBC	= triple-negative breast cancer

Conclusion de la publication 9:

Dans cette étude, le test de cytotoxicité des deux inhibiteurs chimiques de TIP60 nous a permis de déterminer la dose d'inhibiteur et le temps d'incubation optimal pour avoir un meilleur effet des inhibiteurs dans les différentes lignées de cellules du sein. On a obtenu :

-Lignées continues de cancer du sein triple négatif.

- MDA-MB-231 : pour TH1834 (56.71 μ M à 48h) et NU9056 (29.40 μ M à 72h).
- MDA-MB-453 : pour TH1834 (48.68 μ M à 48h) et NU9056 (21.14 μ M à 72h).
- MDA-MB-468 : pour TH1834 (33.34 μ M à 48h) et NU9056 (31.30 μ M à 72h).

-Lignées continues de cancer du sein luminales.

- T47D : pour TH1834 (40.53 μ M à 24h) et NU9056 (11 μ M à 72h).
- MCF-7 : pour TH1834 (66.92 μ M à 48h) et NU9056 (11.38 μ M à 72h).

-Lignées continues de la dystrophie fibrokystique (non cancéreuse).

- MCF-10A : pour TH1834 (111.9 μ M à 48h) et NU9056 (6.83 μ M à 48h).

Après avoir déterminé les doses et temps d'incubation des différents inhibiteurs, nous avons mesuré l'expression ou l'accumulation protéique de TIP60, de la marque H3K4ac et de la marque H3K4me3 utilisée comme témoin négatif car elle n'est pas une cible de TIP60. Ces accumulations ont été observées par western blot où l'on a pu voir dans les différentes lignées continues étudiées qu'après inhibition de TIP60 par TH1834 et NU9056, il n'y a pas de variation d'accumulation de H3K4me3 dans les différentes lignées continues étudiées. Cela confirme que TIP60 ne joue aucun rôle sur cette marque et que l'utilisation des inhibiteurs de TIP60 n'affecte pas l'expression de cette dernière.

Ensuite, on a regardé l'accumulation de TIP60 dans les différentes lignées continues étudiées et on a pu voir qu'il n'y avait pas de variation d'accumulation de TIP60 après l'inhibition de son activité par TH1834 et NU9056. Cela confirme que les inhibiteurs pourraient agir seulement sur l'activité de TIP60 et non sur son expression.

Enfin, on a pu voir que l'accumulation de la marque H3K4ac diminuait dans les lignées TNBC (MDA-MB-231, MDA-MB-453 et MDA-MB-468) et la lignée non cancéreuse (MCF-10A), alors que dans les lignées continues de cancer du sein luminales (T47D et MCF-7) on n'observe pas de grandes variations. La diminution observée dans les lignées TNBCs et fibrokystique confirme belle et bien que les deux inhibiteurs TH1834 et NU9056 agissent bien sur l'activité de TIP60.

Après avoir montré que l'utilisation des inhibiteurs TH1834 et NU9056 entraînait une diminution d'accumulation de la marque H3K4ac dans les différentes lignées continues étudiées, on a voulu voir si cette diminution était bien due à l'inhibition de l'activité de TIP60. Pour cela, nous avons effectué une ChIP-qPCR ciblant la marque H3K4ac sur le promoteur

des gènes de l'hétérochromatine (Myoglobine) et euchromatine (GAPDH) connus pour être des gènes cibles de TIP60 et la marque H3K4me3 est utilisée comme contrôle négatif. Cette expérimentation nous a permis de voir qu'il y a une grande diminution de la marque H3K4ac sur les différents promoteurs de gènes après inhibition de TIP60 par TH1834 et NU9056 dans les différentes lignées continues étudiées. Cela confirme l'efficacité des deux inhibiteurs vis-à-vis de l'activité de TIP60.

La validation de l'efficacité des inhibiteurs TH1834 et NU9056 sur l'activité de TIP60 dans les lignées continues de cancer du sein nous a poussé à regarder l'effet de cette inhibition sur l'expression des récepteurs nucléaires connus pour être dérégulée dans le cancer du sein. Cela a été réalisé par TLDA puis mesuré par qPCR et analysé par étude statistique. La qPCR nous a permis d'établir une heat-map montrant la variation d'expression d'un gène dans les cellules après inhibition par rapport aux cellules sans inhibition. Plus le gène est surexprimé plus l'expression tend vers le rouge, moins le gène est exprimé plus l'expression tend vers le vert. Sur les 48 récepteurs nucléaires étudiés seul les gènes *AR* et *NR3C2* ont montré une variation flagrante dans les lignées continues TNBCs après inhibition de l'activité de TIP60 par TH1834 et NU9056.

La heat-map de sous famille des récepteurs des hormones stéroïdes nous montre une surexpression de *NR3C2* dans les lignées continues TNBCs (MDA-MB-231 et 468) et une sous-expression dans la lignée continue MDA-MB-453 seulement après traitement avec TH1834. Pour les lignées continues de cancer du sein luminales (T47-D et MCF-7) et la lignée non cancéreuse (MCF-10A), nous observons une sous-expression du récepteur *NR3C2* seulement après traitement avec NU9056.

Alors que pour le gène du récepteur aux androgènes *AR*, on observe une sous-expression dans les lignées continues TNBCs (MDA-MB-231 et 468) et une surexpression dans la lignée continue MDA-MB-453 après le traitement par le TH1834.

1.2 Aperçu de l'inhibition chimique de l'histone acétyl-transférase TIP60 *in vivo*.

Dans l'étude précédente, l'efficacité des inhibiteurs chimiques TH1834 et NU9056 sur l'activité de TIP60 a été confirmée et validée *in vitro* dans les lignées continues de cancer du sein. Dans cette nouvelle étude, nous avons voulu voir l'effet de cette inhibition sur la croissance tumorale du sein.

Pour cela, nous avons utilisé des souris femelles nudes BALB/c dans lesquelles nous avons injecté des lignées continues de cancer du sein TNBC (MDA-MB-231) et luminal (MCF-7) co-exprimant le gène de la luciférase et le gène de résistance à la néomycine. Cette injection a été suivie d'une injection de TH1834 l'un des inhibiteurs de TIP60 ou de solution saline comme témoin pendant 10 jours consécutifs. Chaque semaine, la croissance tumorale a été mesurée et déterminée à l'aide de l'imagerie par bioluminescence, la réponse au traitement a été déterminé par western blot en regardant l'accumulation de H3K4ac. Les souris ont été sacrifiées 29 jours après l'inoculation des cellules MDA-MB-231-Luc et 55 jours après l'inoculation des cellules MCF-7-Luc.

Publication 10:

TIP60 Inhibitor TH1834 Reduces Breast Cancer Progression in Xenografts in Mice.

Idrissou M, Judes G, Daures M, Sanchez A, El Ouardi D, Besse S, Degoul F, Penault-Llorca F, Bignon YJ, Bernard-Gallon D. OMICS. 2019 Sep;23(9):457-459.

AU1 ▶

TIP60 Inhibitor TH1834 Reduces Breast Cancer Progression in Xenografts in Mice

AU2 ▶

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Keywords: breast cancer, drug targets and development, TIP60, TH1834, xenografts, *in vivo* animal research

To the Editor:

Breast cancer is one of the most common cancers worldwide and a leading cause of mortality in women. Innovation in diagnostics and rational therapeutics of breast cancer is a public health and women's health priority (Poma et al., 2017; Wang and Zhou, 2017).

TH1834 is a histone acetyltransferase (HAT) inhibitor that has a molecular structure very similar to pentamidine (Fig. 1A), and has shown antitumor effects in many studies. Its mode of action in breast cancer cell lines has been noted through the inhibition of HAT "TIP60" activity (Gao et al., 2014), which results in apoptosis and deregulation of DNA repair in cells. Moreover, the inhibition appears to be specific to TIP60 because the HAT "MOZ" activity is not affected (Gao et al., 2014).

We report in this study the effects of TH1834 *in vivo* on growth of breast cancer cell xenografts in nude mice. For this purpose, MDA-MB-231 (triple negative breast cancer [TNBC]) and MCF-7 (Luminal) cells (obtained from Cell Biolabs, Inc.) stably expressing the luciferase gene and resistant for neomycin have been used. Six-week-old female athymic mice (nu/nu genotype, BALB/c) were purchased from Janvier Labs, France, and housed under aseptic conditions. The Ethics Committee approved all protocols of this study (No. 2016010505184547091).

To test the effect of inhibiting TIP60 activity, 5 million tumor cells were injected into the right flank of the mice. This injection was followed by an injection of TH1834 (50 mg/kg) or saline as a control in 50 μ L cell culture medium and 50 μ L BD Matrigel (BD Biosciences) for 10 consecutive days. Tumor growth and response to treatment were determined once a week using bioluminescence imaging, caliper, and western blot for TIP60 activity corresponding to accumulation of H3K4ac (Fig. 1B, C). Mice were sacrificed 29 days after MDA-MB-231-Luc cell inoculation and 55 days after inoculation for MCF-7-Luc cells.

The tumor growth of xenografts in the group of mice that received MCF-7-Luc cells measured by bioluminescence and calipers showed a significant decrease in tumor area in mice treated with TH1834 compared with control mice ($p < 0.01$) (Fig. 1B-1, 2). Mice that received MDA-MB-231-Luc cells also showed a significant decrease in tumor area in mice treated with TH1834 compared with control mice ($p < 0.05$) (Fig. 1C-1, 2). The effects of the treatment was evaluated further by western blot analyses that allowed observing the protein accumulation of H3K4ac in the xenografts of both cell lines (Fig. 1B-3, C-3). A slight decrease in H3K4ac accumulation was exhibited in MCF-7 xenografts treated with TH1834 compared with control with saline (Fig. 1B-3). In contrast, in MDA-MB-231 xenografts there was no evident difference in the accumulation of the mark H3K4ac compared with the controls (Fig. 1C-3).

Interestingly and unexpectedly, we observed that the inhibition of TIP60 resulted in reduced tumor growth profiles in the two different mammary xenografts in mice. Some studies have reported that TIP60 deletion promotes mammary tumorigenesis (Bassi et al., 2016). The group of mice that received the MCF-7-Luc cells not only showed a significant decrease in tumor growth after TIP60 inhibition by TH1834, but also showed a decrease in the H3K4ac marker in the corresponding xenografts (Fig. 1B). These results corroborate previous results in the laboratory by depletion of TIP60 with anti-TIP60 ShRNAs (Judes et al., 2018), MCF-7 is characterized by hormone receptor expression and present no mutation of the p53 gene. In contrast to its function in DNA damage repair processes by ATM acetylation and activation of p53-dependent genes, the role of TIP60 in the estrogen receptor (ER) signaling pathway seems to be involved in growth reduction. Hence, Jeong et al. (2011) have shown, for example, that TIP60 is necessary for hormonal induction of certain target genes of ER α and also on transcription of ER α . It might follow, then, that TIP60 intervenes in a mechanism

AU3 ▶

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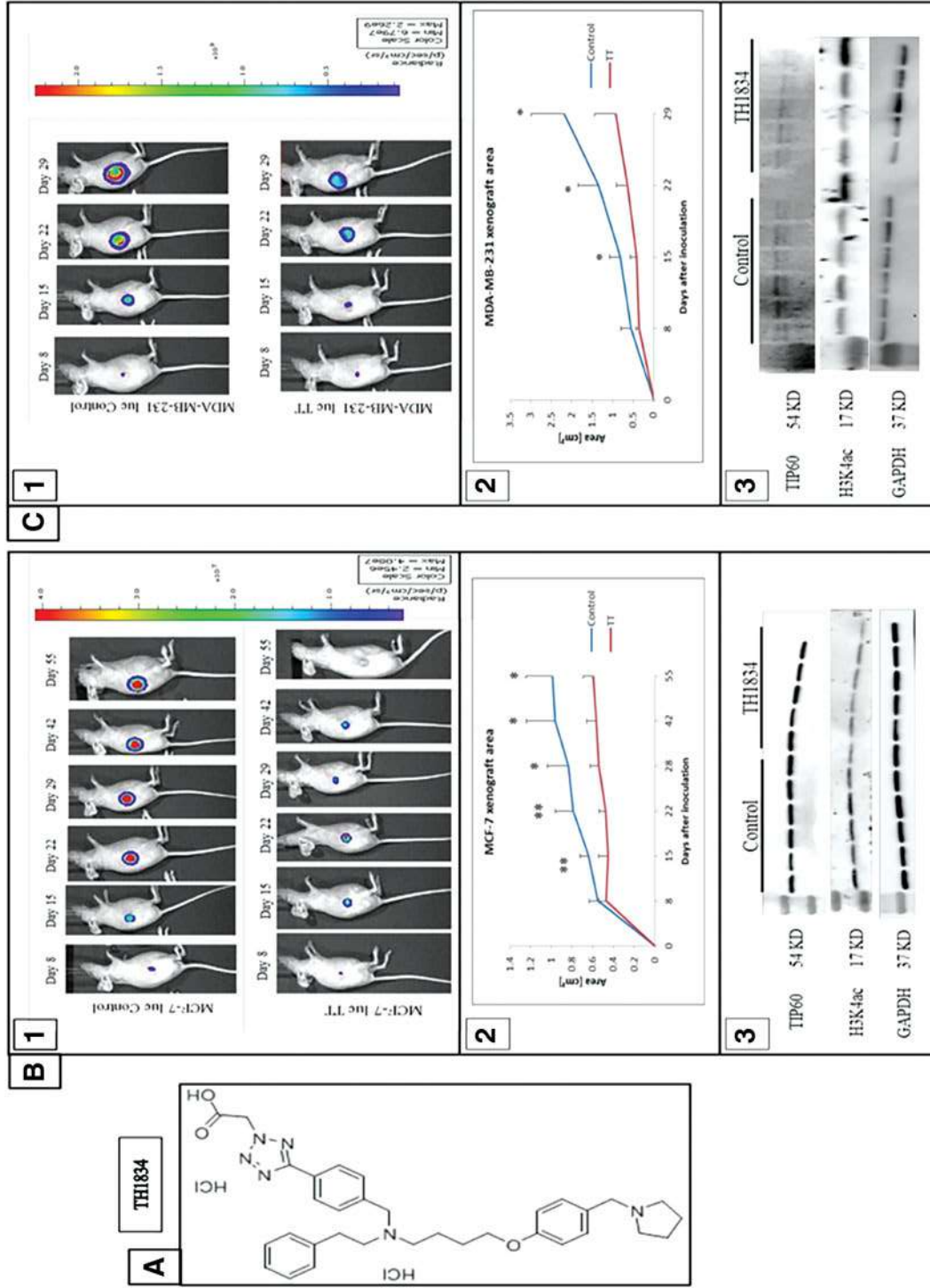


FIG. 1. (A) Molecular structure of TH1834. (B) Measure of tumor growth by bioluminescence, the size of tumors by the caliper and western blot of xenograft protein extracts of MCF-7-Luc. (B1) BALB/c, nu/nu mice received 5×10^6 MCF-7-Luc breast tumor cells injected into the right flank. On days 8, 15, 22, 29, 42, and 55, mice were injected with 2 mg of luciferin. Acquisition of bioluminescence images. (B2) Measure of tumor area on mice after inoculation of MCF-7 cells treated with TH1834 ($n=6$) and control ($n=6$). Data are expressed as means for each group, and error bars indicate the 95% confidence interval of the mean value. Data were analyzed by paired *t*-test ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$). TIP60 inhibition reduces development of (MCF-7) luminal breast cancer xenografts. (B3) Western blots to detect TIP60 and H3K4ac in breast cancer xenografts (MCF-7). Representative quantification was expressed as relative fold change in protein expression of TIP60 and H3K4ac after normalization to actin density. (C) Measure of tumor growth by bioluminescence, measuring the size of tumors with the caliper and western blot of xenograft protein extracts of MDA-MB-231-Luc. (C1) BALB/c, nu/nu mice received 5×10^6 MDA-MB-231-Luc breast tumor cells injected into the right flank. On days 8, 15, 22, and 29, mice were injected with 2 mg of luciferin. Acquisition of bioluminescence images. (C2) Measure of tumor area on mice after inoculation of MDA-MB-231 cells treated with TH1834 ($n=4$) and control ($n=4$). Data are expressed as means for each group, and error bars indicate the 95% confidence interval of the mean value. Data were analyzed by paired *t*-test ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$). TIP60 inhibition reduces development of (MDA-MB-231) triple-negative breast cancer *in vivo*. (C3) Western blots to detect TIP60 and H3K4ac in breast cancer xenografts (MDA-MB-231). Representative quantification was expressed as relative fold change in protein expression of TIP60 and H3K4ac after normalization to actin density.

AU6

AU7

of chromatin remodeling by methylation allowing the recruitment of HMT and acetylation to activate the transcription of different target genes.

In contrast, the group of mice that received the MDA-MB-213-Luc cells displayed a slowdown in tumor growth after TIP60 inhibition by TH1834 (Fig. 1C). These results contrasted with expected and previous results demonstrating a significant increase in tumor growth in mice injected with ER negative MDA-MB-231 cells where expression of TIP60 was inhibited by ShRNA anti-TIP60 (Judes et al., 2018).

In this study, TIP60 was inhibited by a chemical, TH1834, through deprivation of acetyl-CoA. Thus, the different levels of transcriptional and translational inhibition could explain the different profiles observed between Sh and chemical inhibition of TIP60. Kai et al. (2015) have reported that inhibition or removal of acetylation in TNBC cell lines led to an increase in tumor growth. For example, histone deacetylase (HDAC) inhibition by inhibitors such as panabinstat and salinomycin in TNBC cell lines expressing ALDH1 led to decrease in tumor growth, apoptosis, cell cycle arrest, and regulation of epithelial mesenchymal transition (Kai et al., 2015). Further research is warranted to better explore other putative mechanisms in MDA-MB-231 xenografts.

In conclusion, TH1834 inhibitor of TIP60 reduces the progression of breast cancer cell xenografts supporting the idea that TIP60 is involved in breast cancer tumorigenesis and induces a decrease of tumor growth. TH1834 appears to act like an epidrug and thus might open new vistas in novel drug discovery and development for breast cancer.

AU4 ► Acknowledgment

Gaëlle Judes was a laureate for the thesis prize of “La Ligue Contre Le Cancer, Comité du Puy de Dôme” in 2018.

Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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Abbreviations Used

ALDH1 = aldehyde dehydrogenase 1 family
ATM = ataxia telangiectasia mutant
ER = estrogen receptor
H3K4ac = histone 3 lysine 4 acetylated
HAT = histone acetyltransferase
HDAC = histone deacetylase
MOZ = monocytic leukemia zinc finger protein
p53 = protein 53 kDa
ShRNA = short hairpin RNA
TIP60 = TAT-interactive protein 60 kDa
TNBC = triple negative breast cancer

Conclusion de la publication 10:

Les résultats obtenus dans cette étude nous ont permis de clarifier un peu plus le rôle ou l'impact de TIP60 dans le cancer du sein *in vivo*. Grâce au western-blot, on a pu valider l'efficacité de l'inhibiteur TH1834 sur l'activité de TIP60 dans le cancer sein. Cette inhibition a été observée par la diminution de l'accumulation d'H3K4ac dans les souris qui ont reçu les cellules MCF-7-Luc. Par contre, dans les souris injectées avec les cellules MDA-MB-231-Luc on n'observe pas de variation d'accumulation de la marque.

De plus, on a pu voir que l'inhibition de l'activité de TIP60 avait un effet sur la croissance tumorale des xénogreffes. Dans les xénogreffes MCF-7-Luc (luminal) on observe une diminution importante de la croissance tumorale, alors que dans les xénogreffes MDA-MB-231-Luc (TNBCs) on observe un ralentissement de la croissance tumorale. Cela laisse suggérer que TIP60 joue un rôle de suppresseur de tumeurs dans les différents types moléculaires de cancer du sein *in vivo*.

2 Rôle du complexe Tip60/P400/H4K12ac dans la plasticité et l'intégrité chromatinienne impliquée dans la Carcinogénèse

Pour la deuxième question posée qui est : « Quel est le rôle de TIP60 dans la plasticité et l'intégrité chromatinienne lors de la carcinogénèse du sein ? », nous avons utilisé plusieurs stratégies pour répondre à cette question.

Notre première stratégie consiste à déterminer le taux d'enrichissement de H4K12ac dans les différents compartiments chromatiniens dans les tumeurs du sein (*ex vivo*) et dans les lignées continues mammaires tumorale ou non (*in vitro*), parce que dans la littérature, la marque H4K12ac est présente dans l'hétérochromatine dans les cancers comme le cancer colorectal.

La seconde stratégie est de confirmer une co-localisation entre TIP60 et H4K12ac *ex vivo* et *in vitro*, ce qui laissera à penser que TIP60 est responsable du dépôt de cette marque dans le cancer du sein.

La dernière stratégie consiste à observer l'accumulation des différents acteurs formant le complexe de maintien de la plasticité et de l'intégrité chromatinienne qui sont TIP60, P400 et H4K12ac. Cette accumulation est comparée entre les différents types moléculaires de cancer du sein *ex vivo* ou *in vitro*. L'observation de l'accumulation permettra aussi de voir s'il y a une corrélation entre l'expression de TIP60 et celle de H4K12ac.

Enfin une dernière stratégie a été utilisée pour voir s'il y avait une influence fonctionnelle entre TIP60 et P400. Pour cela nous avons inhibé l'expression de TIP60 ou P400 par SiRNA et ensuite nous avons inhibé l'activité de TIP60 par les inhibiteurs chimiques de ce dernier TH1834 et NU9056. Cela pour suivre l'effet de ces différentes inhibitions sur l'expression de P400 et de H4K12ac.

2.1 Le rôle de TIP60 dans la plasticité et l'intégrité chromatinienne lors de la carcinogénèse du sein ex vivo.

Dans la première étude de cet axe, pour pouvoir répondre à la question posée, nous avons utilisé 77 tumeurs de sein et leurs tissus sains adjacents. Ces tissus proviennent de patientes qui n'ont reçu ni chimiothérapie, ni radiothérapie. Ces tumeurs sont de trois sous types moléculaire de cancer du sein différents qui sont Luminal A, Luminal B et les TNBCs.

La première stratégie consistait à regarder l'enrichissement en H4K12ac dans les différents compartiments chromatiniens et dans les différents sous types moléculaire de cancer du sein. Pour cela, une ChIP-qPCR a été réalisée sur les tissus de sein et les niveaux d'enrichissement en H4K12ac des gènes de l'hétérochromatine (*Myoglobine*, *SATa* et *SAT2*) et de l'euchromatine (*GAPDH* et *ADH5*) ont été déterminés.

Ensuite, nous avons réalisé une ChIP-re-ChIP comme seconde stratégie pour voir si on a une Co-localisation de TIP60 et H4K12ac aux niveaux de ces gènes et si l'enrichissement observé est bien dû à une activité de TIP60.

Enfin, nous avons regardé l'accumulation protéique TIP60, P400 et H4K12ac par western blot, mais aussi mesuré l'expression ARN de TIP60 et P400 par RT-qPCR. Dans le but de comparer la variation d'expression des différents acteurs dans les différents sous types moléculaires de tumeur de sein.

Publication 11:

TIP60/P400/H4K12ac play a role as a heterochromatin back-up skeleton in breast tumor. **Idrissou M**, Boisnier T, Sanchez A, Houfak Khoufak F Z, Penault-Llorca F, Bignon YJ and Bernard-Gallon D. Cancer Genomics Proteomics. 2020 Nov-Dec;17(6):687-694. doi: 10.21873/cgp.20223.

TIP60/P400/H4K12ac Plays a Role as a Heterochromatin Back-up Skeleton in Breast Cancer

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Abstract. *Background/Aim:* In breast cancer, initiation of carcinogenesis leads to epigenetic dysregulation, which can lead for example to the loss of the heterochromatin skeleton SUV39H1/H3K9me3/HP1 or the supposed secondary skeleton TIP60/P400/H4K12ac/BRD (2/4), which allows the maintenance of chromatin integrity and plasticity. This study investigated the relationship between TIP60, P400 and H4K12ac and their implications in breast tumors. *Materials and Methods:* Seventy-seven patients diagnosed with breast cancer were included in this study. Chromatin immunoprecipitation (ChIP) assay was used to identify chromatin modifications. Western blot and reverse transcription and quantitative real-time PCR were used to determine protein and gene expression, respectively. *Results:* We verified the variation in H4K12ac enrichment and the co-localization of H4K12ac and TIP60 on the euchromatin and heterochromatin genes, respectively, by ChIP-qPCR and ChIP-reChIP, which showed an enrichment of H4K12ac on specific genes in tumors compared to the adjacent healthy tissue and a co-localization of H4K12ac with TIP60 in different breast tumor types. Furthermore, RNA and protein expression of TIP60 and P400 was investigated and overexpression of TIP60 and P400 mRNA was associated with tumor aggressiveness. *Conclusion:* There is a potential interaction between H4K12ac and TIP60 in heterochromatin or euchromatin in breast tumors.

Breast cancer is the most frequent cancer (24.2% new cases in 2018) and the leading cause of cancer-related death (15% in 2018) (1). The majority of breast cancers are sporadic (non-hereditary), representing 80 to 90% of breast cancers (2). Deregulation of chromatin integrity and plasticity is among the causes of sporadic cancer (3). They occur at the epigenetic level in the different compartments of chromatin, which are heterochromatin (compacted chromatin) that inhibits gene expression, and euchromatin (relaxed chromatin) that promotes gene transcription. These chromatin states are maintained by so-called epigenetic proteins, which, by placing groups or marks on the histones, allow entry or exit from one state to another (4-6).

Among these proteins, SUV39H1, which is a histone methyltransferase (HMT) that adds the H3K9me3 marker on the site where the HP1 protein will bind, thus forming the SUV39H1/H3K9me3/HP1 skeleton which keeps the chromatin closed and inhibits transcription in the heterochromatin region (7, 8). Heterochromatin houses inactive genes such as numerous oncogenes; the loss of this structure would lead to the transcription of oncogenes. Several studies have shown a loss of the SUV39H1 protein in cancer, but also a loss of the SUV39H1/H3K9me3/HP1 skeleton during the onset of carcinogenesis (7, 9).

The acetyl-transferases (HAT) such as TIP60 are able to add acetyl groups on the N-terminal tails of histones resulting in the opening of the chromatin leading to euchromatin (10). TIP60 is also capable of acetylating non-histone proteins such as p53 and ATM allowing their activation (11-13). Many studies have shown that histone acetylation by TIP60 is observed in euchromatin and heterochromatin, allowing the maintenance of these structures (14). Indeed, TIP60 can be recruited at the heterochromatin due to its chromo-domain capable of binding to H3K9me3 or H3K9me groups (15), but can also be recruited via its complex with the H2Az variant into the heterochromatin by the ATPase P400 (16, 17). This recruitment would compensate for the loss of the heterochromatin maintenance

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Key Words: Breast cancer tumor, TIP60, H4K12ac, P400, heterochromatin, euchromatin.

skeleton (SUV39H1/H3K9me3/HP1) by the overexpression of histone demethylase. Grézy *et al.* found that treatment of SUV39H1/2 and NIH3T3 cells with azacytidine resulted in an enrichment of H4K12ac, TIP60, P400 and BRD2 on the heterochromatin (16). This enrichment revealed the existence of a back-up skeleton for the heterochromatin that comprised TIP60/ H4K12ac/BRD2. Indeed, bromo-domain proteins such as BRD2 and BRD4 are able to bind to the H4K12ac mark preventing the detachment of chromatin (18). In breast cancer, a decrease in the protein levels and a residual activity of TIP60 has been observed (19).

In this study, we hypothesized that the H4K12ac modification in heterochromatin and euchromatin is realized by TIP60 and its complex especially with P400, thus allowing maintenance of chromatin stability and integrity. This recruitment of TIP60 could explain its presence in heterochromatin and thus, the dysregulation of the acetylation of p53 and tumor suppressors.

The study of this residual activity of TIP60 in the maintenance of chromatin structures will allow a better understanding of its role in the aggressiveness of cancers and especially of breast cancer.

Materials and Methods

Clinical samples. This study included 77 patients admitted to the Centre Jean Perrin from September 2008 to February 2019, and diagnosed with breast cancer (Table I). Patients were informed about the study and gave informed consent prior to inclusion. All 77 tumors and their adjacent normal breast tissues were obtained from the Centre Jean Perrin tumor bank, Biological Resource Center (CRB), accredited under No.BB-0033-00075, where they were stored in liquid nitrogen at -196°C . The 77 patients selected did not receive chemotherapy and/or radiotherapy, were not predisposed to breast cancer and had no family members with breast cancer.

Chromatin immunoprecipitation (ChIP) assays and Quantitative real-time PCR method and data analysis. ChIP assays were performed on chromatin extracted from breast tumors using the Auto iDeal ChIP-seq kit for Histones (C01010171, Diagenode, Seraing, Belgium) according to manufacturer's instructions and the experimental setup developed in our laboratory (20, 21). The antibodies used were 3 μg of anti-H4k12ac Abs (C15200218, Diagenode) and 1 μg non-immune rabbit IgG (Kch-504-250, Diagenode) serving as a negative control.

For Re-ChIP assays, the immunoprecipitated DNA from the first ChIP assay was used. The second ChIP assay (re-ChIP) was then carried out using 3 μg of anti-TIP60 Abs (SC-166323, Santa Cruz Biotechnology, Dallas, TX, USA) according to the experimental setup developed in our laboratory (20, 21). The quality control and efficacy of all ChIP assays performed in this study were verified using positive and negative controls provided in the manufacturer's kit and according to their instructions (Diagenode). Control of ChIP analysis was performed prior to direct H4K12ac ChIP assays and prior to H4K12ac and TIP60 ChIP and re-ChIP assays.

Real-time qPCR was performed in triplicate at 25 μl final reaction volume that included 5 μl of IP or input, 1 \times de SYBR[®]

Table I. Characteristics of the breast cancer patients included in this study.

	Age		Grade			Ki67	
	<50	>50	I	II	III	≤20	>20
Luminal A	2	26	7	13	8	18	10
Luminal B	1	27	19	5	4	17	9
TNBC	1	20	0	6	15	4	17

Green PCR Master Mix (Applied Biosystems, CA, USA), 200 nM for each GAPDH (C17011047, Diagenode) and Myoglobin Exon 2 primers (C17011006, Diagenode), on a 7900 HT Fast Real Time PCR System (Applied Biosystems).

Protein extraction and immunoblot analysis. Breast cancer tumors and their adjacent healthy tissues were crushed with the crusher (french press) and protein extracts were obtained using NucleoSpin[®] RNA/Protein, Mini kit for RNA and protein purification (Macherey-Nagel, Hoerd, France). About 25-40 μg of extracted proteins were resolved by electrophoresis on 8-15% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) (Bio-Rad, Hercules, CA, USA). Electro-transfer, blockage of membranes, immunoblotting and immunolabeling were performed using the manufacturer's protocol and the experimental methods developed in our laboratory (20, 22). The primary antibodies (Abs) used were: anti-TIP60 Abs (1/500, GTX112197) and anti-P400 Ab (1/500, GTX116689) were purchased from GenTex (CA, USA), anti-H4k12ac Abs (1/750, C15200218, Diagenode) and anti- GAPDH Abs (1/5000, sc-47724, Santa Cruz Biotechnology). The secondary Abs: anti-mouse IgG (1/2000, S3721) and anti-rabbit IgG (1/2000, S3738) were purchased from Promega (Madison, WI, USA).

RNA extraction, reverse transcription (RT) and quantitative real-time PCR analysis. Breast tumors and their adjacent healthy tissues were crushed at the french press. RNA extracts were obtained using NucleoSpin[®] RNA/Protein, Mini kit for RNA, protein purification (Macherey-Nagel, France) and reverse transcription were performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and Synthesized cDNAs were amplified using TaqMan Gene expression PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol and the experimental methods developed in our laboratory (20, 22). The TaqMan Gene Expression assay-on-demand used were TIP60 [Hs00197310_m1] or P400 [Hs01566078_m1] and endogenous control 18S Rna [Hs99999901_s1].

Statistical analyses. Relative expression levels of TIP60, p400 and H4K12ac protein assayed by immunoblotting were quantified using ImageJ software. Data analyses were performed using Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was calculated by One-Way ANOVA test with a multiple comparison test to assess statistical significance between groups. A p -value <0.05 (*) was considered significant, $p < 0.01$ (**) very significant and a $p < 0.001$ (***) highly significant, as stated in the figure legends.

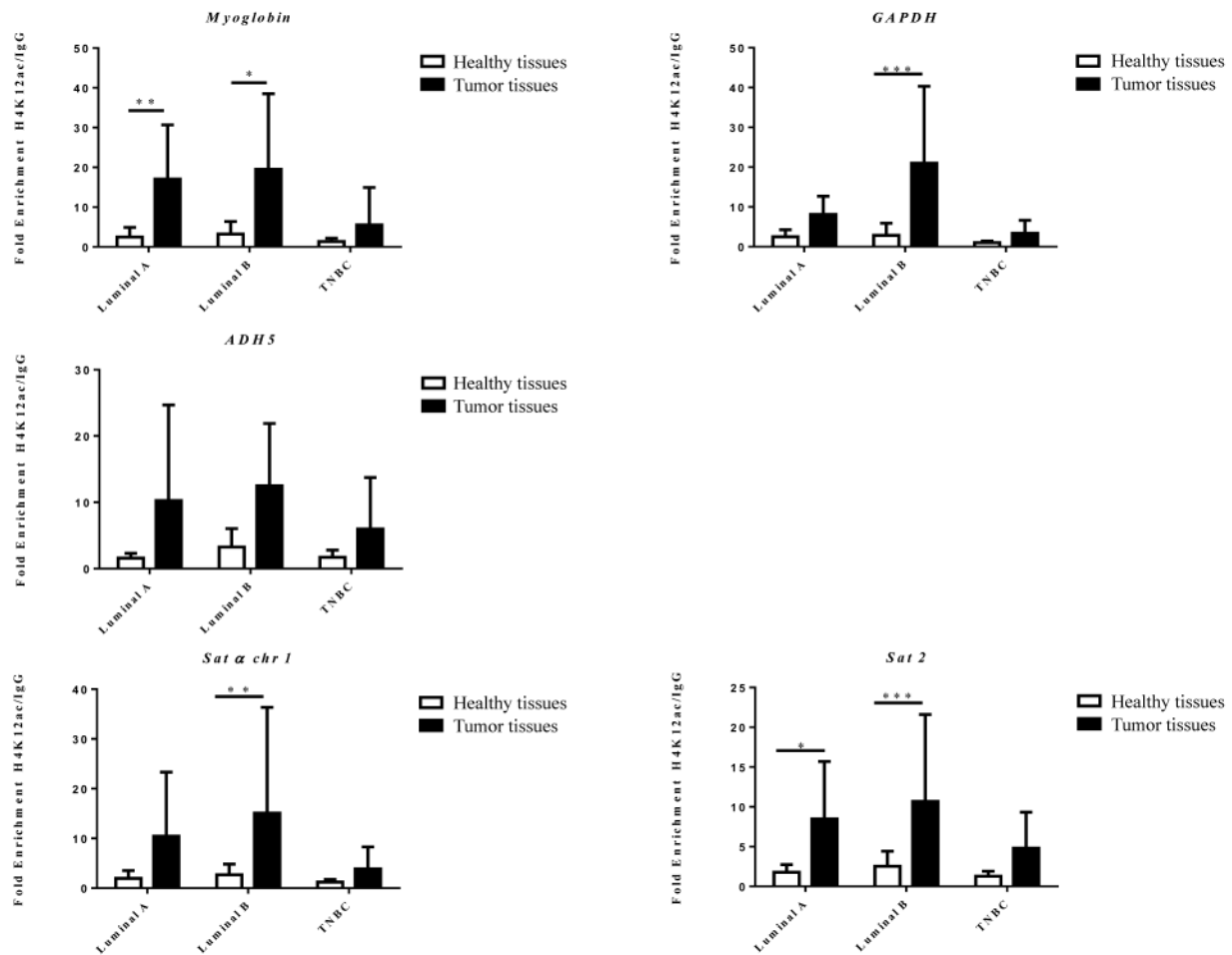


Figure 1. Fold enrichment in H4K12ac over IgG (illustrating the presence of H4K12ac on promoters in euchromatin genes (GAPDH and ADH5) and in heterochromatin (Myoglobin, SAT α and SAT 2 genes) in adjacent healthy breast tissues and Luminal A, Luminal B and TNBC breast tumors. Results are expressed as fold increase \pm SD of (N=19, N=19 and N=12, for Luminal A, Luminal B and TNBC, respectively). p-values were calculated by two-tailed students t-test (* indicating $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). Black bar: breast tumors and white bar: healthy breast tissues.

Results

H4K12ac enriched on genes in euchromatin and heterochromatin in breast tumors. To observe H4K12ac enrichment on the promoters in euchromatin (GAPDH and ADH5) and heterochromatin (Myoglobin, SAT α and SAT2) genes in breast cancer, a ChIP-qPCR was performed on 19 Luminal A, 19 Luminal B and 12 triple negative breast cancer (TNBC) tumors and their adjacent healthy tissues (Figure 1). An enrichment of H4K12ac was found in different euchromatin and heterochromatin genes in almost equal proportions in healthy breast tissues (Figure 1), whereas this enrichment was significantly increased in Luminal A and B tumors compared to their adjacent healthy tissues (Figure 1). In TNBC tumors, this enrichment increased slightly compared to healthy tissues, and a decrease in enrichment can be seen

in TNBC compared to Luminal tumors (Figure 1). These results suggest that there is an equal distribution of H4K12ac in the different chromatin compartments in healthy breast tissues, but this marker is highly increased in Luminal tumors and slightly in TNBC tumors.

Co-localization of H4K12ac and TIP60 in breast tumors. To determine whether TIP60 is responsible for the H4K12ac modification, we performed ChIP-reChIP and examined whether they co-localized in euchromatin (GAPDH and ADH5 genes) and in heterochromatin (Myoglobin, SAT α and SAT 2 genes) (Figure 2). A significant high co-localization was obtained in healthy tissues compared to tumors and this co-localization decreased significantly in luminal A and TNBC tumors. In luminal B tumors, the co-localization of H4K12ac and TIP60 was more important than

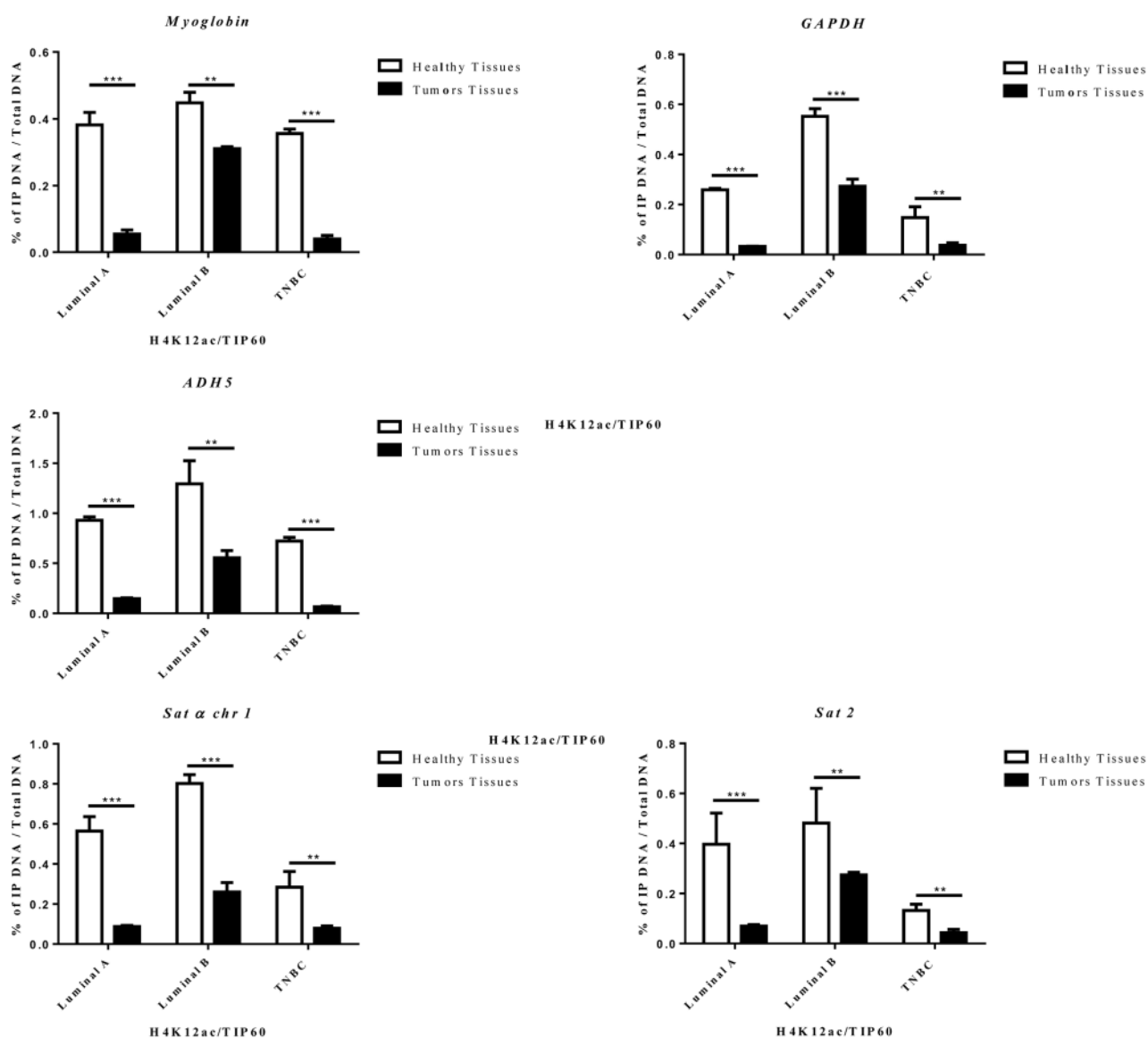


Figure 2. Co-localization of H4K12ac and TIP60 in promoter regions of target genes on euchromatin (GAPDH and ADH5) and heterochromatin (Myoglobin, SATα and SAT 2) in adjacent healthy breast tissues and breast tumors Luminal A, Luminal B and TNBC. Results are expressed as percentage ±SD of six tumor and healthy tissues. p-values calculated by two-tailed students t-test (* indicating $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). Black bar: breast tumors and white bar: healthy breast tissues.

in other types of tumors. This could suggest that TIP60 is recruited during the carcinogenesis induction, because in the less aggressive tumors there is an increase in H4K12ac mark and co-localization of TIP60 and H4K12ac from Luminal A to Luminal B tumors. This recruitment is lost in the most aggressive TNBC tumors, which could be due to the high decrease in TIP60 expression in these aggressive tumors.

Variations in the expression of TIP60 and P400 in breast tumors. After observing the variations in the enrichment of

the H4K12ac mark and the co-localization of TIP60 with this mark, TIP60 and P400 mRNA and protein accumulation in breast tumors and adjacent healthy tissues was examined. RNA expression was studied by RT-qPCR, which demonstrated a significantly lower expression of the mRNA of TIP60 and P400 in Luminal tumors, while a significant overexpression of TIP60 and P400 mRNA was observed in TNBCs tumors (Figure 3). The expression of TIP60 and P400 proteins as well as the H4K12ac modification in breast tumors and their adjacent healthy tissues were studied by

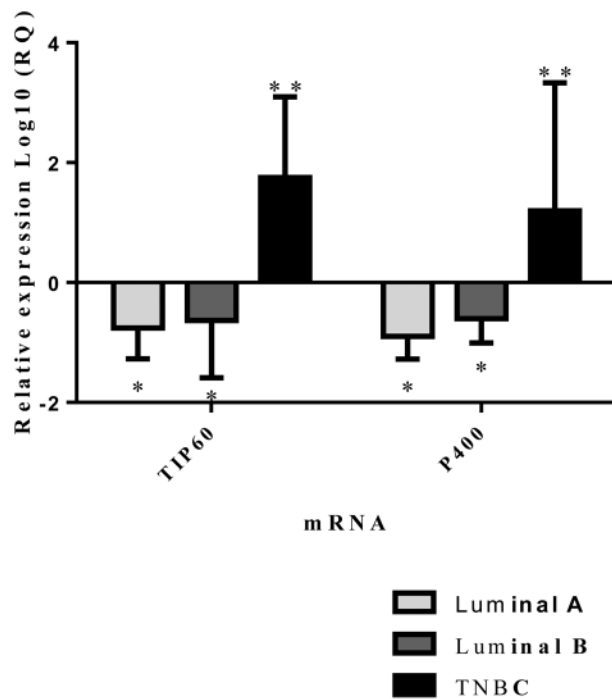


Figure 3. Relative RNA expression of TIP60 and P400 of breast tumors (Luminal A, Luminal B and TNBC) compared to their healthy adjacent tissues. Results are expressed as relative expression $\text{Log}_{10} \pm \text{SD}$ of triplicates, the significance is represented by (* if the expression is twice more or less important or ** if the expression is ten times more or less important). Black bar: TNBC breast tumors, grey bar: Luminal B breast tumors and white bar: Luminal A breast tumors.

western blot (Figure 4). A decrease in protein and mark levels between tumors and healthy tissues were observed. These observations demonstrated a difference in the transcription and translation of TIP60 and P400. In fact, transcription of TIP60 and P400 was decreased in the least aggressive tumors (Luminal) and increased in the most aggressive tumors (TNBC) (Figure 3). However, their translation into proteins progressively decreased with the aggressiveness of the tumor (Figure 4). This may suggest that there is a deregulation of the translation of these proteins confirming the decrease in the co-localization between H4K12ac and TIP60 observed by re-ChIP.

Discussion

In breast cancer, downregulation of TIP60 is observed early in carcinogenesis (19, 23), which seems to affect the activities of TIP60 in cancer. TIP60 is a pleiotropic protein that plays a role in several cellular mechanisms involved in tumor development and growth due to its ability to acetylate non-histone proteins such as p53 and ATM (12, 13, 24).

Recruitment of TIP60 in heterochromatin to compensate for the loss of the SUV39H1/H3K9me3/HP1 skeleton, in early carcinogenesis, may decrease the activation of tumor suppressors such as p53 (24-26). To confirm this recruitment, we first examined the enrichment of H4K12ac in heterochromatin and euchromatin. We found that the levels of H4K12ac in non-tumor tissues did not vary across chromatin species (Figure 1). However, in tumors, particularly luminal tumors, there was a strong increase in H4K12ac levels, which were similar to those in healthy tissues of TNBC tumors (Figure 1). Knowing that H4K12 is a target for TIP60 (27), we investigated the co-localization of H4K12ac and TIP60 in the different chromatin species in breast tumors.

High co-localization of H4K12ac and TIP60 was observed in healthy tissues compared to tumors (Figure 2). However, a decrease in co-localization of H4K12ac and TIP60 was observed in Luminal A and TNBCs, whereas in intermediate Luminal B tumors there was an increase in this co-localization (Figure 2). These results could suggest that TIP60 was already present in the different chromatin species to deposit the H4K12ac mark because there was a higher co-localization of H4K12ac and TIP60 in the corresponding healthy tissues compared to the tumors. However, the increased levels of H4K12ac and the co-localization with TIP60 observed in Luminal B tumors compared to Luminal A and TNBCs could be the result of a concentration or recruitment of TIP60 to compensate for the progressive loss of the SUV39H1/H3K9me3/HP1 skeleton. This loss of H4K12ac enrichment and co-localization with TIP60 in TNBCs is due to the progressive loss of TIP60 protein expression in these tumors observed by western blotting (Figure 4). The loss of TIP60 protein during breast cancer progression has been demonstrated (23). Under-expression of TIP60 and P400 mRNA was observed in Luminal tumors and over-expression in TNBCs (Figure 3). This could suggest that the increased expression of TIP60 RNA was disrupted at the translational level, resulting in reduced levels of the protein. This study showed that during carcinogenesis, TIP60 is highly solicited in heterochromatin but also in euchromatin probably to maintain chromatin stability and integrity. During the progression and depending on the aggressiveness of carcinogenesis, translational perturbation of TIP60 leads to its loss but also to the loss of the TIP60/P400/H4K12ac skeleton and chromatin instability.

To better understand this mechanism (Figure 5), TIP60 or P400 depletion could be induced *in vitro* in different breast cancer and non-cancer cell lines. This will allow us to examine how H4K12ac levels vary in the different species of chromatin after this depletion. The depletion of TIP60 or P400 will also allow us to investigate how TIP60 acts on P400 and *vice versa*.

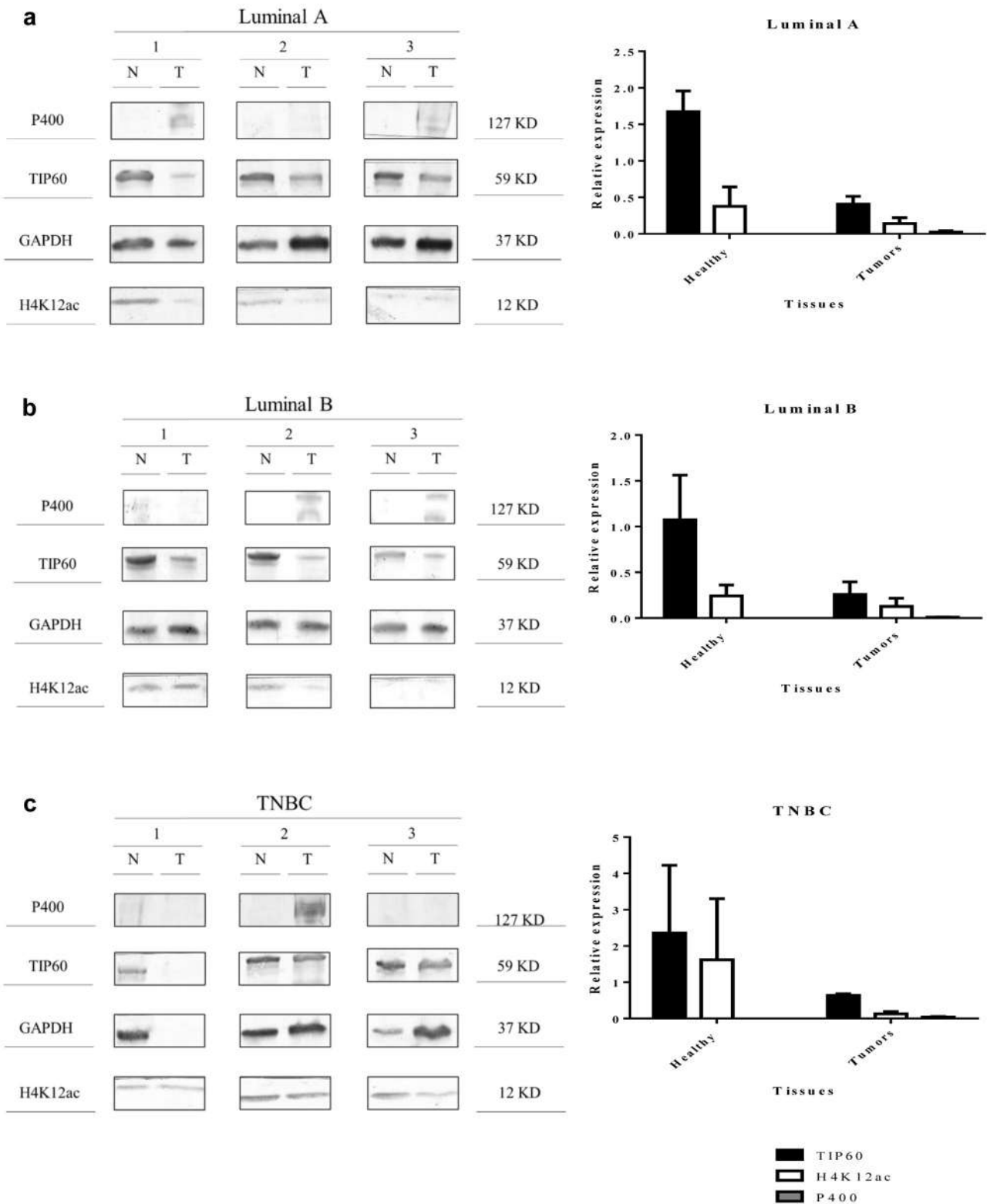


Figure 4. *TIP60*, *P400*, *H4K12ac* and *GAPDH* protein expression in the different breast tumors (Luminal A, Luminal B and TNBC) and their corresponding healthy adjacent tissues. (a) Shows the immunoblot of extracts from Luminal A breast tumors and their corresponding healthy tissues. (b) Shows the immunoblot of extracts from Luminal B breast tumors and their corresponding healthy tissues. (c) Shows the immunoblot of extracts from TNBC tumors and their corresponding healthy tissues. Quantification was performed by Image J.

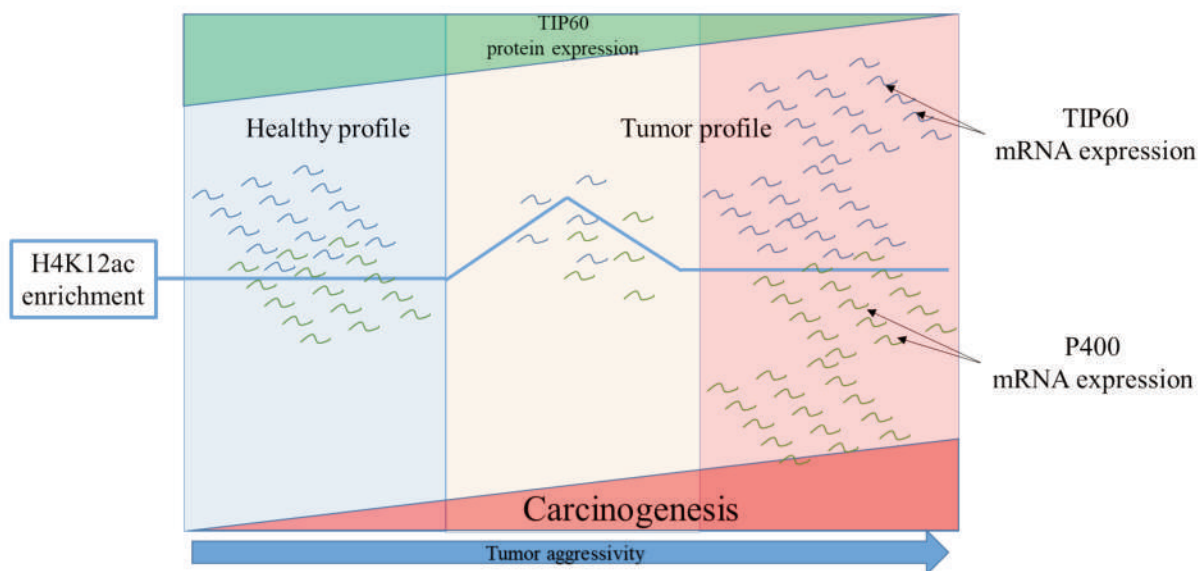


Figure 5. Illustration of the variation of H4K12ac enrichment as a function of tumor aggressiveness, protein expressions of TIP60 and P400 but also the expression of TIP60 and P400 RNA in heterochromatin and euchromatin.

Conclusion

In this study, the presence of TIP60 and H4K12ac modification was confirmed and a decrease of these two proteins was observed in breast tumors compared to healthy tissue. TIP60 and H4K12ac are present in euchromatin and heterochromatin. Finally, interaction between TIP60, P400 and H4K12ac was observed. The understanding of TIP60/P400/H4K12ac skeletal formation, the variations in its different components and the impact they have on each other will allow us to better understand carcinogenesis. This will undoubtedly allow us to use TIP60 or H4K12ac as a biomarker for the aggressiveness of the tumor, but also to use TIP60 as a therapeutic target against breast cancer.

Conflicts of Interest

There are no conflicts of interest to declare in relation to this study.

Authors' Contributions

Writing-review & editing, Mouhamed Idrissou; Methodology, Tiphane Boisnier, Anna Sanchez and Fatma Zohra Houfah Khoufah; Resources, Frédérique Penault-Llorca, Yves-Jean Bignon.; Supervision, project administration, Writing-review & editing, Dominique Bernard-Gallon.

Acknowledgements

This work was supported by a grant from the French Ligue Régionale Contre Le Cancer – Comités du Puy-de-Dôme et de l'Allier.

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Received August 26, 2020

Revised September 8, 2020

Accepted September 10, 2020

Conclusion de la publication 11:

Les différentes stratégies utilisées dans cette étude nous ont permis tout d'abord de confirmer la présence de la marque H4K12ac dans les différents compartiments chromatiniens (hétérochromatine et euchromatine), ceci quelque soit le tissu (tumoral ou sain). De plus la ChIP-qPCR nous a montré qu'il y a une forte augmentation de la marque H4K12ac dans les tissus tumoraux par comparaison avec leurs tissus sains correspondants. Cette augmentation est observée dans tous les différents sous types moléculaires mais aussi dans tous les compartiments chromatiniens.

La ChIP-re-ChIP utilisée en deuxième stratégie nous montre une co-localisation de TIP60 et de H4K12ac au niveau de ces différents gènes de l'hétérochromatine (*Myoglobine*, *SATα* et *SAT2*) et de l'euchromatine (*GAPDH* et *ADH5*). Par contre, cette Co-localisation entre TIP60 et H4K12ac se perd en fonction de l'agressivité de la tumeur.

L'observation de l'accumulation des différents acteurs formant le complexe TIP60/P400/H4K12ac est faite par western blot. Cela nous permet de voir une diminution d'accumulation de TIP60 et de H4K12ac dans les différents sous types moléculaires par rapport aux tissus sains, mais aussi une augmentation de P400 dans les tumeurs. Ensuite la mesure de l'expression ARN de TIP60 et P400 nous montre que les deux protéines ont un même profil d'expression ARN qui est sous exprimé dans les tumeurs luminales et surexprimé dans les TNBCs.

2.2 Le rôle de TIP60 dans la plasticité et l'intégrité chromatinienne lors de la carcinogénèse du sein in vitro.

La deuxième étude de cet axe est réalisée in vitro pour vérifier les observations obtenues dans la première étude, mais aussi de pouvoir moduler certains paramètres pour voir les effets ou les interactions des principaux acteurs du complexe TIP60/P400/H4K12ac dans la carcinogénèse du sein.

Tout d'abord nous avons suivi les mêmes stratégies que celles utilisées dans l'étude ex vivo, à savoir regarder le taux d'enrichissement de H4K12ac dans les différents lignées continues de cellules tumorales du sein (MDA-MB231, MCF-7 et T47D) ou non cancéreuse (MCF-10A). Ensuite, nous avons observé la co-localisation de TIP60 et H4K12ac dans les différentes lignées continues étudiées. Puis, nous avons mesuré l'expression des ARNm de TIP60 et P400 comme dans l'étude précédente.

Par contre, dans cette étude nous avons inhibé l'expression de TIP60 par un SiRNA, mais aussi son activité grâce aux inhibiteurs TH1834 et NU9056. Ceci pour suivre l'effet de cette inhibition sur l'expression de TIP60 et P400, mais aussi sur l'accumulation de H4K12ac. Ce qui nous permettra de voir l'influence de TIP60 sur les autres acteurs du complexe TIP60/P400/H4K12ac.

Publication 12:

Implementation of the TIP60/P400/H4K12ac structure in breast cancer cell lines.

Idrissou M, Boisnier T, Sanchez A, Houfah Khoufah F Z, Penault-Llorca F, Bignon YJ and Bernard-Gallon D. (soumise en 2020)

Implementation of the TIP60/P400/H4K12ac structure in breast cancer cell lines

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Short title: *TIP60/P400/H4K12ac and breast cancer*

Keywords: breast cancer; cell culture; TIP60; H4K12ac; P400; heterochromatin; euchromatin

To the Editor:

TIP60, a histone acetyl-transferase, acts as a transcription factor in maintenance of plasticity and chromatin integrity. RNA and protein expressions of TIP60 have been shown to be reduced in breast cancer. These expressions are used to stratify breast cancers from the least to the most aggressive (Gorrini et al., 2007; McGuire et al., 2019). TIP60 can be recruited into heterochromatin by ATPase P400 when it has incorporated the H2Az variant into heterochromatin, as TIP60 is able to bind to mono- or tri-methylated H3K9 (Rajagopalan et al., 2018). This recruitment would compensate for the loss of the heterochromatin maintenance skeleton (SUV39H1/H3K9me3/HP1) by overexpression of histone demethylase. This chromatin maintenance activity could unbalance the residual activity of TIP60 in breast cancer. Some studies have shown that inhibition of the residual activity of TIP60 in breast cancer would lead to tumor cell death by apoptosis (Brown et al., 2016).

An earlier study in different molecular types of breast tumor was performed in our laboratory (Idrissou et al., 2020a). We demonstrated that there is an interaction between TIP60 and H4K12ac in heterochromatin and euchromatin in breast tumors. In order to better understand these observations and the process of formation of the second skeleton TIP60/P400/H4K12ac of heterochromatin, the current study was performed in breast cancer cell lines as shown and described in the figure legends. Specifically, we analyzed the variation of H4K12ac and its co-location with TIP60 in different chromatin compartments.

Firstly, enrichment obtained by ChIP-qPCR showed that we have a higher enrichment of H4K12ac in MCF-10A non-cancerous cells compared to cancerous cells in heterochromatin and euchromatin. In the TNBC cell line, a high level of enrichment compared to luminal cell lines on the promoters of the genes was obtained in a different chromatin compartments (Figure 1A). ChIP-re-ChIP demonstrates that there is co-

localization of TIP60 and H4K12ac that does not vary in the studied breast cell lines (Figure 1B).

The depletion induced by SiRNA-TIP60 showed an under-expression of TIP60, which suggests that the inhibition was successful, but also an under-expression of P400 which suggests that TIP60 influences the expression of P400 in breast cell lines, except for the TNBC cell line (MDA-MB-231) where no great variation is observed (Figure 2. Panel 1). Depletion of P400 by SiRNA-P400 shows a decrease in P400 expression but no change in TIP60 expression. This suggests that the expression of P400 does not influence the expression of TIP60 in the different breast cell lines (Figure 2. Panel 1). These depletions are observed by western blot and we can see that there is a decrease in accumulation of TIP60, H4K12ac and P400 after SiRNA-TIP60 which confirms the observations obtained by RT-qPCR. On the other hand, a decrease in accumulation of P400 after SiRNA-P400 was observed which is not followed by a change in accumulation of TIP60 or H4K12ac (Figure 2. Panel 2).

To confirm if these observations are due to the activity of TIP60 or to its protein expression, we inhibited the activity of TIP60 by TH1834 or NU9056. This inhibition does not show any variation in expression in the MCF-10A cell line except for the expression of P400 after inhibition with NU9056 where an under-expression was exhibited (Figure 2, Panel 3). For the inhibitor TH1834, we can see that we have the same expression profile between TIP60 and P400 in breast cancer cell lines. There is no variation in the expression of TIP60 and P400 in the MCF-7 cell line.

In the T47D cell line, we observed an overexpression of TIP60 and P400, but also an under-expression in the TNBC cell line (Figure 2, Panel 3). For inhibitor NU9056 in breast cancer cell lines, the same expression profile is observed for TIP60 and P400. In the MCF-7 cell line, an under-expression of TIP60 and P400 is observed, while in the T47D cell line, an over-expression is found. No variation in expression is revealed in the TNBC cell line (Figure 2, Panel 3).

Finally, the inhibition of TIP60 activity induced by TH1834 and NU9056 is shown by western blot and demonstrates that there is no variation in TIP60 or P400, but only a

decrease in the accumulation of H4K12ac in the different breast cell lines as seen in a previous study ((Idrissou et al., 2020b) (Figure 2, Panel 4).

These findings collectively show that there is an interaction between TIP60 and P400, but also a link between TIP60 and H4K12ac in the different chromatin compartments, which could suggest its role in maintaining plasticity and chromatin integrity.

Acknowledgments

This work was supported by a grant from the French Ligue Régionale Contre Le Cancer – Comités du Puy-de-Dôme et de l’Allier.

Author disclosure statement

The authors have no competing financial interest to declare.

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Figure legends

Figure 1. A. *Enrichment on H4K12ac in fold enrichment over IgG (illustrating the presence of H4K12ac on gene promoter) in euchromatin (GAPDH and ADH5) and heterochromatin (Myoglobin, SAT α and SAT 2) genes in breast cancer cell lines (T47D, MCF-7 and MDA-MB-231) and the fibrocystic cell line (MCF-10A). Data are presented with SD of triplicate, and p-values according to two-tailed student t-test (* indicating $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). (Black bar = breast cancer tumors and White bar = healthy breast tissue). B.* *Co-localization of H4K12ac and TIP60 in promoter regions of target genes in euchromatin*

(*GAPDH* and *ADH5*) and heterochromatin (*Myoglobin*, *SAT α* and *SAT 2*) in breast cancer cell lines (T47D, MCF-7 and MDA-MB-231) and the fibrocystic cell line (MCF-10A). Errors are SD of quadruplicate of breast cancer cell lines and fibrocystic cell line, and p-values according to two-tailed student t-test (* indicating $p < 0.05$ and ** $p < 0.01$). (Black bar = TNBC cell line (MDA-MB-231), grey bar = Luminal breast cancer cell lines (T47D and MCF-7) and white bar = non-cancerous cell line (MCF-10A)).

Figure 2. mRNA and protein expressions of TIP60 and P400 in breast cancer cell lines (MCF-7, T47D and MDA-MB-231) and the fibrocystic breast cell line (MCF-10A). **1)** the relative RNA expression of TIP60 and P400 on Log 10 of breast cancer cell lines and fibrocystic breast cell line treated with or without siRNA (TIP60 or P400). **2)** the immunoblot of TIP60, P400, H4K12ac and GAPDH of breast cancer cell lines (MCF-7, T47D and MDA-MB-231) and fibrocystic breast cell line (MCF-10A) treated with or without siRNA (TIP60 or P400). **3)** the mRNA and protein expression of TIP60 and P400 in breast cancer cell lines and fibrocystic breast cell line treated with or without chemical inhibitors of TIP60 (TH1834 and NU9056. **4)** the immunoblot of TIP60, P400, H4K12ac and GAPDH of breast cancer cell lines (MCF-7, T47D and MDA-MB-231) and fibrocystic breast cell line (MCF-10A) treated with or without chemical inhibitors of TIP60 (TH1834 and NU9056). Errors are SD of triplicate, the significance is represented by (* the expression is twice more or less, ** the expression is ten times more or less). (Black bar = TNBC cell line (MDA-MB-231), grey bar = Luminal breast cancer cell lines (MCF-7 and T47D) and white bar = fibrocystic breast cell line (MCF-10A)) (1 = MCF-10; 2 = MCF-7; 3 = T47D and 4 = MDA-MB-231).

Abbreviations:

H3K4ac: Histone 3 lysine 4 acetylated

HAT: Histone acetyltransferase

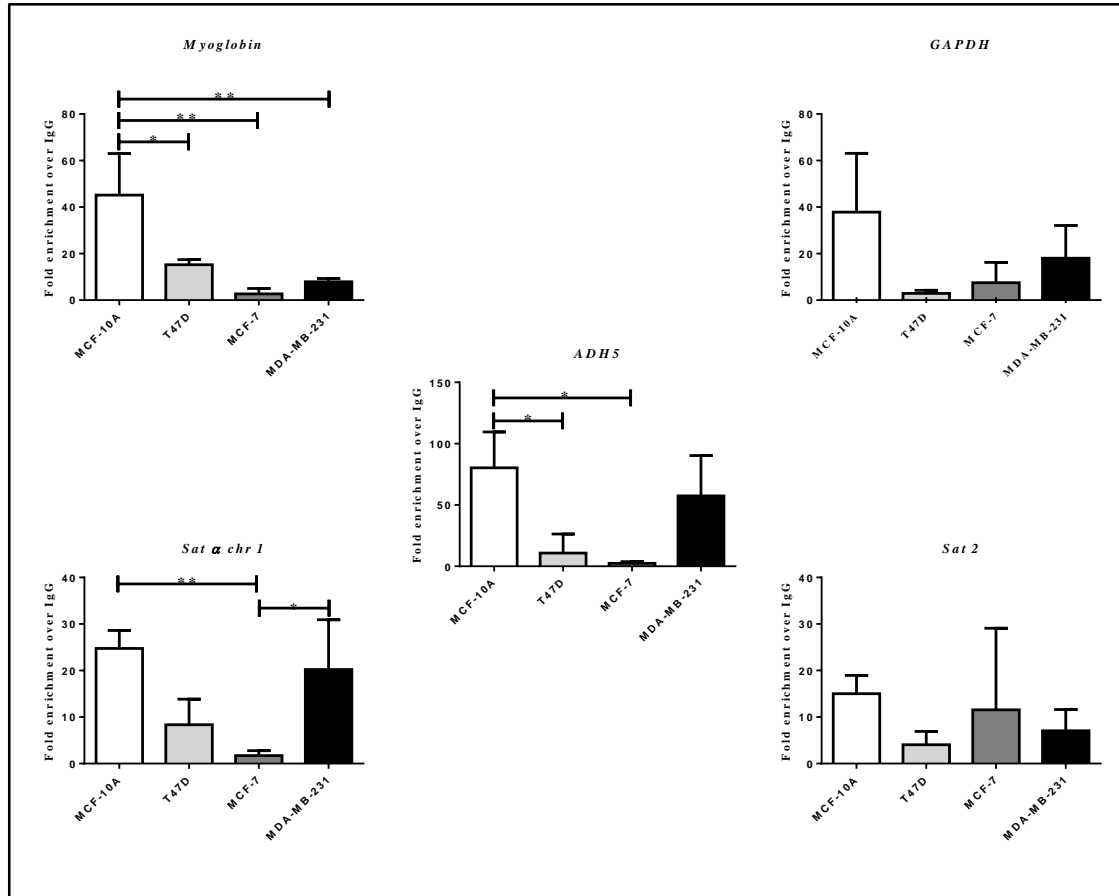
HDAC: Histone deacetylase

SiRNA: small interfering RNA

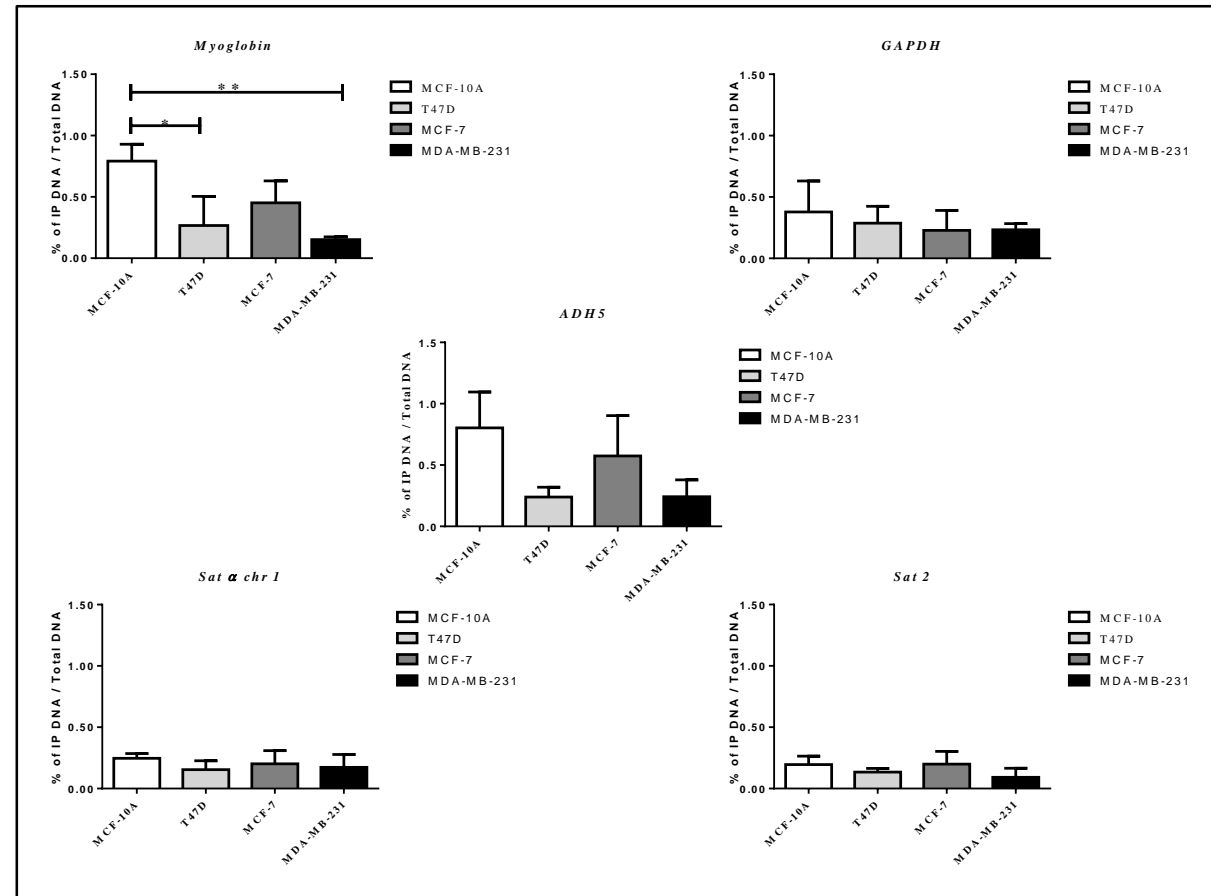
TIP60: TAT-interactive protein 60 kDa

TNBC: Triple negative breast cancer

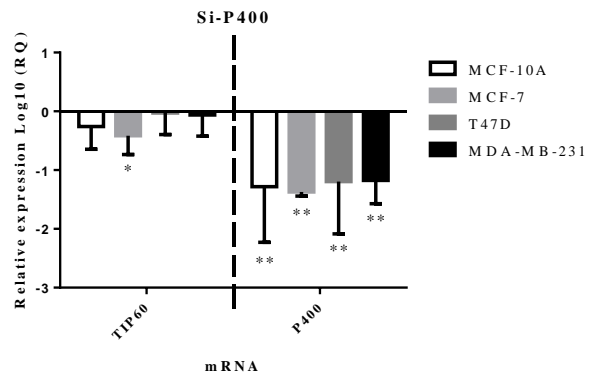
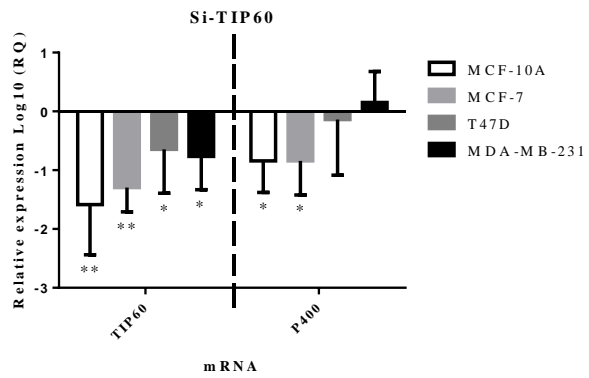
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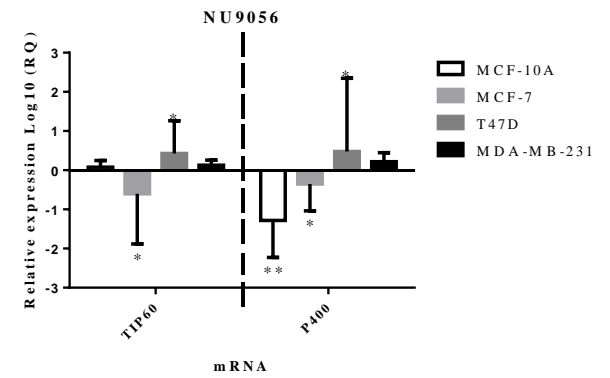
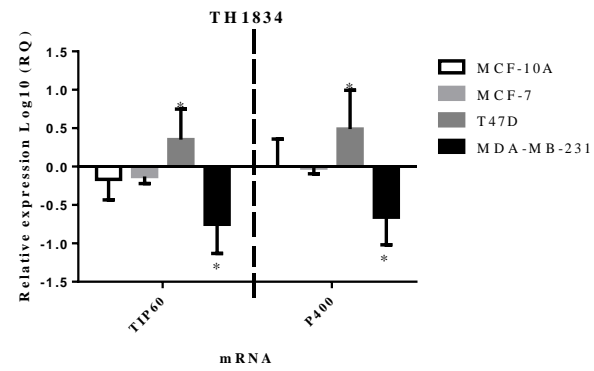
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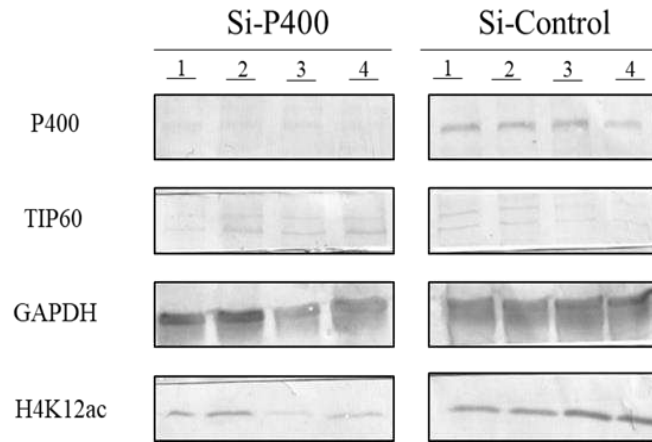
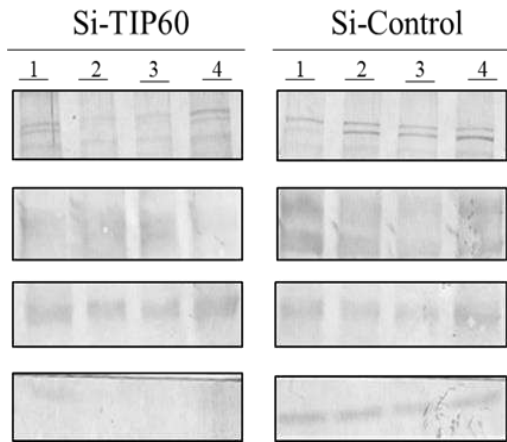
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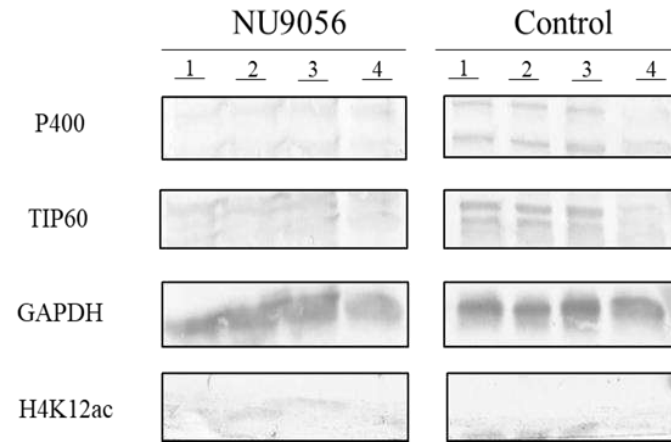
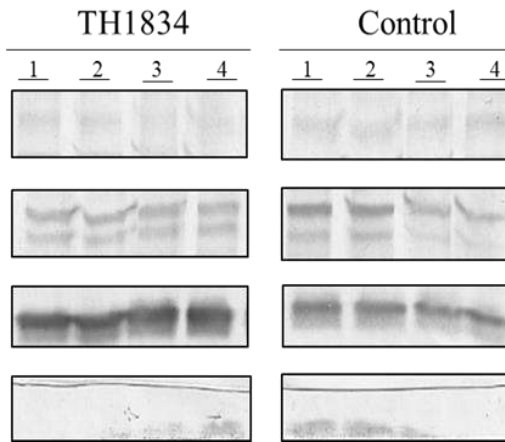
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Conclusion de la publication 12:

Dans cette étude, les mêmes stratégies que celles de la première étude sont réalisées. Tout d'abord, l'enrichissement obtenu par CHIP-q PCR nous permet de voir qu'on a un plus fort enrichissement en H4K12ac dans les cellules non cancéreuses MCF-10A par rapport aux cellules cancéreuses dans l'hétérochromatine et l'euchromatine. Dans les cellules cancéreuses, on peut remarquer que les cellules TNBC ont un grand taux d'enrichissement comparé aux cellules luminales sur les promoteurs des gènes dans les différents compartiments chromatiniens.

L'observation de la co-localisation de TIP60 et H4K12ac par CHIP-re-CHIP dans les lignées continues du sein étudié nous montre qu'il y a une co-localisation des acteurs, mais aussi que cette co-localisation ne varie pas entre ces lignées continues.

La déplétion induite de TIP60 par SiRNA-TIP60 nous montre une sous-expression de TIP60, ce qui montre que l'inhibition a été réussie, mais aussi on a pu observer une sous expression de P400 ce qui suggère que TIP60 influe sur l'expression de P400 dans les cellules du sein sauf pour la lignée continue TNBC (MDA-MB-231) où l'on n'observe pas de grande variation.

D'autre part, la déplétion de P400 par SiRNA-P400 montre une diminution d'expression de P400 mais pas de variation d'expression de TIP60. Ce qui voudrait dire que l'expression de P400 n'influe pas sur celle de TIP60 dans les différentes cellules du sein.

Ces déplétions sont observées en western blot et on peut voir qu'on a effectivement une diminution d'accumulation de TIP60, H4K12ac et P400 après SiTIP60 ce qui confirme les observations obtenues par RT-qPCR, mais aussi une diminution d'accumulation de P400 après le SiP400, qui n'est pas suivie de variation d'accumulation de TIP60 ou H4K12ac.

Pour confirmer si ces observations sont dûe à l'activité de TIP60 ou juste à son expression protéique, nous avons inhibé l'activité de TIP60 par TH1834 et NU9056. Cette inhibition ne montre pas de variation l'expression dans la lignée non cancéreuse sauf pour l'expression de P400 après inhibition au NU9056 ou l'on voit une sous expression de ce dernier.

Pour l'inhibiteur TH1834, nous pouvons voir qu'on a le même profil d'expression entre TIP60 et P400 dans les cellules cancéreuses. On n'observe pas de variation expression de TIP60 et P400 dans la lignée continue MCF-7, par contre dans la lignée T47D on observe une surexpression des TIP60 et P400, mais aussi une sous-expression dans la lignée TNBC.

Pour l'inhibiteur NU9056 dans les lignées cancéreuses, on observe le même profil d'expression pour TIP60 et P400. Dans la lignée continue MCF-7 on observe une sous expression de TIP60 et P400, alors que dans la lignée continue T47D on observe une surexpression. Par contre, on n'observe pas de variation d'expression dans la lignée continue TNBC.

L'inhibition de l'activité de TIP60 induit par TH1834 et NU9056 est observée par western blot et nous montre que l'on n'a ni variation de TIP60 ni de P400, mais uniquement une diminution de l'accumulation de H4K12ac dans les différentes lignées continues mammaires.

V. DISCUSSION ET PERSPECTIVES

Le cancer est un problème de santé publique qu'il fait partie des maladies les plus mortelles dans le monde et en France. Parmi ces cancers, le cancer du sein est le cancer le plus fréquent et le plus mortel chez la femme. Ce cancer voit son taux d'incidence en hausse de 0.6%, alors que son taux de mortalité est en baisse de -1.3% en France. Ces chiffres nous montrent qu'il y a eu de bonnes avancées sur la prise en charge et le dépistage rapide des patientes. Par contre le traitement de cette maladie est encore peu efficace et très invasif, cette difficulté de traitement est dû à l'hétérogénéité moléculaire de cette maladie.

Cette hétérogénéité a permis l'élaboration des classifications des cancers du sein en fonction de leur profils d'expression génique tels que la classification moléculaire du consensus de St. Gallen dans laquelle l'expression de certain gènes mutés tels que *p53* est prise en considération.

L'épigénome est l'ensemble de toutes les molécules, protéines et de tous les mécanismes capables de modifier l'expression d'un gène sans toucher à son intégrité. Ces mécanismes capables de moduler l'expression d'un gène sont : les micro-ARN, la méthylation de l'ADN et les modifications post-traductionnelles des histones. En effet, dans le cancer du sein, de nombreux dérèglements dans les mécanismes épigénétiques sont observés comme par exemple le déséquilibre entre les HATs et les HDACs qui entraîne une modification du profil épigénétique dans les cellules cancéreuses modifiant l'expression de certains gènes impliqués dans la croissance cellulaire, l'apoptose et la migration cellulaire.

Hormis la classification des cancers du sein en fonction des paramètres histologiques (cancer non invasif et invasif) et moléculaires (cancer luminal A, luminal B, HER2+ et TNBC). De nouveaux moyens de classification ont vu le jour grâce à la mise en place des bases de données OMICS qui permettent d'identifier un cancer du sein en fonction de son profil épigénétique. Cette nouvelle classification permettra une meilleure prise en charge des patientes atteintes du cancer du sein. En effet, des études sur l'expression des micro-ARN dans 1091 tumeurs et 104 tissus sains du sein montrent qu'il y a une augmentation d'expression de certains micro-ARN tels que (mir-21, mir-96, mir-183, mir-592, and mir-182) considérés comme oncogènes, mais aussi une diminution d'autres tels que (mir-39, mir-10b, mir-99a, mir-145, and let-7c) considérés comme suppresseurs de tumeur dans le cancer du sein (Lu et al., 2020). Ce qui a permis de stratifier les différents types de cancer du sein en associant l'expression des micro-ARN aux sous types moléculaires et histologiques. Dans les cancers à mauvais pronostic, tels que les sous types moléculaires HER2+ ou TNBCs, mais aussi le stade III on a une surexpression du mi-130a et une sous expression du mir-1247 (Lu et al., 2020). D'autres études sur le profil de méthylation de l'ADN ont permis de classifier les différents types moléculaires de cancer du sein. En effet, en 2016, Holm et al, ont identifiés 7 types de profil de méthylation de l'ADN correspondant aux sous types moléculaires de cancer du sein. Cette étude a été faite avec 188 tumeurs de sein et comparées à 669 profils de méthylation issus de la base de données de The cancer Genome Atlas data, ces différents profils sont appelés épitypes allant de 1 à 7. L'épitype 1 correspond à un profil normal de méthylation de l'ADN et correspondant au tissu normal du sein. L'épitype 2 et 3 tend vers une hypo-méthylation et correspond au sous type luminaux A, l'épitype 4 est fortement caractérisé par une hypo-méthylation global de l'ADN et correspond aux sous types luminaux A et B. l'épitype 5 est caractérisé par une hyperméthylation des îlots CPG des promoteurs de gènes et correspond au sous type luminal B. l'épitype 6 correspond au lui le sous type

moléculaire de cancer du sein HER2+, alors que l'épitype 7 est caractérisé par une hypométhylation des îlots CPG des promoteurs de gènes correspondants au sous type moléculaire TNBC (Holm et al., 2016). De plus des études comme celle de Elsheikh et al, montre une corrélation entre l'agressivité des cancers du sein et les modifications de l'histone. En effet, cette étude a été réalisée sur 880 tumeurs du sein et a pu montrer que les tumeurs à mauvais pronostic tels que les TNBCs et les HER2+ présentent un faible taux global d'acétylation et de méthylation, alors que les tumeurs de type luminal présentent un taux plutôt élevé (Elsheikh et al., 2009). Malgré cette caractérisation des modifications des histones dans le cancer du sein, une stratification plus élaborée des cancers du sein en fonction des différentes modifications des histones devient nécessaire pour mieux lutter contre ce fléau.

Des études menées dans notre laboratoire sur la méthylation des histones nous ont permis de caractériser certains types histologiques et moléculaires de cancer du sein en fonction de l'enrichissement de certains gènes en marques H3K27me3 et H4R3me3. Karsli et al, ont montré que dans les cancers du sein TNBCs, on avait une augmentation de l'enrichissement en H3K27me3, alors que dans les cancers luminaux et HER2+ on avait plutôt une diminution de cet enrichissement (Karsli-Ceppioglu et al., 2017). La marque H4R3me3 est montrée aussi diminuée dans les tumeurs de grande taille et cette marque est diminuée dans les tumeurs à statut ganglionnaire faible (Karsli-Ceppioglu et al., 2014). L'acétylation des histones a été peu utilisée pour classifier ou stratifier les cancers du sein, cette acétylation dépend de l'équilibre qu'il existe entre les HDCs et les HATs. Dans notre laboratoire, l'étude menée sur l'histone déacétylase SIRT1 a permis d'élaborer une caractérisation des cancers du sein en fonction de leurs niveaux d'expression de cette protéine. Cette étude a montré que dans les cancers luminaux A, B et les HER2+, on a une surexpression de SIRT1 et que dans les cancers TNBCs on observe plutôt une sous-expression de SIRT1. Ce qui a permis de constater que l'expression de SIRT1 diminuait avec l'agressivité des tumeurs (Rifai et al., 2017, 2018b). La classification et la stratification des différents types moléculaires de cancer du sein en fonction du profil d'acétylation permettront sans doute d'enrichir les bases de données OMICS mais aussi de mieux lutter contre cette maladie.

C'est dans cette optique que s'inscrivent les travaux effectués dans cette thèse sur l'équilibre des protéines épigénétiques SIRT1 (HDAC) et TIP60 (HAT) agissant sur le profil d'acétylation des différents types moléculaires de cancers du sein, mais aussi sur leurs rôles dans le cancer du sein. Nos premiers travaux se sont focalisés sur le rôle de l'acétyl-transférase TIP60 et de l'effet de l'inhibition de son activité dans le cancer du sein. Dans un premier temps, l'effet de l'inhibition de TIP60 par deux inhibiteurs chimiques spécifiques TH1834 qui prive TIP60 d'acétyl CoA et NU9056 qui bloque son site actif (Brown et al., 2016; Gao et al., 2014). Cette inhibition est observée sur une lignée continue cellulaire non cancéreuse (MCF-10A) et cinq lignées continues de cancer du sein différentes. Ces cinq lignées cellulaires représentent les cancers luminaux (MCF-7 et T47D) exprimant les récepteurs aux œstrogènes et à la progestérone (ER+ et PR+) et n'expriment pas le récepteur des facteurs de croissance épidermiques humains (HER2-), les cancers triples négatifs (MDA-MB-231, MDA-MB-453 et MDA-MB-468) n'expriment aucun de ces récepteurs (ER-, PR- et HER2-). Les récepteurs nucléaires sont connus pour jouer des rôles d'activateurs de la transcription des gènes impliqués dans la croissance cellulaire ou l'apoptose qui sont dérégulées dans le cancer du sein (Fu et al., 2004b) (Bakshi et al., 2017; Fu et al., 2004; Lee et al., 2013; Xie et al., 2009). TIP60 fait partie de ces protéines capables de se fixer sur les récepteurs nucléaires et de jouer

le rôle de co-activateur transcriptionnel en les acétylant induisant ainsi la transactivation des gènes cibles (Figure 22).

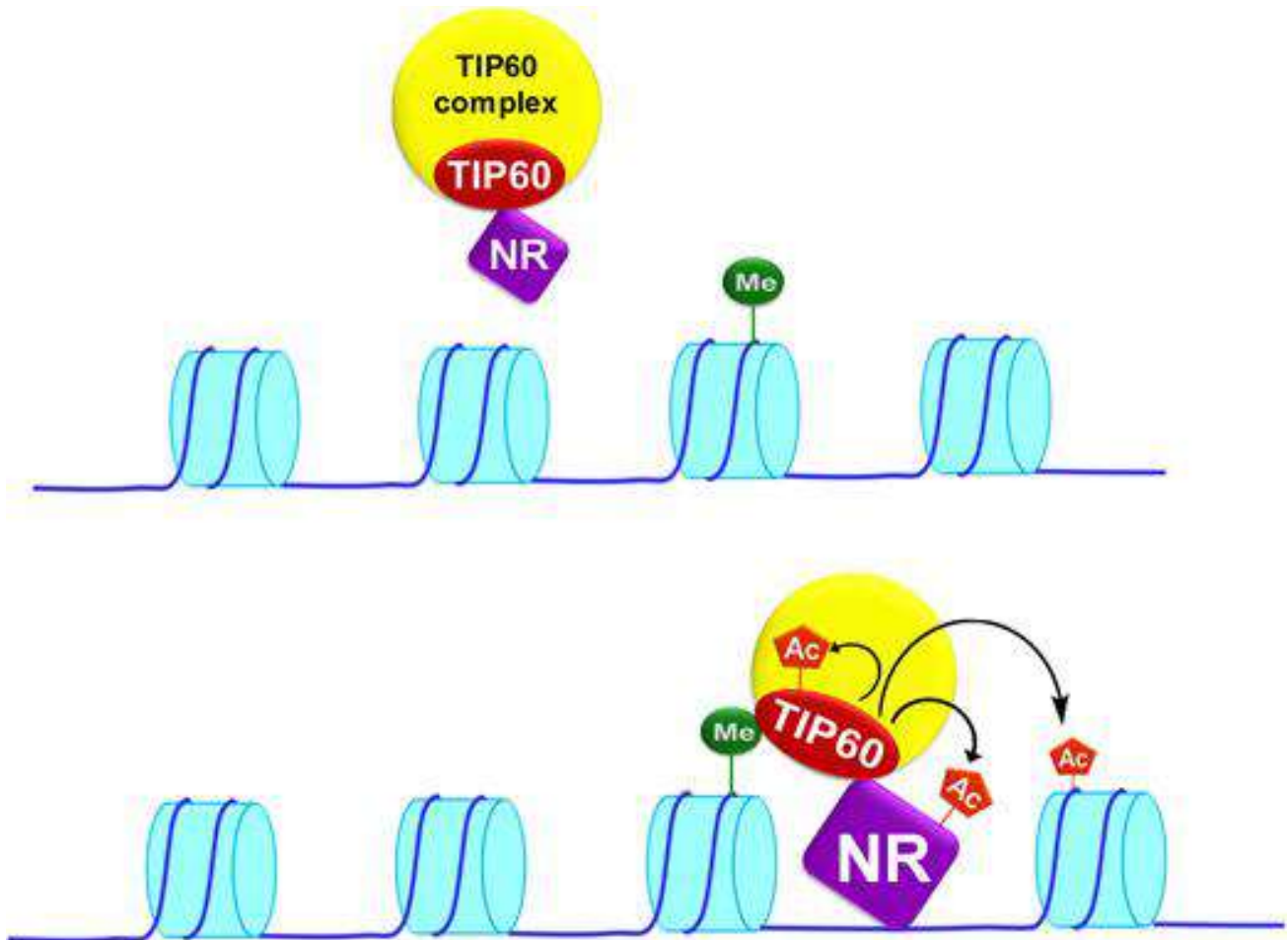


Figure 22 : Représentation de l'activation des récepteurs nucléaires et de la transcription par TIP60 et son complexe. Fixation de TIP60 sur la méthylation des histones grâce à son chromo-domaine ce qui lui permet d'acétyler les récepteurs nucléaires et les histones entraînant l'activation de la transcription des gènes cibles. Figure tirée de(Jaiswal and Gupta, 2018)

Cette étude a démontré que l'inhibition de l'activité de TIP60 par les deux inhibiteurs chimiques est bien efficace car on observe une diminution de la marque H3K4ac dans les différentes lignées continues cellulaires étudiées. En effet H3K4ac est une marque spécifique de TIP60 qui se retrouve dans les deux compartiments chromatinien, la diminution de cette marque montre que l'activité de TIP60 est inhibée (Judes et al., 2016). Après avoir vérifié l'efficacité de l'inhibition de l'activité de TIP60, l'expression des 48 récepteurs nucléaires qui

sont présents chez l'homme a été déterminée. Ces récepteurs nucléaires qui permettent de caractériser les cancers du sein par leurs expressions sont analysés par TLDA en comparant les différentes cellules du sein traitées par le TH1834 ou le NU9056 par rapport à des cellules du sein sans traitement. Aucun de ces récepteurs nucléaires ne voit son expression varier après l'inhibition au NU9056 dans toutes les différentes lignées continues étudiées. Par contre, pour le traitement au TH1834, seul les récepteurs nucléaires *AR* et *NR3C2* voient leurs expressions variées dans les lignées TNBCs (MDA-MB-231, MDA-MB-453 et MDA-MB-468). Le récepteur *NR3C2* pour (Nuclear Receptor Subfamily 3, Group C, Member 2), voit son expression augmentée dans les lignées MDA-MB-231 et MDA-MB-468, alors que dans la lignée MDA-MB-453 on observe une diminution d'expression de *NR3C2*. *NR3C2* est un récepteur nucléaire impliqué dans les mécanismes cellulaires tels que l'apoptose. Son rôle dans le cancer du sein est encore méconnu et son interaction avec TIP60 dépend sûrement de son acétylation par la plupart des acétyl-transférases (Nan et al., 2015).

Pour le récepteur aux androgènes *AR*, on a observé une expression inverse que celle du récepteur *NR3C2* dans les différentes lignées continues TNBCs. Dans les lignées continues MDA-MB-231 et MDA-MB-468, on observe une augmentation d'expression d'*AR* alors que dans la lignée continue MDA-MB-453 on observe une diminution de son expression par rapport aux tissus sains correspondants. Cette augmentation d'expression suggère que TIP60 influe sur l'expression de ce récepteur dans ces deux lignées continues. Dans la lignée MDA-MB-453 représentant le sous type luminal androgen receptor (LAR), le gène *AR* est surexprimé et la diminution de son expression après traitement au TH1834 peut suggérer que TIP60 est impliquée dans la surexpression d'*AR* dans cette lignée. Il est connu dans la littérature que TIP60 partage un lien étroit avec le récepteur *AR*, en effet, TIP60 est capable d'activer *AR* induisant ainsi son activité d'activateur transcriptionnelle des gènes impliqués dans la survie et la croissance cellulaire (Fu et al., 2004a) (Figure 23). L'inhibition de l'activité de TIP60 diminue le taux d'acétylation d'*AR* entraînant son inactivation, cette inactivation pourrait entraîner la mort cellulaire et arrêt de la croissance. L'inhibition de TIP60 pourrait donc être utilisée pour lutter contre les cancers du sein de sous type LAR, car dans cette lignée TIP60 se comporte comme un oncogène en activant le récepteur aux androgènes induisant la transcription des oncogènes impliqués dans la croissance et la survie cellulaire (Tostain et al., 2004). Par contre dans les lignées basal like (MDA-MB-468) et mésenchymal stem-like (MDA-MB-231) TIP60 semble jouer un rôle de suppresseur de tumeur car son inhibition entraîne une surexpression d'*AR*.

Cette étude nous a permis de démontrer que l'activité acétyl-transférase de TIP60 est impliquée dans l'expression de certains récepteurs nucléaires tels que *AR* et *NR3C2* dans les lignées continues TNBCs mesenchymal stem-like (MDA-MB-231), basal like (MDA-MB-468) et luminal androgen receptor (MDA-MB-453). La modulation de cette acétylation induite par TIP60 dans les différentes lignées continues TNBCs qui sont les plus agressives pourrait permettre de lutter contre le cancer du sein.

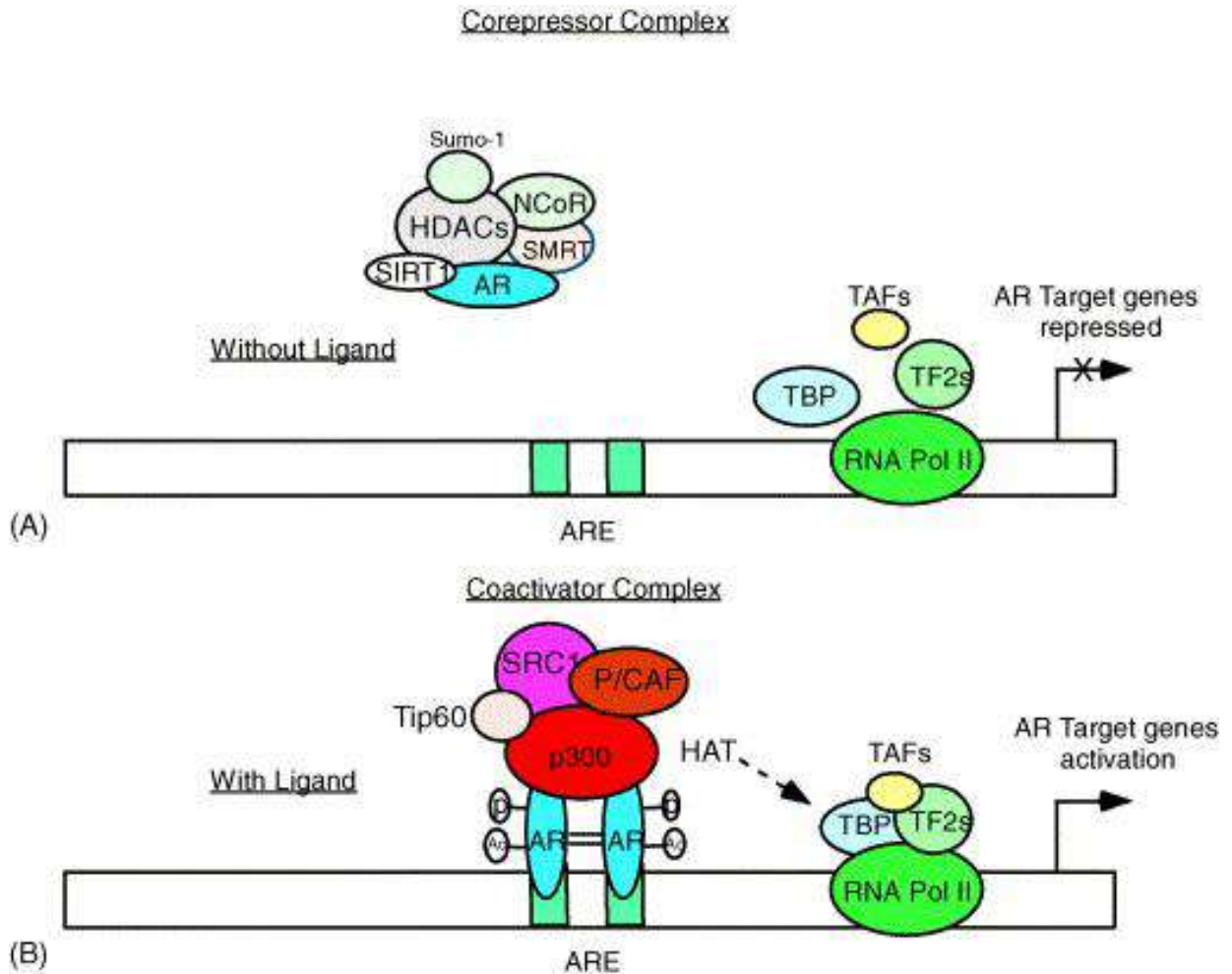


Figure 23 : Représentation de l'activation de la transcription des gènes cibles d'AR. A) le récepteur AR est capturé par le complexe répresseur de SIRT1 entraînant ainsi la répression des gènes cibles de AR. B) l'acétylation de AR par le complexe TIP60 entraîne son activation et la transcription des gènes cibles de AR. Figure tirée de (*Fu et al., 2004a*).

Ensuite, nous nous sommes intéressés à l'effet de l'inhibition de TIP60 par l'un de ses inhibiteurs chimiques spécifiques TH1834, *in vivo* dans les souris nude BALB/c injectées avec les lignées continues de cancer du sein (MDA-MB-231 et MCF-7). Ces deux lignées continues sont couplées avec de la luciférase pour permettre de suivre leur croissance dans l'organisme des souris. L'injection de ces lignées continues est suivie d'une injection de TH1834 ou de solution saline utilisée comme témoin pendant 10 jours consécutifs. La croissance de la tumeur est mesurée au pied à coulisse et par bioluminescence chaque semaine pendant 29 jours pour les cellules MDA-MB-231-Luc et 55 jours pour les cellules MCF-7. L'efficacité de l'inhibition est vérifiée par western-bot en regardant l'accumulation de la marque H3K4ac spécifique à TIP60. Le western-blot confirme bien une diminution importante de la marque H3K4ac dans la lignée continue MCF-7-Luc, alors que la diminution est moins marquée dans la lignée continue MDA-MB-231-Luc. De plus les mesures de la croissance tumorale montrent une diminution importante de la croissance dans les tumeurs des souris MCF-7-Luc, alors que dans les souris MDA-MB-231 on observe un ralentissement de cette croissance tumorale, ce qui suggère un rôle de suppresseur de tumeur de TIP60 dans ces xénogreffes et a un effet plus important dans les xénogreffes MCF-7 que dans les xénogreffes MDA-MB-231. Ce qui caractérise ces tumeurs est l'expression des récepteurs aux œstrogènes ER et à la progestérone PR, qui sont exprimés dans les cellules MCF-7 et ne sont pas exprimés dans les cellules MDA-MB-231. Cette diminution de la croissance tumorale observée dans les xénogreffes MCF-7 peut être dû à l'interaction qui existe entre TIP60 et les récepteurs nucléaires (Jaiswal and Gupta, 2018). En effet, TIP60 est capable d'acétyler les récepteurs aux œstrogènes ER entraînant ainsi leurs activations et la transcription des gènes cibles d'ER impliqués dans la croissance et la prolifération cellulaire. De plus, TIP60 peut activer le récepteur à la progestérone PR et permettre son interaction avec le récepteur ER, cette interaction active la voie PI3K/AKT et la voie SRC/RAS/ERK conduisant à la prolifération et le détachement cellulaire dans le cancer du sein (Hopp et al., 2004; Jeong et al., 2011; Wiebe, 2006). Ce lien entre TIP60, ER et PR dans les mécanismes de prolifération ou de différenciation cellulaire observés dans le cancer du sein pourrait expliquer qu'on a une forte diminution de la croissance tumorale dans les xénogreffes MCF-7 car l'activité de ces deux récepteurs est inhibée par l'absence d'activité de TIP60. Par contre, la diminution de la croissance tumorale observée dans les xénogreffes MDA-MB-231 ne s'explique pas par l'inactivation des récepteurs ER et PR car ce sous types moléculaire de cancer du sein ne les exprime pas. Cette diminution de la croissance tumorale pourrait donc venir de l'absence d'acétylation au niveaux de certains oncogènes impliqués dans les mécanisme cellulaires tels que la croissance et la prolifération cellulaire.

On peut conclure que l'inhibition de l'activité de TIP60 par TH1834 dans les xénogreffes MCF-7 et MDA-MB-231 pourrait être utilisée comme traitement « epidrugs » dans la lutte contre le cancer du sein, mais aussi que TIP60 a un rôle très important dans la croissance tumorale des tumeurs luminales via sont interaction avec les récepteurs ER et PR.

La deuxième partie de nos travaux est concentrée sur la formation du complexe TIP60/P400/H4K12ac dans l'hétérochromatine et son rôle dans le maintien de la plasticité et l'intégrité chromatinienne lors de la carcinogenèse du sein. En effet, dans l'initiation de la carcinogénèse, l'intégrité et la plasticité chromatinienne se voient modifiées par la perte des structures de maintien de la chromatine. Dans la plupart des cancers, le squelette de maintien

de l'hétérochromatine constituée de SUV39H1/H3K9me3/HP1 est perdue, cette perte est due à une diminution importante de la protéine SUV39H1, ce qui entraîne le relâchement de l'hétérochromatine et l'accessibilité aux oncogènes ou à la polyploïdie. Cependant, un second squelette se formerait pour pouvoir combler l'absence de SUV39H1/H3K9me3/HP1 au niveau de l'hétérochromatine. En effet, ce mécanisme a été observé dans le cancer colorectal où TIP60 était recruté par P400 au niveau de l'hétérochromatine ou il permettrait l'incorporation de la variante histone H2Az à la place de H2A. La présence de TIP60 dans l'hétérochromatine est suivie par une acétylation de ces cibles telles que les histones H3K4, H3K9 et H4K12. L'acétylation de H4K12 permet la fixation des protéines à bromo-domaines tels que BRD2 et BRD4, la fixation de ces protéines aurait pour effet de consolider et de maintenir la structure de l'hétérochromatine. Ce qui nous a permis de poser une hypothèse sur la mise en place de ce mécanisme au cours de la carcinogénèse du sein exposée dans une lettre à l'éditeur (Idrissou et al., 2018).

Dans le but de répondre à cette hypothèse, nous avons analysé certains paramètres nous permettant de suivre la formation du complexe TIP60/P400/H4K12ac et la variation de ses différents constituants dans les sous types moléculaires de tumeurs du sein (*ex vivo*). Ces tumeurs proviennent de patientes ayant développé un cancer du sein et n'ayant subi aucun traitement, ni de chimiothérapie, d'hormonothérapie ni de radiothérapie. La chromatine de ces tumeurs et leurs tissus sains correspondants ont été analysés par ChIP-qPCR pour déterminer l'enrichissement de H4K12ac dans les différents compartiments de la chromatine. D'après notre hypothèse posée, on devait avoir une augmentation de cet enrichissement lorsqu'on entre dans un profil cancéreux et une diminution dans un profil cancéreux avancé. Nos analyses nous ont permis de valider cette hypothèse car nous observons une forte augmentation d'enrichissement en H4K12ac dans les tumeurs lumineuses et un enrichissement moins élevé dans les tumeurs TNBCs correspondant à un profil cancéreux avancé. La forte augmentation de l'acétylation dans les cancers *ER+* avait déjà été observée par l'équipe de Nagarajan et al. en 2015 où ils ont pu montrer un enrichissement global de H4K12ac et surtout au niveau du promoteur du gène *ER* (Nagarajan et al., 2015). Leur étude met en lumière aussi une co-occupation de la marque H4K12ac et de la protéine BRD4 sur le promoteur de *ER* et cette association aurait pour rôle d'entraîner une surexpression du gène *ER* et ainsi une augmentation de la prolifération des cellules cancéreuses (Liu et al., 2019; Nagarajan et al., 2015). Cela pourrait expliquer l'augmentation moins importante de H4K12ac dans les tumeurs TNBCs car elle n'exprime pas le gène *ER* qui semblerait être responsable de cet enrichissement. Par contre, l'enrichissement en H4K12ac est observé non seulement dans l'hétérochromatine mais aussi dans l'euchromatine des différentes tumeurs.

Ensuite, nous avons analysé la Co-occupation ou Co-localisation de la marque H4K12ac avec TIP60 par ChIP-re-ChIP pour vérifier si TIP60 était bien responsable de l'acétylation de H4K12. Cette analyse nous montre que TIP60 est bien présent sur les promoteurs de gènes de l'hétérochromatine et de l'euchromatine dans les profils tumoraux ou sains. Cette Co-occupation diminue dans les profils tumoraux par rapport aux profils sains, mais la diminution est plus forte dans la tumeur TNBC que dans les tumeurs lumineuses. Cela peut être dû à une diminution d'expression de TIP60 dans les tumeurs du sein. En effet, plusieurs études ont montré que la protéine TIP60 diminuait avec l'agressivité du cancer, cette diminution serait plus importante dans les cancers du sein TNBCs et moins importante dans les cancers lumineux (Mattera et al., 2009; McGuire et al., 2019; Pandey et al., 2015). De plus, d'autres études ont pu démontrer que la marque H4K12ac est une marque cible de TIP60 dans

le cancer du sein mais aussi dans d'autres cancers (Grezy et al., 2016; Parira et al., 2017). Ces deux premiers paramètres nous permettent déjà de dire que H4K12ac est placée dans tous les compartiments de la chromatine cela grâce à l'activité de TIP60. L'enrichissement de cette marque dépend du profil tumoral du tissu, ainsi un fort enrichissement est obtenu pour les tumeurs lumineales et un faible enrichissement pour les TNBCs.

Les paramètres suivants, à savoir l'expression ARNm et protéique de TIP60 et P400 nous permettent de suivre la variation des deux protéines et de déduire leur interaction. Tout d'abord, l'analyse d'expression de l'ARNm de TIP60 et P400 par RT-qPCR nous a permis de voir que TIP60 et P400 partagent le même profil d'expression dans les différents sous types moléculaires de tumeur du sein. Dans les tumeurs lumineales, on observe une sous-expression de TIP60 et P400, alors que dans les tumeurs TNBCs on observe plutôt une surexpression de TIP60 et P400. L'expression ARNm de TIP60 et P400 dans le cancer du sein est peu étudiée, la seule étude sur l'expression ARNm de TIP60 a été effectuée dans les différentes lignées continues de cancers du sein. Cette étude montre que l'ARNm de TIP60 est surexprimé dans les lignées continues lumineales, mais aussi pour les lignées continues TNBCs sauf pour la lignée MDA-MB-231 ou son expression est sous-exprimée.

Pour l'expression protéique, on observe qu'on a une diminution d'accumulation de TIP60 dans les différents sous types moléculaires et que cette diminution était progressive avec l'agressivité de la tumeur. De plus l'accumulation de P400 augmente dans les différents sous types de tumeurs du sein par rapport aux tissus normaux et cette accumulation augmente aussi avec l'agressivité de la tumeur. Ce qui nous laisse penser que l'accumulation de TIP60 est inversement proportionnelle à celle de P400. En effet, de nombreuses études montrent que l'accumulation de TIP60 diminuerait avec l'agressivité du cancer du sein, alors que celle de P400 tend à augmenter avec l'agressivité du cancer du sein. Ce déséquilibre dans l'accumulation de TIP60 et de P400 fait partie du processus de carcinogénèse du sein (Figure 24) (Grezy et al., 2016; Mattera et al., 2009).

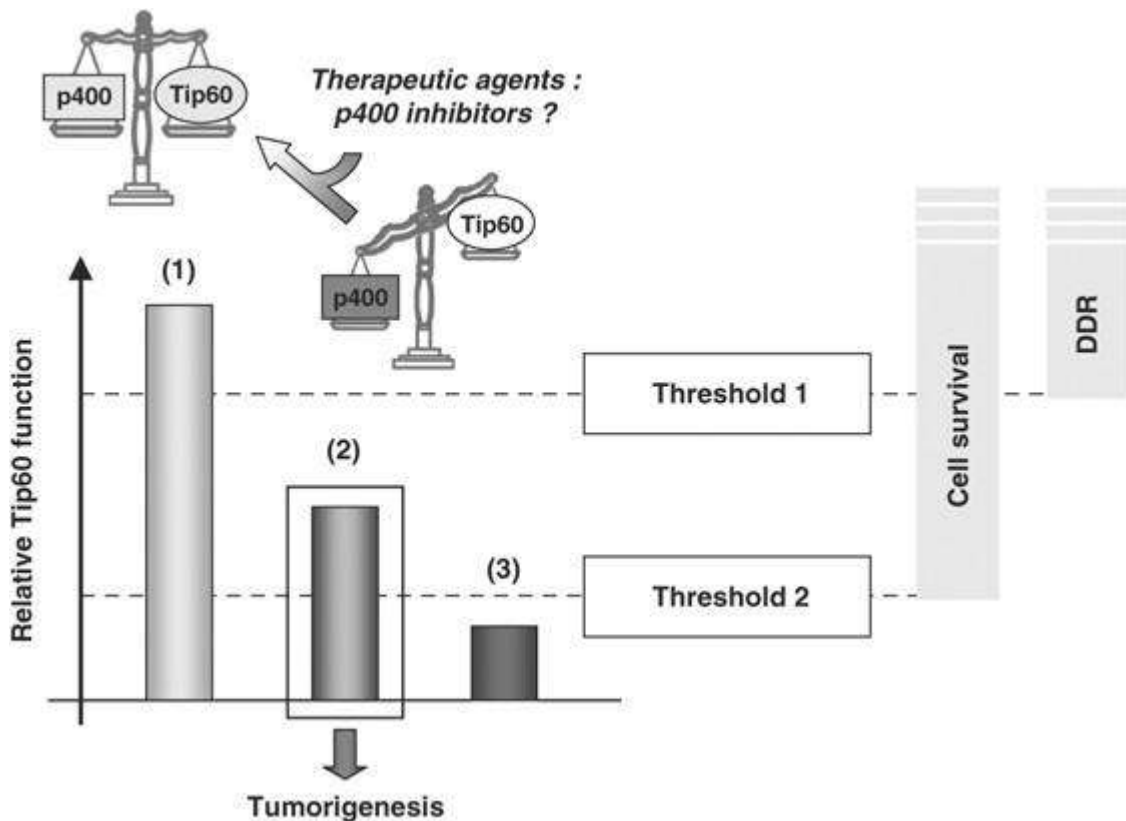


Figure 24: Représentation de la balance TIP60/P400 dans l'initiation de la tumorigénèse : 1) Lorsque TIP60 et P400 ont une expression équilibrée, la cellule est dans un profil normal. 2) Lorsque TIP60 et P400 ont une expression en faveur de P400, la cellule est dans un profil tumoral. 3) Lorsque TIP60 et P400 ont une expression très en faveur de P400, la cellule est dans un profil tumoral très agressif. Figure tirée de (Grezy et al., 2016)

Tous les paramètres analysés dans ces travaux nous ont permis de conclure que TIP60 est responsable de l'acétylation de l'histone H4 sur sa lysine 12 et qu'il existe un équilibre entre TIP60 et P400 qui lorsqu'il est perturbé, pourrait entraîner l'initiation de la carcinogénèse du sein, il nous a permis aussi d'établir un profil expression des différents acteurs constituant le squelette de maintien de l'hétérochromatine.

Par contre, les travaux effectués sur les tumeurs ne nous renseignent toujours pas sur l'interaction possible entre TIP60 et P400. D'où notre dernière étude dans laquelle on regardera les mêmes paramètres que la précédente mais cette fois dans les lignées continues de cancer du sein. Cela nous permettra de faire varier certains paramètres pour mieux comprendre le mécanisme de mise en place du squelette de maintien de l'hétérochromatine.

Pour cette étude, nous avons utilisé quatre lignées continues de cellules représentant les différents sous types moléculaires de cancer du sein, une lignée non cancéreuse (MCF-10A), deux lignées lumineales (MCF-7 et T47D) et une lignée TNBC (MDA-MB-231). Comme pour l'étude précédente, l'enrichissement de la marque H4K12ac a été observé dans les différentes lignées continues étudiées. L'enrichissement de H4K12ac dans les différents compartiments de la chromatine est plus important dans la lignée continue non cancéreuse (MCF-10A) par rapport aux lignées continues cancéreuses, mais aussi la lignée continue

TNBC (MDA-MB-231) présente un enrichissement plus important que celui des lignées continues lumorales (MCF-7 et T47D). De plus, l'analyse de la co-localisation de TIP60 et de la marque H4K12ac dans l'hétérochromatine et l'euchromatine nous a montré qu'il n'y avait pas de variation de cette co-localisation dans les différentes lignées continues étudiées. Ces résultats diffèrent de ceux obtenus dans la précédente étude sur les tumeurs, où l'on avait plutôt une augmentation de l'enrichissement et une diminution de la co-localisation dans les profils tumoraux. Cela pourrait être dû au microenvironnement de la tumeur qui pourrait influencer sur le dépôt de la marque ou sur l'activité des HATs. En effet, dans une étude *in vitro*, plusieurs paramètres sont perdus comme le microenvironnement tumoral qui pourrait encore exister si l'on faisait une étude *in vivo* ou *ex vivo*.

Dans cette dernière étude, au lieu de suivre la variation d'expression des différents acteurs du complexe TIP60/P400/H4K12ac, nous avons plutôt modulés l'expression de TIP60 ou de P400 en utilisant des SiRNAs de TIP60 ou P400 mais aussi les inhibiteurs de TIP60, TH1834 ou NU9056. La déplétion de TIP60 et P400 par leurs SiRNAs respectifs nous a permis de dire que l'expression de l'un influe sur l'expression de l'autre seulement dans la lignée continue non cancéreuse (MCF-10A) et la lignée continue lumorale (MCF-7). En effet, la déplétion de TIP60 entraîne une sous-expression en ARNm et protéine P400 et la déplétion de P400 entraîne aussi une sous-expression en ARNm et protéine TIP60 dans les lignées continues MCF-10A et MCF-7. Cela s'expliquerait par le fait que TIP60 et P400 font partie du même complexe donc la diminution d'expression de l'un entraîne la diminution d'expression de l'autre. Par contre, on a pu observer aussi que la déplétion de TIP60 entraîna une plus forte sous-expression de P400 alors que celle de P400 entraîna une sous-expression de TIP60 moins importante. Cela peut s'expliquer par le fait que TIP60 peut agir seule sans le complexe NUA4 dont fait partie P400 (Obri et al., 2014). La sous expression observée dans les lignées continues MCF-10A et MCF-7 est sûrement dû à la grande présence TIP60 dans ces lignées continues, car l'expression de TIP60 diminue avec l'agressivité du cancer du sein ce qui explique la faible variation observée dans les lignées continues les plus agressives (T47D et MDA-MB-231) (Krukenberg et al., 2014; McGuire et al., 2019). D'autre part, l'accumulation de la marque H4K12ac ne varie pas après déplétion de P400, mais diminue après déplétion de TIP60. Ce qui est logique car P400 ne possède pas d'activité acétyl-transférase et surtout que TIP60 peut effectuer son activité acétyl-transférase sans le complexe NUA4.

L'inhibition de l'activité de TIP60 par TH1834 et NU9056 nous permet de voir que l'activité de TIP60 n'a aucun effet sur l'expression de TIP60 et P400. Par contre, l'accumulation de H4K12ac dans les différentes lignées continues étudiées diminue. Ces observations sont tout à fait normales car l'activité de TIP60 n'influe ni sur sa transcription, ni sur celle de P400.

En perspectives, pour pouvoir répondre plus clairement à nos interrogations il serait nécessaires d'effectuer d'autres expérimentations supplémentaires nous permettant de mieux cerner les différents mécanismes étudiés.

Pour la question posée dans notre premier axe « Quel est l'effet de l'inhibition de l'activité de TIP60 dans les différents sous types moléculaires de cancer du sein ? », il serait intéressant d'étudier les xénogreffes et lignées continues étudiées par immunohistochimie.

Cela nous permettra de suivre l'accumulation des différentes protéines étudiées dans la cellule ou le tissu, mais surtout de voir les compartiments cellulaires les plus affectées par l'inhibition de l'activité de TIP60. Il serait aussi intéressant de suivre la variation d'autres cibles non-histones de TIP60 telles que : p53, p21 ou ATM, qui sont impliquées dans les mécanismes cellulaires dérégulés dans le cancer du sein comme l'apoptose, la réparation des dommages de l'ADN, la croissance et la différenciation cellulaire. Le traitement des xénogreffes sur des souris avec l'inhibiteur NU9056 serait une expérimentation intéressante à réaliser car NU9056 n'a pas le même mode d'action que TH1834 et pourrait agir différemment comme nous l'avons observé dans les lignées continues étudiées. Il serait aussi intéressant de faire une analyse transcriptomique par TLDA des récepteurs nucléaires sur les xénogreffes traitées avec TH1834 et NU9056.

Dans notre second axe pour la question « Quel est le rôle de TIP60 dans la plasticité et l'intégrité chromatinienne lors de la carcinogénèse du sein ? ». Il serait aussi intéressant d'effectuer avec les anticorps correspondants par immunohistochimie ou par immunofluorescence l'étude des différents constituants du complexe TIP60/P400/H4K12ac, pour voir leur accumulation dans la cellule ou le tissu, les compartiments où ils sont exprimés, mais aussi de pouvoir superposer leurs expressions en formant un merge. Il serait aussi intéressant de faire aussi une co-immunoprécipitation pour suivre l'interaction physique qu'il pourrait avoir entre les différentes protéines étudiées (TIP60/P400, TIP60/H4K12ac et P400/H4K12ac). Une ChIP-re-ChIP entre P400 et H4K12ac serait aussi intéressante à faire. Enfin, une étude *in vivo* serait intéressante à réaliser en utilisant les xénogreffes des souris (nudes) réalisées par injection des différentes lignées continues de cancer du sein. Ces xénogreffes seront analysées par les différentes techniques utilisées lors de cette thèse, mais aussi celles citées en perspectives.

En conclusion, cette thèse a permis de démontrer l'impact de TIP60 dans la croissance tumorale du sein, mais aussi de valider l'efficacité des inhibiteurs chimiques de TIP60, TH1834 et NU9056. Elle a aussi permis de mettre en lumière un nouveau rôle de TIP60 dans la plasticité et l'intégrité chromatinienne via l'acétylation de l'histone H4K12 mise en place lors de la carcinogénèse mammaire. Cette thèse met encore plus en avant une possible utilisation de TIP60 comme biomarqueur ou cible thérapeutique du cancer du sein.

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VII. WEBOGRAPHIE

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RESUME

De nos jours, les mécanismes épigénétiques ont clairement été identifiés comme étant impliqués dans la carcinogénèse du sein. L'avancéE des recherches dans le domaine a mis en lumière une possible utilisation des protéines régulant les mécanismes épigénétiques comme cible thérapeutique dans la lutte contre le cancer du sein. La variation de l'équilibre de ces protéines épigénétiques est un facteur marquant dans l'initiation et la progression tumorale du cancer du sein. L'histone acétyl-transférase TIP60 et histone déacétylase SIRT1 sont des protéines épigénétiques antagonistes qui voient leurs équilibres grandement impacté dans le cancer du sein, la compréhension des mécanismes aboutissant à ce déséquilibre permettra de mieux comprendre la carcinogénèse du sein. C'est dans cette optique qu'on s'est intéressé au rôle de l'histone acétyl-transférase TIP60 dans le cancer du sein. Pour déterminer le rôle de TIP60 dans le cancer du sein, on a suivi l'effet de l'inhibition de l'activité de TIP60 dans le cancer du sein *in vivo* et *in vitro*. En effet, cette inhibition de TIP60 induite par les deux inhibiteurs spécifiques de TIP60 (TH1834 et NU9056), nous a permis de voir que l'utilisation de ces inhibiteurs impactait l'acétylation des cibles de TIP60 telles que la marque H3K4ac impliquée dans la transcription des gènes, mais aussi dans l'expression de certains récepteurs hormonaux tels que AR et NR3C2 *in vitro*. De plus, cette inhibition entraîne une diminution de la croissance tumorale du sein *in vivo*. En parallèle, on s'est intéressés aussi au rôle de TIP60 dans l'intégrité et la plasticité chromatinienne dans le cancer sein. Pour cela, nous avons suivi la variation des acteurs du complexe que constitue TIP60/P400/H4K12ac dans la progression du cancer du sein *ex vivo* et *in vitro*. Cela nous a permis de voir qu'il y a l'installation d'un squelette constitué de TIP60/P400/H4K12ac pour maintenir l'intégrité et la plasticité de la chromatine suite à la perte de son squelette principale SUV39H1/H3K9me3/HP1. En effet, nos travaux ont mis en évidence le rôle fondamental de TIP60 dans le cancer du sein, mais aussi sa possible utilisation comme cible thérapeutique dans la lutte contre le cancer du sein.

Mot clés : Epigénétique, cancer du sein, TIP60, H4K12ac, TH1834, NU9056.

ABSTRACT

Nowadays, epigenetic mechanisms have been clearly identified as being involved in breast carcinogenesis. Advances in research in the field have highlighted the possible use of proteins regulating epigenetic mechanisms as a therapeutic target in the fight against breast cancer. The variation in the balance of these epigenetic proteins is a significant factor in the initiation and progression of breast cancer. The histone acetyl-transferase TIP60 and the histone deacetylase SIRT1 are epigenetic antagonist proteins which equilibrium is greatly impacted in breast cancer. Understanding the mechanisms leading to this imbalance in carcinogenesis, the role of the histone acetyl transferase TIP60 in breast cancer has been investigated in this thesis. To determine the role of TIP60 in breast cancer, the effects of TIP60 inhibition in breast cancer *in vivo* and *in vitro* were investigated. Indeed, this inhibition of TIP60 induced by two specific inhibitors of TIP60 (TH1834 and NU9056), demonstrated the impacts of these inhibitors on the acetylation of TIP60 targets such as the H3K4ac mark involved in gene transcription, but also in the expression of specific hormone receptors such as *AR* and *NR3C2 in vitro*. In addition, this inhibition leads to a decrease in breast tumor growth *in vivo*. In parallel, the role of TIP60 in chromatin integrity and plasticity in breast cancer has also been investigated. For this, we have followed the variation of the actors in the complex that constitutes TIP60/P400/H4K12ac in the progression of breast cancer *ex vivo* and *in vitro*. This revealed that there is the installation of a TIP60/P400/H4K12ac constituent skeleton to maintain the integrity and plasticity of the chromatin, following the loss of its principal skeleton SUV39H1/H3K9me3/HP1. Indeed, our work has highlighted the fundamental role of TIP60 in breast cancer, but also its possible use as a therapeutic target in the fight against breast cancer.

Keywords: Epigenetics, breast cancer, TIP60, H4K12ac, TH1834, NU9056.