# Cellular Metabolism Regulates Anti-Oxidant Response Through ERK5-MEF2 Pathway 

Abrar Ul Haq Khan

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## THĖSE

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> Cellular Metabolism Regulates Anti-Oxidant Response Through ERK5-MEF2 Pathway
> Rôle de la voie ERK5-MEF2 dans la régulation de la réponse anti-oxydante par le métabolisme cellulaire

## Soutenue le 27 /06/2017 devant le jury composé de



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## Abbreviations

| $\boldsymbol{\alpha - K G}$ | $\alpha$-ketoglutarate | AIF | apoptosis-inducing factor |
| :---: | :---: | :---: | :---: |
| ALT | Alanine aminotransferase | AML | Acute myelogenous leukemia |
| AMPK | AMP-activated protein kinase | APL | Acute promyelocytic leukemia |
| ARE | antioxidant responsive element | ATP | Adenosine triphosphate |
| BACE1 | $\beta$-site Amyloid Precursor Protein Cleaving Enzyme 1 | BAFF | B cell-activating factor |
| BBR | Berberine | Bcl-2 | Bcell lymphoma 2 protein |
| BMK-1 | Big MAP kinase 1 | BSA | Bovine serum albumin |
| CD | Common docking domain | CDK | Cyclin dependent kinase |
| CLL | Chronic lymphocytic leukemia | CNS | Central nervous system |
| COT | Cancer Osaka thyrpoid | CREB | cAMP response element binding protein |
| CSC | Cancer stem cell | CVS | Cardiovascular system |
| DGCR8 | DiGeorge critical protein | ECs | Endothelial cells |
| EGF | Epitheliuem Growth Factor | ERK | Extracellular Regulated kinases |
| ESC | Embryonic stem cells | ETC | Electron transport chain |
| F6P | Fructose-6-phosphate | FAs | Fatty acids |
| FH | Familial hypercholesterolaemia | FLT3 | Fms-like tyrosine kinase-3 |
| FOXO | Forkhead boxcontaining protein type O | G6P | Glucose-6-phosphate |
| GADP | Glyceraldehyde 3 phosphate | GAPDH | Glyceraldehyde phosphate dehydrogenase |
| G-CSF | Granulocyte ColonyStimulating Factor | GDH | Glutamine dehydrogenase |
| GLS | Glutaminase | GSH | Glutathione |
| HDAC | Histone deacetylase | HDL | High density lipoprotein |
| HIC1 | Hypermethylated in cancer 1 | HIF-1 $\alpha$ | Hypoxia Inducible Factor $1 \alpha$ |
| HIV-1 | Human immunodeficiency virus type 1 | H3K4me3 | Trimethylation of Lys 4 of histone 3 |
| HO-1 | HMOX1 (heme oxygenase (decycling) 1) | JNK | C-Jun amino (N)- terminal kinases |
| LIF | Leukemia inhibitory factor | LDL | Low density lipoprotein |
| LDLR | Low density lipoprotein | LPS | Lipopolysaccharide |


|  | receptors |  |  |
| :---: | :---: | :---: | :---: |
| MAPK | Mitogen activated protein kinases | MAPKKK | MAPKK kinase |
| MAPKAPK | MAPK activated protein kinases | MAF | Maf avian musculoaponeurotic fibrosarcoma oncogene homolog |
| MEF | Myocyte enhancer factor | miRNA | MicroRNA |
| MTTS1 | Metastasis suppressor 1 | NES | Nuclear export signal |
| NGF | Nerve growth factor | NLS | Nuclear localization signal |
| NQO-1 | NAD(P)H dehydrogenase [quinone] 1 | OXPHOS | Oxidative phosphorylation |
| PCSK9 | Proprotein convertase subtilisin/kexin type 9 | PDGF | Platelet derived growth factor |
| PEP | Phosphophenolipyruvat e | PFK | Phosphofructokinase |
| PGC1 $\alpha$ | Peroxisome proliferators-Activated receptor gamma coavtivator 1 alpha | PGK | Phosphoglycerate kinase |
| PK | Pyruvate kinase | PKA | Protein kinase A |
| PML | Promyelocytic leukemia protein | Pol II | Polymerase II |
| PPAR $\alpha$ | Peroxisome proliferator-activated receptor $\alpha$ | Pri-miRNA | primary-miRNA |
| Q | Ubiquinone | RARA | Retinoic acid receptor alpha |
| RISC | RNA-induced silencing complex | RNAi | RNA interference |
| ROS | Reactive oxygen species | SFN | Sulforaphane |
| SOD | Superoxide dismutase | SRE | Sterol regulating elements |
| RT | Room temperature | SH3 | Src-homology 3 |
| Sh | Short Hairpin | SIRT1 | Sirtuin 1 |
| TCA | Tricarboxylic acid cycle | TF | Transcriptional factors |
| TG | triglycerides | TGF- $\beta$ | Transforming Growth factor- $\beta$ |
| TNF- $\boldsymbol{\alpha}$ | Tumor Necrosis Factor$\alpha$ | TSS | Transcription start site |
| UCP | Uncoupling protein | UTR | Untranslated region |
| UCB | Unconjugated bilirubin | VEGF | Vascular endothelial growth factor |
| XRE | Xenobiotic response element |  |  |


#### Abstract

Cellular metabolism is the main source of energy and cancer cells has different metabolism than non-transformed cells. Tumor cell tends to avoid mitochondrial activity and oxidative phosphorylation (OXPHOS) and prefer glycolysis for energy production (Warburg effect). This alteration in metabolism is beneficial for growing cells in many ways that promote tumor growth and suppress the anti-cancer immune response. This specific metabolism is an auspicious target for the development of cancers chemotherapies.


My thesis work comprises two parts. The first portion describes that when cancer cells are forced to utilize their mitochondria in order to obtain the energy from OXPHOS they initiate an antioxidant mechanism to cope with the deleterious effects of reactive oxygen species (ROS) produced during mitochondrial activity. Mitochondrial stimulation leads to activation of ERK5-MEF2 signaling pathway, which triggers the antioxidant mechanism by at least two ways.

Initially we observed that MEF2 up regulates the expression of miR23a, which inhibits KEAP1 expression. This protein is responsible for ubiquitinational degradation of NRF2, a master regulator of the antioxidant response in cells. The inhibition of KEAP1 prevents the NRF2 cytoplasmic degradation. This results in high built up of NRF2 in cytoplasm that translocates to nucleus where it binds to ARE (antioxidant response element) in the upstream promoter region of many antioxidant genes and initiates their transcription. Latter we observed that activation of ERK5MEF2 pathway directly results in de novo synthesis of NRF2, resulting in nuclear translocation and triggering of the antioxidative mechanism. Inhibition of ERK5MEF2 pathway impairs the cellular antioxidant response, thus sensitizing cells towards oxidative stress.

The second part of my work explored the mechanism behind the lipid lowering effects of dichloroacetate (DCA). DCA is a small molecule, which inhibits the PDK1 and enables pyruvate to enter the mitochondria. It was used clinically in past to lower the plasma cholesterol level but the underlying mechanism was not clear and we uncover it. DCA forces cells to perform OXPHOS, which activates the ERK5-MEF2 pathway.

This pathway directly up-regulates the expression of Low Density Lipoprotein Receptors (LDLR) that are mainly involved in the endocytosis of cholesterol-rich low density lipoproteins, which are responsible for the majority of cardiovascular diseases. Inhibition of this pathway suppresses lipid influx and hence, it would be an interesting target of future investigation since high cholesterol level is the main cause of various life threatening diseases and the development of atherosclerosis.

Our next goal is to exploit other possible cellular mechanism regulated by ERK5MEF2 pathway. Based on our preliminary data, we propose that this pathway not only regulate the LDLR expression but many other genes, which are directly or indirectly involved in lipid metabolism.

Key words: Tumor, Metabolism, Warburg effect, OXPHOS, Dichloroacetate, LDLR, KEAP1, NRF2, Cholesterol.

## Résumé

Le métabolisme cellulaire est la source principale d'énergie et les cellules cancéreuses ont un métabolisme différent des cellules non transformées. La cellule tumorale a tendance à éviter l'activité mitochondriale et ainsi la phosphorylation oxydative, pour lui préférer la voie de la glycolyse pour la production d'énergie (Effet Warburg). Cette altération du métabolisme est si bénéfique pour les cellules en croissance que cela favorise la croissance tumorale et supprime la réponse immunitaire anticancéreuse. La spécificité de ce métabolisme en fait une cible intéressante pour le développement de thérapies anticancéreuses.

Mon travail de thèse comporte deux parties. La première partie décrit que lorsque les cellules cancéreuses sont forcées à utiliser la voie mitochondriale comme source d'énergie à travers l'oxydation phosphorylative, elles initient un mécanisme antioxydant pour tolérer les effets délétères des espèces oxygénées réactives (EOR ou ROS pour reactive oxygene species) produites au cours de l'activité mitochondriale. La stimulation mitochondriale entraîne l'activation de la voie de signalisation ERK5MEF2, et cette dernière engendre un mécanisme antioxydant de deux façons.

Initialement, nous avons observé que MEF2 régule positivement l'expression de miR23a, et ce dernier inhibe l'expression de KEAP1. Cette protéine est responsable de la dégradation ubiquitine dépendante de NRF2, un régulateur clé de la réponse antioxydante cellulaire. L'inhibition de KEAP1 empêche la dégradation cytoplasmique de NRF2. Consécutivement à cela la concentration cytoplasmique en NRF2 augmente ce qui engendre sa translocation dans le noyau où il se lie à une séquence élément de réponse antioxydant (ARE) dans la région promotrice de nombreux gènes antioxydants, initiant ainsi leur transcription. Plus tard nous avons observé que l'activation de la voie ERK5-MEF2 induisait directement la synthèse de novo de NRF2, induisant sa translocation nucléaire et un mécanisme antioxydant. L'inhibition de la voie ERK5-MEF2 altère la réponse antioxydante, sensibilisant ainsi les cellules au stress oxydant.

La seconde partie de mon travail a exploré les mécanismes à l'origine des effets hypolipémiants du dichloroacétate (DCA). Le DCA est une petite molécule qui inhibe la PDK1 et permet au pyruvate d'entrer dans la mitochondrie. Il a été utilisé en
clinique dans le passé pour baisser les taux plasmatiques de cholestérol mais le mécanisme n'était pas clair et nous l'avons décris. Le DCA force les cellules à entrer en oxydation phosphorylative ce qui active la voie ERK5-MEF2. Cette voie augmente directement l'expression du LDLR (Low Density Lipoprotein Receptor ; récepteur aux lipoprotéines de basse densité) qui permet l'endocytose des LDL riches en cholestérol qui sont responsables de la plupart des maladies cardiovasculaires. L'inhibition de cette voie supprime l'afflux de lipides et par conséquent serait une cible intéressante pour de futures recherches puisque de hauts taux de cholestérols sont directement corrélés avec une augmentation du risque d'athérosclérose et de toutes les complications mortelles qu'il entraine.

Notre prochain objectif est d'explorer les autres mécanismes cellulaires régulés par la voie ERK5-MEF2. Sur la base de nos résultats préliminaires, nous proposons que cette voie non seulement régule l'expression du LDLR mais aussi celle de nombreux autres gènes qui sont impliqués directement ou indirectement dans le métabolisme des lipides.

Mots clés: Tumeur, Métabolisme, Effet Warburg, OXPHOS, Dichloroacétate, LDLR, KEAP1, NRF2.

## General Introduction

## 1. Metabolism

Growing cells need energy for all their basic functions like; growth, division, hemostasis and proper effector functions through metabolism. Chemical and enzymatic reactions are turned on and off or sped up and slowed down according to the cell's immediate needs and overall functions. Metabolism is a composite set of chemical reactions used for production of energy that is used for building the cellular components. Metabolism is divided in; 1) Catabolism; break down of complex organic materials like proteins, fats and glucose to produce energy, 2) Anabolism; construction of cellular component like nucleic acid, protein by utilizing energy produced in catabolism. Production of energy involved a chain of chemical reactions where bigger molecules are degraded into smaller molecules, which release energy to fuel cellular activity. These coordinated series of chemical reactions interlink and form a complex network called metabolic pathways, which are regulated by enzymes. Cells must balance their catabolic and anabolic pathways in order to control the levels of critical metabolites and to ensure the availability of sufficient energy. Interestingly metabolic pathways are strikingly similar across diverse species indicating their effectiveness (Alberts et al., 2013; O'Connor, Adams, \& Fairman, 2010; Pace, 2001).

Fundamental molecules used for energy production in metabolism are carbohydrates, proteins and lipids. Carbohydrates like glucose are the main biomolecule involved in cellular respiration (Lehninger, Nelson, \& Cox, 2005) but these are not the only option for cells as fatty acids and amino acids (like Glutamine) are also suitable for catabolic as well as anabolic reactions. The fatty acids through beta-oxidation in mitochondria produce acetyl-CoA, which can enter the Krebs cycle. Amino acids are oxidized to urea and carbon dioxide for energy production. Initially, larger molecules are transformed into smaller ones during digestion, which occurs extracellularly. Subsequently, these smaller molecules are taken up by cells and degraded into further smaller molecules, which produce a small amount of energy. Finally, these smaller molecules are used to produce energy by cellular respiration, which is the main phenomenon producing energy for cells. The process, e.g. for glucose, can be divided into many steps: glycolysis, pyruvate oxidation, Krebs or Tricarboxylic acid cycle (TCA), oxidative phosphorylation (OXPHOS) ending in energy synthesis (Alberts et al., 2013; O'Connor et al., 2010).

### 1.1. Cellular Respiration

A complex set of catabolic reactions within cells is responsible for energy production and is termed as cellular respiration (Niknamian, 2016). Energy is commonly stored in the form of adenosine triphosphate (ATP) for further usage (Bailey). Living cells obtain energy through two different ways; 1) aerobic respiration is when oxygen is used as oxidizing agent and electron acceptor and 2) anaerobic respiration is when oxygen is not present as a terminal electron acceptor (Fig. 1). Aerobic metabolism is more effectual than anaerobic metabolism and yields higher energy. In anaerobic glycolysis lactate can be produced from initial metabolism of glucose along with other toxic metabolites, whereas in aerobic glycolysis pyruvate is oxidized to acetyl-CoA and ends in Krebs cycle and OXPHOS inside mitochondria (Nancharaiah, Mohan, \& Lens, 2016; Simon \& Klotz, 2013; Smeitink, Sengers, \& Trijbels, 2005).


Fig. 1. Cellular respiration. Aerobic verses anaerobic respiration. Adopted from (Niknamian, 2016).

### 1.1.1. Glucose Metabolism

Glucose is converted in pyruvate inside cytoplasm with the release of 2 ATPs, which involves ten chemical reactions involving nine intermediate compounds (Fig. 2).

Glucose $+2 \mathrm{NAD}^{+}+2 \mathrm{ADP}+2 \mathrm{P}_{\mathrm{i}} \rightarrow 2$ pyruvate $+2 \mathrm{NADH}+2 \mathrm{H}^{+}+2 \mathrm{ATP}+2 \mathrm{H}_{2} \mathrm{O}$

At the start ATP is consumed to convert glucose into intermediate compounds with the participation of various enzymes. Firstly, glucose converted into glucose-6-phosphate (G6P) in the presence of hexokinase at the cost of 1 ATP. This irreversible reaction maintains low glucose concentration inside cells promoting its transport. Then G6P is converted into fructose-6-phosphate (F6P) with phosphate isomerase. Another ATP molecule is used when F6P converts in fructose 1,6-biphosphate via phosphofructokinase (PFK). Reaction catalyzed by hexokinase and PFK are irreversible rendering these enzymes as regulatory points. Citrate and ATP inhibit PFK. At this point aldolase splits 6 -carbon molecule into 2 triose sugars, dihydrooxyacetone phosphate and glyceraldehyde 3-phosphate (GADP). Triphosphate isomerase rapidly converts dihydroxyacetone phosphate into glyceraldehyde 3-phosphate. This is also called preparatory phase.


Fig. 2. Glycolysis. Adopted from (www.wikipedia.org).
Glucose is degraded through 10 enzymatic reactions to produce 2 molecules of pyruvate and two molecules of ATP. Every chemical reaction (red box) is regulated by different enzyme. Steps 1 and 3 consume ATP (blue) and steps 7 and 10 produce ATP (yellow). Since steps 610 occur twice per glucose molecule, this leads to a net production of ATP.

The second phase is called pay-off phase as it yields 2 nicotinamide adenine dinucleotide (NAD) and 4 ATP molecules hence producing a net gain of 2 ATP molecules from glycolysis. Glyceraldehyde 3-phosphate is converted into 1,3-biphosphoglycerate (1,3BPG) with the addition of inorganic phosphate and this reaction is catalyzed by glyceraldehyde phosphate
dehydrogenase (GAPDH). Each triose gives two hydrogen atoms to two molecules of NAD ${ }^{+}$ producing $\mathrm{NADH}^{+}$. Phosphoglycerate kinase (PGK) regulates the enzymatic transfer of a phosphate group from 1,3-biphosphoglycerate to ADP yielding 2 ATP molecules. This 3phosphoglycerate is converted in phosphophenolpyruvate (PEP) via catalytic action of mutase and enolase. Finally, pyruvate kinase (PK) converts PEP into pyruvate and another ATP molecule is yielded at this final step of glycolysis. PK also regulates glycolysis as ATP and glucagon inhibit PK during low plasma level of glucose (Lithaw, 2009).

### 1.1.2. Fructose Metabolism

Fructose catabolism is called fructolysis. Fructose enters in cells through glucose transporter 2 (GLUT2) (Antoine et al., 1997) and phosphorylated into fructose 1-phosphate, which can directly enters into glycolysis except in liver cells (Fig. 3). Whereas in liver cells fructose is initially phosphorylated into triose sugar which than enters into glycolysis (Lithaw, 2009).


Fig. 3. Fructose Metabolism. Adopted from (www.wikipedia.org).
Fructose is metabolized through a series of reaction catalyzed by Fructokinase, aldolase B and finally Triose phosphate isomearse to produce Glyceraldehyde-3-phosphate, which enters in glycolysis.

Mannose, another hexose sugar, undergoes phosphorylation and isomeration by hexokinase and isomerase enzyme respectively before entering into glycolysis pathway. Hydrolysis of lactose produced another hexose sugar i.e. galactose which is metabolized by Leloir Pathway. This involves a series of reactions catalyzed by distinctive enzymes to convert galactose into
glucose 6-phosphate for submission to glycolysis. In culture conditions, cells mainly use galactose for nucleic acid production while glutamine as an energy source (Reitzer, Wice, \& Kennell, 1980; Lawrence J Reitzer, Burton M Wice, \& David Kennell, 1979).

### 1.1.3. Galactose Metabolism

Galactose is metabolized by enzymes; GALK, GALT and GALE. A secondary metabolic pathway catalyze by the enzyme UDP galactose pyrophosphorylase (GALPP), ensures the conversion of galactose-1-phosphate into UDP-galactose, used for glycosylation of proteins and lipids. Glucose-1-phosphate converts into Glucose-6-phosphate and enters into glycolysis (Fig. 4).


Fig. 4. Galactose Metabolism. Entry of galactose to glycolytic pathway. Adopted from (Cuthbert, Klapper, \& Elsas, 2008).

The end product of glycolysis is pyruvate, which is decarboxylated to acetyl-CoA, releasing NADH and carbon dioxide (Fig. 5). This occurs in mitochondria linking glycolysis and Krebs cycle. This reaction is catalyzed by pyruvate dehydrogenase complex that is negatively regulated by NADH and acetyl-CoA.


Fig. 5. Pyruvate Decarboxylation. Adapted from (Jennifer Agnes Hilary Lucy).

1) Carboxyl functional group removed as $\mathrm{CO}_{2}$ - decarboxylation catalyzed by pyruvate decarboxylase 2) Redox reaction that converts $\mathrm{NAD}^{+}$to NADH and $\mathrm{H}^{+}$, pyruvate is oxidized and $\mathrm{NAD}^{+}$is reduced 3) CoA is attached to acetate compound to form Acetyl CoA for participation in Krebs Cycle.

### 1.1.4. The Krebs cycle (Citric acid cycle or Tricarboxylic acid cycle)

The Krebs cycle is also known as Tricarboxylic acid cycle occurs inside the mitochondria where acetate is oxidized for energy production through a series of chemical reactions (Fig. 6). Acetate, in the form of acetyl-CoA serves to reduce $\mathrm{NAD}^{+}$to $\mathrm{NADH}^{+}$and as a result carbon dioxide is produced. Amino acids, which are produced through protein catabolism, are converted into acetyl-CoA to enter into the Krebs cycle (Wagner, 2014). Similarly, fat catabolism yields glycerol and fatty acids. Glycerol through gluconeogenesis pathway can be converted into glucose while fatty acids undergo beta-oxidation to produce acetyl-CoA to be used in Krebs cycle. The overall cycle can be summarized as follows.

1 Glucose $+10 \mathrm{NAD}^{+}+2 \mathrm{CoQ}+2 \mathrm{ADP}+2 \mathrm{GDP}+4 \mathrm{Pi}+2 \mathrm{H}_{2} \mathrm{O} \rightarrow 6 \mathrm{CO}_{2}+10 \mathrm{NADH}, \mathrm{H}^{+}+$ $2 \mathrm{CoQH}_{2}+2 \mathrm{ATP}+2 \mathrm{GTP}$

Availability as well as inhibition of certain substrates and products influence and regulate the Krebs cycle. For instant, abundance of NADH and ATP inhibit pyruvate dehydrogenase, isocitrate dehydrogenase and $\alpha$-ketoglutarate dehydrogenase to regulate this cycle. Similarly, ADP activates citrate synthase but ATP subdues citrate (Smeitink et al., 2005). Calcium also regulates many steps in the cycle by activating pyruvate dehydrogenase, isocitrate
dehydrogenase and $\alpha$-ketoglutarate dehydrogenase (Denton, 2009; Ivannikov \& Macleod, 2013).


Fig. 6. Krebs cycle or TCA cycle. Adapted from (www.wikipedia.org).
Acetyl CoA is degraded to $\mathrm{CO}_{2}$ and $\mathrm{H}^{+}$and electrons are transported to the transport chain by NADH and $\mathrm{FADH}_{2}$. The first step of the cycle is to transfer an acetyl group on the oxaloacetate to form citrate. The rest of the cycle consists of catalytic transformations. The final step produces oxaloacetate, which can then receive a new acetyl and repeat the cycle.

### 1.1.5. Oxidative phosphorylation (OXPHOS)

Majority of aerobic organisms obtain their energy through a metabolic pathway named as oxidative phosphorylation (Peter HGM Willems, Rodrigue Rossignol, Cindy EJ Dieteren, Michael P Murphy, \& Werner JH Koopman, 2015). In this pathway electrons are transferred from electron donor to electron acceptors (Oxygen) through a series of redox reactions. The end product of glycolysis and TCA cycle are two coenzymes, are NADH and $\mathrm{FADH}_{2}$ containing electrons, which are oxidized to produce large amount of energy. Electrons are removed from NADH and $\mathrm{FADH}_{2}$ through a series of enzymes producing a proton
electrochemical gradient that is utilized for ATP production via ATP synthase. The series of enzymes which act as electron donors and acceptors in electron transport comprises complex I to complex V and are also called the electron transport chain (ETC) that resides in the inner membrane of mitochondria (Fig. 7).

Electrons are passed from an electron donor to an electronegative acceptor, which further passes them to another acceptor and finally electrons are passed to oxygen (most electronegative and terminal acceptor in the chain). Energy is released when electrons are passed between acceptor and donor, which are employed to create a proton gradient in mitochondrial membrane (Smeitink et al., 2005). The overall electron transport chain;


## Complex I (NADH dehydrogenase)

Two electrons pass from NADH to ubiquinone (Q) results in translocation of four protons from the mitochondrial matrix to the inter membrane space creating proton gradient. The complex I is inhibited by barbiturates, rotenone and metformin.

## Complex II (Succinate dehydrogenase)

This enzyme is important and critical as it is involved in both Krebs cycle and ETC. Succinate is oxidized into fumerate by reducing ubiquinone. No proton is transported across the membrane. Complex II is constrained by thenoyltrifluoroacetone (TTFA), 3-Nitropropionic acid and malonate, an analogue of succinate.

$$
\text { Succinate }+\mathrm{CoQ} \longrightarrow \text { Fumerate }+\mathrm{CoQH}_{2}
$$

## Complex III (Q-cytochrome c oxidoreductase)

Proton gradient is produced by this complex through the oxidation of one molecule of ubiquinol and reduction of two molecules of cytochrome c. Antimycin A and Myxothiazol inhibits this complex:

$$
\mathrm{CoQH}_{2}+2 \mathrm{CytCFe}^{3+} \longrightarrow \mathrm{CoQ}+2 \mathrm{CytCFe}^{2+}
$$



Fig. 7. Electron Transport Chain. Adapted from (www.wikimedia.org).
Main reactions involve electron acceptors of both sides of the inner mitochondrial membrane in 5 complexes. Parallel to the transport of electrons, protons are pumped into the space between the membranes of the mitochondrion. The chemical gradient is formed that is used to produce ATP by oxidative phosphorylation.

## Complex IV (Cytochrome c oxidase)

This complex reduce $\mathrm{O}_{2}$ molecule into $\mathrm{H}_{2} \mathrm{O}$ and expels proton of matrix toward the mitochondrial intermembrane space resulting in proton gradient. This complex is impaired by cyanide, azide, sulfide and carbon monoxide, which bind to cytochrome c oxidase and competitively inhibits its function.

$$
4 \mathrm{CytFe}^{2+}+\mathrm{O}_{2}+4 \mathrm{H}^{+} \longrightarrow 4 \mathrm{CytFe}^{3+}+2 \mathrm{H}_{2} \mathrm{O}
$$

## Complex V (ATP synthase)

The final enzyme involved in OXPHOS utilizes the proton gradient to convert ADP and inorganic phosphate for ATP synthesis. A single molecule of ATP synthesis requires three to four protons. The function of this complex is compromised by oligomycine and dicyclohexylcarbodiimide (DCCD).

$$
\mathrm{ADP}+\mathrm{Pi}+4 \mathrm{H}_{\text {intermembrane }}^{+} \quad \longrightarrow \mathrm{ATP}+4 \mathrm{H}_{\text {matrix }}^{+}
$$

### 1.2. Lactic acid Fermentation

In anaerobic conditions lactic acid fermentation is a redox reaction utilized as alternative path to obtain energy. In anaerobic organism primarily energy is produced through glycolysis by reducing $\mathrm{NAD}^{+}$to NADH and resulting in 2 net ATPs. In limited $\mathrm{NAD}^{+}$supply, NADH is oxidized and regenerates $\mathrm{NAD}^{+}$. NADH gives its extra electron to pyruvate molecule produced during anaerobic glycolysis and regenerate NAD for further glycolysis resulting in lactic acid production through reduction of pyruvate (Fig. 8). Fermentation can produce only 2 ATP however this process is speedy than OXPHOS but the end product of fermentation is lactic acid which is not metabolized and should be expelled from the cells to external medium (Hsu \& Sabatini, 2008; Tortora, Case, \& Funke, 2016).

The lactic acid fermentation can be summarized as;

$$
\mathrm{C}_{6} \mathrm{H}_{12} \mathrm{O}_{6}+2 \mathrm{ADP}+2 \mathrm{P}_{\mathrm{i}}+2 \mathrm{NAD}^{+} \rightarrow 2 \mathrm{CH}_{3} \mathrm{COCOO}^{-}+2 \mathrm{ATP}+2 \mathrm{NADH}+2 \mathrm{H}_{2} \mathrm{O}+2 \mathrm{H}^{+}
$$



Fig. 8. Lactic Acid Fermentation. Adopted from (www.slideplayer.com).
In the absence of oxygen the cells undergo fermentation where glucose is converted to pyruvate by glycolysis, which does not enter the Krebs cycle but produce lactic acid by the enzyme lactate dehydrogenase (LDH).

### 1.3. Glutaminolysis

Glutamine is the most abundant naturally occurring non-essential amino acid, which is also the most profuse free amino acid in human plasma with the ability to cross blood-brain barrier (H. Kim, 2011) and is broadly used as medical food supplement (Marini, 2016; Moe - Byrne,

Brown, \& McGuire, 2016). Glutamine from extracellular medium is transported in the cells through highly specific transporters like SLC1A5 (Matés et al., 2009 \{Wise, 2010 \#732; Wise \& Thompson, 2010). It is broken down into glutamate, aspartate, lactate, alanine, pyruvate and citrate by glutaminolysis inside mitochondria. Glutaminase (GLS) metabolizes it into glutamate that is than converted into $\alpha$-ketoglutarate $(\alpha-\mathrm{KG})$ by dehydrogenase (GDH), which can directly enter into the TCA cycle. Human genome consists of two distinct izozymes of GLS, which are; GLS1 encodes the kidney-type izozyme and GLS2 encodes liver-type izozyme (Shanware, Mullen, DeBerardinis, \& Abraham, 2011). In growing cells glutamine is also used for the production of glutathione (GSH), one of the main cellular endogenous antioxidant along with other amino acids (H. Kim, 2011).

Certain tissues rely heavily on glutamine like immune cells, kidney, intestine, liver and spleen. It assists in cellular homeostasis by acting as osmolyte in intestine and liver tissues while in kidney acts as a non-toxic nitrogen carrier (Wischmeyer, 2007). Activation of T-cells require high amount of energy coming mainly from glutamine metabolism that leads to high glutamine transport and GLS activity. The ERK/MAPK pathway regulates glutamine high uptake and metabolism during T-cell activation (Carr et al., 2010). Glutamine and alanine, by producing NADPH maintains redox potential and speeds up post operational healing process. Similarly, NADPH acts as free radicals assisting immune cells in pinocytosis and phagocytosis (Soeters \& Grecu, 2011).

### 1.3.1. Glutamine metabolism and cancer

Rapid and continues proliferation of cancer cells requires constant energy source in order to regulate the production of macromolecules which make them dependent on glutamine (Moeez G Rathore et al., 2012). This requirement is accomplished by glutamine by acting as carbon source for intermediates of the TCA cycle and nitrogen source for the production of other vital molecules like nucleotides, hexosamine and non-essential amino acids (Shanware et al., 2011). De novo synthesis of purine and pyrimidine is assisted by glutamine in form of amido nitrogen provision since in low glutamine condition cells tends to delay S-phase transit (Gaglio, Soldati, Vanoni, Alberghina, \& Chiaradonna, 2009). The production of lactate, alanine and ammonia in tumor cells is glutamine dependent, which maintains non-essential amino acid pools. Activation of mTOR signaling in cancer cells for growth and autophagy is also sustained by glutamine (Durán et al., 2012). Several types of tumors depend greatly on glutamine as observed in pancreatic cancer (Paca-2 cell line) and breast cancer (MCF-7 cell
line). Glutamine analogs like acivicin and 6-diazo-5-oxo-l-norleucine (DON) are used in clinics but their deleterious effects on CNS make their use questionable (Dang, 2009).

Tumor cells growing in glutamine containing media utilize glutamate for TCA cycle (DeBerardinis et al., 2007; Lawrence J Reitzer et al., 1979; Rodrigue Rossignol et al., 2004). Therefore targeting glucose metabolism as cancer therapy permits these cells for their survival rendering them resistance. Similarly, p65 activation in tumor targets miR-23a expression facilitating glutamine consumption (Moeez G Rathore et al., 2012).

Activated oncogenes exert stress in early cell transformation, which accompanied with vigorous metabolism results in elevated reactive oxygen species (ROS) leading to DNA damage or cell death. This protective defense mechanism hinders the proliferation of transformed tumor cells. Glutamine metabolism benefits cancer cells to evade from deleterious effect of ROS through production of GSH while NAPDH production by glutamine maintains GSH in its reduced state. This assumption is supported with the inhibition of GLS which prevented the Rho GTPases induce transformation of NIH-3T3 cells (J.-B. Wang et al., 2010). Thus glutamine metabolism protects tumor cells from oxidative damage by acting as buffer in oxidative damage. This is the reason that glutamine metabolism is up regulated by many oncogenic insults and mutations (Table 1) (Altman, Stine, \& Dang, 2016).

Tumor-suppressing role of GLS2 (liver type) was also observed when it is activated by tumor suppressor p53 initiating glutamine metabolism and GSH synthesis and triggers antioxidant activity contributing in tumor suppression function of p53 (W. Hu et al., 2010; S. Suzuki et al., 2010).

Table 1: Influence of oncogenes and tumor suppressor gene loss on glutamine metabolism. Adopted from (Altman et al., 2016).

| Oncogenic change | Role in glutamine metabolism | References |
| :---: | :---: | :---: |
| MYC Up-regulation | Up regulates glutamine metabolism enzymes and transporters | (DeBerardinis et al., 2007; Gao et al., 2009; Wise et al., 2008; M. Yuneva, Zamboni, Oefner, Sachidanandam, \& Lazebnik, 2007; M. O. Yuneva et al., 2012) |
| KRAS mutations | Drives dependence on glutamine metabolism, suppresses $G L U D$ and drives $N A D P H$ generation via $M E 1$ | (Brunelli, Caiola, Marabese, <br> Broggini, \& Pastorelli, 2014; <br> Gaglio et al., 2011; Gaglio et al., 2009; Son et al., 2013; Weinberg et al., 2010) |
| HIF1 $\alpha$ or HIF2 $\alpha$ stabilization | Drives reductive carboxylation of glutamine to citrate for lipid production | (Gameiro et al., 2013; Metallo et al., 2012; Wise et al., 2011) |


| HER2 Up-regulation | Activates glutamine metabolism through MYC and NF - к $B$ | (Z. Chen, Wang, Warden, \& Chen, 2015; Qie, Chu, Li, Wang, \& Sang, 2014) |
| :---: | :---: | :---: |
| p53, p63 or p 73 activity | Activates GLS2 expression | (Arianna et al., 2013; W. Hu et al., 2010; S. Suzuki et al., 2010; Velletri et al., 2013) |
| $\begin{gathered} J A K 2-V 617 F \\ \text { mutation } \\ \hline \end{gathered}$ | Activates $G L S$ and increases glutamine metabolism | (Zhan et al., 2014) |
| mTOR Up-regulation | Promotes glutamine metabolism via induction of MYC and GLUD or aminotransferases | (Coloff et al., 2016; Alfred Csibi et al., 2013; Alfredo Csibi et al., 2014; Haigis et al., 2006) |
| NRF2 activation | Promotes production of glutathione from glutamine | (Mitsuishi et al., 2012) |
| TGF $\beta-W N T$ <br> Up-regulation | Promotes SNAIL and DLX2 activation, which up regulate $G L S$ and activates epithelial to mesenchymal transition | (S. Y. Lee et al., 2016) |
| PKC $\zeta$ loss | Stimulates glutamine metabolism through serine synthesis | (Ma et al., 2013) |
| PTEN loss | Decreased GLS ubiquitylation | (Garcia-Cao et al., 2012) |
| RB1 loss | Upregulates GLS and SLC1A5 expression | (Reynolds et al., 2014) |
| $G L U D$, glutamate dehydrogenase; $G L S$, kidney - type glutaminase; $G L S 2$, liver - type glutaminase; $H I F$, hypoxia - inducible factor; $J A K 2$, Janus kinase 2; ME1, malic enzyme 1 ; $N F-\kappa B$, nuclear factor - $\kappa \mathrm{B}$; $N R F 2$, nuclear factor, erythroid derived 2 , like $2 ; P K C \zeta$, protein kinase $\mathrm{C} \zeta ; R B 1$, retinoblastoma $1 ; T G F \beta$, transforming growth factor $-\beta$. |  |  |

### 1.4. Lipid metabolism

Lipids are naturally occurring molecules mainly used for energy purpose. They include fats, waxes, sterols, fat-soluble vitamins (such as vitamins A, D, E, and K), monoglycerides, diglycerides, triglycerides, phospholipids, etc. They are also a structural component of cell membrane and involved in signaling (Fahy et al., 2009; Subramaniam et al., 2011). Lipid metabolism includes several steps including lipid uptake, transport, synthesis and degradation, through a series of complex process (C. Huang \& Freter, 2015). Since lipids are hydrophobic and insoluble in blood plasma, they are transported by hydrophilic, spherical structures called lipoproteins, which possess surface proteins (Apo proteins or Apo lipoproteins) that also serve as co-factors and ligands for lipid-processing enzymes. Lipoproteins are organized according to size and density; Very low-density lipoproteins (VLDL) low-density lipoproteins (LDL), intermediate-density lipoproteins (IDL) and high-density lipoproteins (HDL). LDLs are produced from VLDL and IDL metabolism and are most cholesterol-rich proteins. About 40 to $60 \%$ of all LDL are cleared by the liver and is mediated by Apo-B and hepatic LDL receptors (LDLR). Lipid catabolism mainly occurs in mitochondria through beta-oxidation, which results in degradation of fatty acids to generate acetyl-CoA (Fig. 9) (Goldberg, 2016).


Fig. 9. Overview of cellular fatty acid metabolism. Adopted from (Currie, Schulze, Zechner, Walther, \& Farese, 2013).

### 1.4.1. Lipid metabolism and cancer

High proliferating cancer cells requires fatty acids in order to synthesis their membranous structure and lipid metabolism is altered in cancer cells with increased de novo fatty acid (FAs) synthesis while limiting FAs availability limit cancer cell proliferation (Currie et al., 2013; C. R. Santos \& Schulze, 2012). Almost all lipids are physiologically important in this issue, but triglycerides (TGs) and cholesterol contribute most to disease (Goldberg, 2016). Alteration in lipid metabolism is also observed in various cancers (DeBerardinis \& Thompson, 2012; C. R. Santos \& Schulze, 2012; Swinnen, Brusselmans, \& Verhoeven, 2006). Nearly in all nonmalignant adult tissues expression and activity of fatty acid synthase
are extremely low while is significantly up regulated in a number of cancers (Lupu \& Menendez, 2006). In ovarian cancer fatty acid binding protein 4 (FABP4) was observed to provide FAs from surrounding adipocytes to meet the need of tumor growth (Nieman et al., 2011). Similarly, CD36, a widely expressed trans membrane protein involved in fatty acid uptake, has been implicated in breast cancer (DeFilippis et al., 2012). Recently it is found that CD36 is involved in metastasis in tumor cells and its inhibition impairs tumor metastasis (Z. Li \& Kang, 2017; Pascual et al., 2017; Villanueva, 2017).

Tumor cells can also obtain lipids from glutamine, since glutamine metabolism is up regulated in cancer cells. Glutamine can be converted to citrate by the reversal of the Krebs cycle reactions catalyzed by isocitrate dehydrogenase (IDH) and aconitase (Metallo et al., 2012; Mullen et al., 2012; Wise et al., 2008), which further can then be used for yielding acetyl-groups for FAs synthesis (Currie et al., 2013). Suppression of Acetyl-CoA carboxylase 1 (ACC1) by siRNA or its chemically inhibition induces apoptosis in prostate cancer (Brusselmans, De Schrijver, Verhoeven, \& Swinnen, 2005), breast tumor (Chajès, Cambot, Moreau, Lenoir, \& Joulin, 2006) and in prostate cancer cells (Beckers et al., 2007) but not in normal cells while chemical inhibition of fatty acid synthase (FASN) execute cancer cells (Lupu \& Menendez, 2006).

Various anti-cancer drugs are lipid-based or effectively regulate lipid metabolism, e.g. cytarabine, etoposide, vinblastine, and vincristine etc (C. Huang \& Freter, 2015). Similarly certain anticancer drugs, e.g. cerulenin, inhibit fatty acid synthase (FASN) and target tumor progression (Kridel, Lowther, \& Pemble IV, 2007) while others significantly lower intracellular cholesterol level by blocking different steps of lipid biosynthesis (Benakanakere et al., 2014; Gorin, Gabitova, \& Astsaturov, 2012; Luu, Sharpe, Gelissen, \& Brown, 2013). Thus blocking lipid synthesis is getting a promising approach in targeting tumor cell proliferation and inducing apoptosis.

### 1.5. Tumor metabolism

Energy metabolism of tumor cells is different than the normal cells as majority of cancer cells prefer to obtain energy from aerobic glycolysis, i.e. glycolysis in the presence of ample amounts of oxygen, rather than respiration, a phenomenon called the Warburg effect (Fig. 10) (Martin Villalba et al., 2013; Warburg, Wind, \& Negelein, 1927). Though the amount of energy produced is less compared to OXPHOS, this process is much quicker offering plenty
of benefits to vigorsly growing tumor (Martin Villalba et al., 2013). Pyruvate dehydrogenase kinase-1 (PDK1) is a master regulator of Warburg effect. It negatively regulate pyruvate dehydrogenase complex ( PDH ) resulting in this metabolic switch from mitochondrial OXPHOS to glycolysis. Tumor cells have highly active PDK1, which ensures energy production from glycolysis.


Fig. 10. Warburg effect. Adopted from (Vander Heiden, Cantley, \& Thompson, 2009).
This alteration of cell metabolism can be an interesting tool in chemotherapy to target specifically cancer cells since suppression of PDK1 activity will force cancer cells to use their mitochondria in order to fully oxidize the glucose instead of producing lactate or other intermediates of glycolysis (Peter W Stacpoole et al., 2008; Martin Villalba et al., 2013).

Tumor cells undergo extraordinarily high rates of replicative cell division that demands proteins, lipids and nucleic acids to cope this process. So tumor cells require the expertise to
obtain high amount of nutrients and speedy conversion of their carbon and nitrogen into macromolecules. Aerobic glycolysis (Warburg effect) enables them to smoothly fulfill the enhanced production of macromolecules (Fig. 11).


Fig. 11. Some metabolic activities are required for tumor growth. Adopted from (DeBerardinis, 2008).

Signaling pathways of oncogenes and tumor suppressor genes directly regulate cancer cell metabolism by controlling glycolysis, OXPHOS, pentose phosphate pathway and glutamine metabolism to support energy and lipid synthesis (Fig. 12) (Altman et al., 2016; Carr et al., 2010; Moeez G Rathore et al., 2012).

Growth factor receptors stimulate phosphoinositide 3-kinase (PI3K) signaling enhancing glucose uptake by hexokinase via AKT and stimulating phosphofructokinase activity in the initial glycolysis phase (DeBerardinis, Lum, Hatzivassiliou, \& Thompson, 2008). Later, tyrosine kinase negatively regulates flux, allowing glycolytic intermediates production and
supports NADPH production (Vander Heiden et al., 2009). Similarly, glutamine metabolism is enhanced by MYC oncogene supporting NADPH production. Thus, MYC dependent cancer cells are sensitive to glutamine scarcity (M. Yuneva et al., 2007), while the majority of genes involved in glutamine metabolism are directly or indirectly regulated by MYC protein (Gao et al., 2009; Wise et al., 2008).


Fig. 12. Metabolism of proliferating cells. Adopted from (Vander Heiden et al., 2009).
AMP-activated protein kinase (AMPK), a metabolic sensor, regulates glucose and lipid metabolism under variations in nutrients and intracellular energy levels and is directly activated by the tumor suppressor gene LKB1 (also known as STK11) (Hawley et al., 2003; Shaw et al., 2004; Woods et al., 2003). The LKB1-AMPK pathway regulates cellular metabolism under low intracellular ATP levels and low nutrient conditions by arresting cell growth. This pathway along with p53 reduces metabolic flux regulating glycolysis in stress
conditions. This results in reduced glycolysis during conditions of low energy availability or oxidative stress (Shackelford \& Shaw, 2009).

## Le Métabolisme Cellulaire

Les cellules proliférantes utilisent en permanence de l'énergie nécessaire à leur croissance et fonctionnement. Le processus par lequel ces cellules obtiennent de l'énergie à partir de diverses sources énergétiques est appelé «métabolisme ». La voie métabolique est une série de réactions chimiques interconnectées et complexes utilisées par la cellule pour dégrader diverses molécules riches en énergie afin de produire de l'énergie cellulaire sous forme d'adénosine triphosphate (ATP) pour activité normale et pour construire de nouveaux constituants cellulaire. Les cellules vivantes obtiennent de l'énergie par la respiration aérobie, qui se produit en présence d'oxygène et permet de produire une grande quantité d'énergie. En l'absence d'oxygène, ces cellules effectuent la respiration anaérobie qui entraîne la production de lactate et autres métabolites toxiques tout en produisant une faible quantité d'énergie en comparaison avec la respiration aérobie.

Les molécules essentielles au métabolisme cellulaire sont les glucides, les protéines et les lipides. Le glucose est la principale source de combustible pour la production d'énergie cellulaire, il est transformé en molécules de pyruvate dans le cytoplasme. Le pyruvate est converti en AcétylCoA qui entre dans le cycle de Krebs pour subir une série de réactions chimiques à l'intérieur des mitochondries. La majorité des organismes aérobies obtiennent leur énergie par phosphorylation oxydative (OXPHOS) où le transfert d'électrons entre un donneur et un accepteur plus électronégatif libère de l'énergie. Cette dernière est employée pour générer un gradient de concentration de protons autour de la membrane mitochondriale. Ce processus appelé chaîne de transport d'électrons implique diverses enzymes, facteurs et co facteurs. Les cellules peuvent également métaboliser d'autres molécules comme le galactose, le fructose, etc. De façon intéressante, les voies métaboliques sont étonnamment semblables dans diverses espèces.

Dans les cellules tumorales, cette voie métabolique est altérée car les cellules préfèrent la glycolyse aérobie à l'OXPHOS (Effet de Warburg). Bien qu'elle fournie moins d'énergie, elle est rapide et offre divers avantages en faveur d'une prolifération continue des cellules. Les cellules tumorales ont un pyruvate déshydrogénase kinase-1 (PDK1) hautement active, qui régulent négativement le complexe pyruvate déshydrogénase ( PDH ) et assurent la production d'énergie à partir de la glycolyse. Les cellules tumorales subissent des taux élevés de division cellulaire réplicative exigeant des protéines, des lipides et des acides nucléiques. Pour faire face à ce processus, les cellules tumorales augmentent leur métabolisme glutaminique et
lipidique pour répondre à la demande de cellules en croissance excessive. Divers oncogenes régulent directement le métabolisme des cellules cancéreuses. L'altération de cette voie métabolique est prometteuse et peut être ciblée afin de contrôler la croissance tumorale.

## 2. Dichloroacetate

It is very much clear now that most cancer cells obtain a large amount of energy from anaerobic glycolysis (Warburg Effect), a cardinal characteristic of tumor, first portrayed by Otto Warburg over 90 years ago (Saunier, Benelli, \& Bortoli, 2016; Warburg et al., 1927). This altered cellular metabolism could be an interesting pharmacological targeting approach specifically for tumor inhibition (Martin Villalba et al., 2014). A key regulator of this metabolic switch is pyruvate dehydrogenase kinase-1 (PDK1), which negative regulate pyruvate dehydrogenase complex (PDC) disconnecting glycolysis from mitochondrial OXPHOS and forcing cancer cells to mainly rely on aerobic glycolysis. Since PDK1 is rightly active in tumor cells so suppressing PDK1 activity could force cancer cells to fully oxidize the glucose by mitochondrial activity, instead of lactate and other intermediates production. Dichloroacetete (DCA) is one of the chemical critical in this approach (Fig. 13-14) due to its land mark ability to stimulate PDC (P. W. Stacpoole, 2012; Whitehouse \& Randle, 1973).


Fig. 13. Mechanism of Action and structure of pyruvate dehydrogenase kinase (PDK) bound with DCA. Adopted from (Sutendra \& Michelakis, 2013).

DCA being a structural analog of pyruvate attaches to PDKs at pyruvate-binding site, subsequently inhibiting the catalytic activity of kinase (Bowker-Kinley, DAVIS, Pengfei, HARRIS, \& POPOV, 1998; Roche et al., 2001). Therapeutic usages of DCA are almost a century ago but since 1970 it's been under intensive investigation (M. O. James \& Stacpoole, 2016; P. W. STACPOOLE, 1969).

Dichloroacetete, a small molecule, is a chemical by-product produced during chlorination process of water. Being smaller in size $(150 \mathrm{Da})$ it has the ability to get across cell membranes and majority of tissue including blood brain barrier (P. W. Stacpoole, 2011). DCA stimulates mitochondrial respiration through resuming pyruvate dehydrogenase (PDH) function (M. O. James \& Stacpoole, 2016; Martin Villalba et al., 2013), thus resulting in ROS production and effecting cellular redox state (NADH/NAD ratio), the adenylate pools (ATP/ADP), mitochondrial membrane potential $(\Delta \Psi)$ and pH gradient. All these factors could trigger several signaling pathways and transcription factors showing the multi-functionality of DCA as an anti-cancer agent. Still DCA is a non-US FDA approved investigational drug but under extensive investigations as a metabolic modulator due to its stimulatory feat of PDC (M. O. James \& Stacpoole, 2016; Martin Villalba et al., 2014).


Fig. 14. Schematic representation of the metabolic differences between differentiated tissues and proliferating tissues. Adopted from (Gonçalves, Baltazar, \& Reis, 2015).
In the presence of oxygen, non-proliferating tissues metabolize glucose to pyruvate and oxidize it in mitochondria through OXPHOS. On the other hand, glucose is metabolized to lactate in the absence of oxygen. But in tumor cells, glucose is metabolized to pyruvate and than converted into lactate even in the presence of oxygen (Warburg effect).

DCA activates PDH, which can trigger the Krebs cycle thus impacting the changes in NADH/NAD ratio. The change in this ratio regulates the deacetylase Sirtuin 1 (SIRT1) activity, which in result regulate the co-transcription factor PGC1 $\alpha$ (Peroxisome proliferatoractivated receptor $\gamma$ coactivator $1-\alpha$ ), hence triggering mitochondrial biogenesis. Active
mitochondria produce ROS that further can modulate PGC1 $\alpha$ activity and ATP/(ADP)(AMP) ratio leading to activation of AMPK. Metformin, another AMPK activator, also demonstrates the anti-tumor activity. Moreover, functional mitochondria and alteration in membrane potential activate mitochondrial apoptotic pathway. These are few of known effects of DCAinduced tumor regression yet exact mechanisms are still to be elucidated (Babu et al., 2011; Bonnet et al., 2007; Brandsma, Dorlo, Haanen, Beijnen, \& Boogerd, 2010; Heshe et al., 2011; Samudio, Fiegl, \& Andreeff, 2009; Stockwin et al., 2010; Martin Villalba et al., 2013). DCA also sensitizes cancer cells to conventional chemotherapy approaches (Nerea Allende-Vega et al., 2015) but the exact understandings of mechanisms are still needed to be explored which further will discover the metabolic pathways used by cancer cell for survival.

Availability of DCA in plasma is still very much unclear. Oral administration is readily absorbed and can be detected in human plasma within 15 minutes with approximately half-life of 1 hour (Kankotia \& Stacpoole, 2014; P. W. Stacpoole, 2011; P. W. Stacpoole, Henderson, Yan, Cornett, \& James, 1998). The plasma drug clearance drops with dose repetition (P. Stacpoole, Harwood Jr, \& Varnado, 1983). In plasma, DCA is converted into glyoxylate by glutathione transferase zeta 1 (GSTZ1-1), through a reaction that requires GSH without its consumption (M. James, Cornett, Yan, Henderson, \& Stacpoole, 1997; Zeen, Philip, \& Anders, 1998). Consequently activity of GSTZ1-1 regulates duration of DCA action (M. O. James \& Stacpoole, 2016). On the other hand DCA inactivates GSTZ1-1 by forming adducts of this protein rendering its inability (Guo et al., 2006). Thus chronic administration reduces its metabolism and elimination (M. O. James \& Stacpoole, 2016). But prolonged dosing cause reversible peripheral neuropathy especially in adults due to oxidative stress (Calcutt et al., 2009; P Kaufmann et al., 2006; P. W. Stacpoole, 2011). DCA was used at baring doses; in vitro experiments of human or rodent cell culture, used doses are between 1 and 50 mM while in healthy cells, for ex vivo, PDC stimulation was observed at $\leq 1 \mathrm{mM}$ dosing (Whitehouse \& Randle, 1973). This can possibly elucidate that higher doses may affect other cellular processes besides activation of PDC. In severe cases, even a 6-log dose range in vitro have been investigated (Yeung, Pan, \& Lee, 2008). However an effective as well as nontoxic dose of DCA is still be identified for its long-term clinical use (M. O. James \& Stacpoole, 2016).

### 2.1. Dichloroacetate in Cancer

Vigorously growing cancer cells need a constant source of energy and biomolecules for the production of macromolecules. Tumor cells meet most of their energy needs through
glycolysis rather by mitochondrial OXPHOS (Martin Villalba et al., 2014; Warburg et al., 1927), which favors their quick growth (Saunier et al., 2016). An array of metabolic transformations in tumor cells results in loss-of-function of tumor suppressor genes while gain-of-function of oncogenes consequently increased glucose consumption, reduced mitochondrial respiration and cell death resistance ensuring cancer progression (Ribas, García-Ruiz, \& Fernández-Checa, 2016). OXPHOS is regulated by PDC and is severely impaired in cancer cells (Gray, Tompkins, \& Taylor, 2014) due to its inhibition, while DCA can reoffend this process (Martin Villalba et al., 2013). Recently, this metabolic shift (Warburg effect) is being reinterpreted in this modern biology with cumulative recognition of critical mitochondrial role in cancer progression (Cairns, Harris, \& Mak, 2011; Cheong, Lu, Lindsten, \& Thompson, 2012) Several metabolic diseases are treated with DCA when its metabolic properties are identified in detail including some clinical trials for the treatment of different cancers (M. O. James \& Stacpoole, 2016; Saunier et al., 2016; Peter W Stacpoole et al., 2008). DCA by activating the mitochondrial metabolism forces cell to unrestraint its original metabolic process and turns on the cell's "suicide switch" in tumor (Misra, Ye, Ostadhossein, \& Pan, 2016).

The anti cancer properties of DCA were first reported in 2007 when in vitro DCA treatment increased glucose oxidation and stimulated mitochondrial production of apoptosis-inducing factor (AIF) and ROS resulting in improved cell death only in tumor cells (Bonnet et al., 2007). DCA treatment decreased lactate production thus rendering tumor cells sensitive to host defense mechanism (Nerea Allende-Vega et al., 2015; Kankotia \& Stacpoole, 2014). DCA delays onset of solid tumor formation restricting tumor size and enhancing apoptosis mediated by decrease in PDK expression (Bonnet et al., 2007; Velpula, Bhasin, Asuthkar, \& Tsung, 2013). DCA alone or in combinatorial therapy is used for the treatment of different cancers (Ectodermal, Mesodermal, Endodermal), with diverse doses (Tables 2-4), (Kankotia \& Stacpoole, 2014). Preclinical investigations reveals that DCA along with various conventional or standard anticancer therapies like Doxorubicin, vincristine, Irradiation, Poly (I: C), Nutlin-3, sorafenib or Bortezomib (Tables 2-4) give promising results (Nerea AllendeVega et al., 2015; Kankotia \& Stacpoole, 2014; Shen et al., 2013). DCA was also used in conjugation with hemoglobin to suppress the growth of monocytic cancer cell line THP-1 (N. Zhang \& Palmer, 2011). Similarly, DCA in combination with metformin promotes apoptosis in leukemic cells while suppresses ovarian tumor (X. Li et al., 2016; Voltan et al., 2016).

Another way used by DCA to subdue cancer resistance is through suppressing ABC drug transporters (Achuthan, Callaghan, \& Blackburn, 2016).

Published clinical trials are very few and for the moment limited to only those patients, who failed to respond standard anticancer therapy; 2 in patients with glioblastoma (Dunbar et al., 2014; E. Michelakis et al., 2010), 1 in patients with advanced non-small cell lung cancer (Garon et al., 2014) and 1 in patients with solid tumors (Q. S.-C. Chu et al., 2015). Additionally, one patient with non-Hodgkin's lymphoma gets cured with using DCA as an auto-medication (Flavin, 2010). Theses results were promising enough to keep investigation for evaluating DCA in co-treatment with conventional therapies against cancer. Reported data includes 74 in vitro studies, 25 in vivo studies, 47 co-treatment studies and 18 analogue studies (Kankotia \& Stacpoole, 2014).

Table 2: Therapy of cancers from ectodermal origin. Adapted from (Kankotia \& Stacpoole, 2014).

| Cell type | In vitro DCA dosage (mM) | $\begin{gathered} \text { In vivo DCA } \\ \text { dosage (mg/kg) } \end{gathered}$ | Co-treatment or analogue | Reference |
| :---: | :---: | :---: | :---: | :---: |
| Ectodermal |  |  |  |  |
| Human breast | 2.5-40 |  | Estradiol derivative | (Stander et al., 2011) |
|  | 2.5-80 | 112 | Bicarbonate | (Robey and Martin, 2011) |
|  | 5 |  | Arsenic trioxide | (Sun et al., 2011) |
|  | 1-30 |  |  | (Feuerecker et al., 2012) |
|  | 0.25-1 |  |  | (Babuet al., 2011) |
|  | 0.001-100 |  | 5-ALA, PDT | (Kwitniewski et al., 2011) |
|  | 0.5 |  |  | (Bonnet et al., 2007) |
|  |  |  | Mitaplatin* (in vivo) | (Johnstone et al., 2013) |
|  | 0.5-5 | 50 |  | (Sutendra et al., 2013) |
|  | 10 |  | Allopurinol | (Lefort et al., 2014) |
| Rat breast | 1-5 | 23-200 |  | (Sun et al., 2010) |
| Human glioblastoma | 10 | 50 | Bevacizumab | (Kumar et al., 2013b) |
|  | 0.5 |  |  | (Bonnet et al., 2007) |
|  | 0.1-1 |  | Radiation | (Velpula et al., 2013) |
|  | 1 | 100 | Fl-EGFR | (Duan et al., 2013) |
| Rat glioma | 1-128 | 25-125 |  | (Park et al., 2013) |
|  |  | 200 |  | (Park et al., 2013) |
|  | 0.25-1 |  | Radiation, etoposide | (Morfouace et al., 2012) |
| $\begin{gathered} \text { Human } \\ \text { medulloblastoma } \end{gathered}$ | 0.1-10 |  | Cisplatin | (Heshe et al., 2011) |


| Human melanoma | 0.05-20 | Varied ( $75 \mathrm{mg} / \mathrm{L}$ of H 2 O ) | Elesclomol | (Kluza et al., 2012) |
| :---: | :---: | :---: | :---: | :---: |
| Mousemelanoma | 2-10 | 25-100 | $\begin{gathered} \text { Poly(IC) } \\ \text { immunotherapy } \end{gathered}$ | (Ohashi et al., 2013) |
|  |  | 100 | Capecitabine | (Vella et al., 2012) |
| Human neuroblastoma | 0.1-10 |  | Cisplatin | (Heshe et al., 2011) |
|  | 0.25-2 |  |  | (Niewisch et al., 2012) |
|  | 5-50 | 2.5-25 |  | (Vella et al., 2012) |
|  | 1-30 |  |  | (Feuerecker et al., 2012) |
|  | 25 |  |  | (Hanberry et al., 2014) |
| $\begin{gathered} \text { Human } \\ \text { squamous } \end{gathered}$ | 1-40 |  | Sulindac | (Ayyanathan et al., 2012) |
|  | 2-8 |  |  | (Sun et al., 2009) |
|  |  |  | N-phe. Dichlo.* (in vitro) | (Li et al., 2012) |
|  | 0.001-100 |  | 5-ALA, PDT | $\begin{aligned} & \text { (Kwitniewski et al., } \\ & 2011 \text { ) } \end{aligned}$ |
|  | 0.05-02 |  | Mitaplatin*, cisplatin | (Xue et al., 2012) |

Table 3: Therapy of cancers from endodermal origin. Adapted from (Kankotia \& Stacpoole, 2014).
\(\left.$$
\begin{array}{|c|c|c|c|c|c|}\hline \text { Cell type } & \begin{array}{c}\text { In vitro DCA } \\
\text { dosage (mM) }\end{array} & \begin{array}{c}\text { In vivo DCA } \\
\text { dosage (mg/kg) }\end{array}
$$ \& \begin{array}{c}Co-treatment or <br>

analogue\end{array} \& Reference\end{array}\right]\)| (Chen et al., 2009) |
| :---: |


|  |  |  | (in vitro) |  |
| :---: | :---: | :---: | :---: | :---: |
|  | 1-40 |  | Oncolytic AV | (Xiao et al., 2010) |
|  | 0.05-0.2 |  | Mitaplatin*, cisplatin | (Xue et al., 2012) |
| Human lung | 2.5-40 |  | Sulindac | (Ayyanathan et al., 2012 ) |
|  |  |  | N -phe. Dichlo.* <br> (in vitro) | (Li et al., 2012) |
|  | 10 |  | Pt drugs* | (Fiebiger et al., 2011) |
|  |  |  | N-phe. Dichlo.* (in vitro) | (Li et al., 2012), <br> (Yang et al., 2010) |
|  | 1-40 |  | Oncolytic AV | (Xiao et al., 2010) |
|  | 0.02 |  | Mitaplatin*, cisplatin | (Dhar and Lippard, 2009) |
|  | 0.5 | 50-100 |  | (Bonnet et al., 2007) |
|  |  |  | Pt drugs* (in vitro) | (Liu et al., 2013) |
|  | 0.5-5 | 50 |  | (Sutendra et al., 2013) |
|  |  | 100 | Capecitabine | (Zheng et al., 2013) |
| Human prostate | 0.01-100 |  | Radiation | (Cao et al., 2008) |
|  | 80 |  | Honokiol DCA* | (Hahm et al., 2014) |
|  |  |  |  | (Lin et al., 2014) |

Table 4: Therapy of cancers origin mesodermal. Adapted from (Kankotia \& Stacpoole, 2014).

| Cell type | $\begin{aligned} & \text { In vitro DCA } \\ & \text { dosage } \\ & (\mathrm{mM}) \end{aligned}$ | In vivo DCA dosage (mg/kg) | Co-treatment or analogue | Reference |
| :---: | :---: | :---: | :---: | :---: |
| Mesodermal |  |  |  |  |
| Human cervical | 2-16 |  | Cisplatin | (Xie et al., 2011) |
|  | 10 |  |  | (Wu et al., 2013) |
|  | 1-40 |  | Oncolytic AV | (Xiao et al., 2010) |
|  | 0.02 |  | Mitaplatin*, cisplatin | (Dhar and Lippard, 2009) |
|  | 1.6-25 |  | 2-Deoxy-D-glucose | $\begin{aligned} & \text { (Anderson et al., } \\ & 2009 \text { ) } \end{aligned}$ |
| Mouse cervical | 1.6-25 | 25 | $\begin{aligned} & \text { DCA-PLA mats* (in } \\ & \text { vivo) } \end{aligned}$ | (Liu et al., 2012) |
| Human lymphoma | 5-40 |  |  | (Kumar et al., 2012) |
| Mouse lymphoma | 2-10 | 25-100 | $\begin{gathered} \text { Poly(IC) } \\ \text { immunotherapy } \end{gathered}$ | (Ohashi et al., 2013) |
|  | 1 | 112 | Cisplatin | $\begin{gathered} \text { (Kumar et al., } \\ \text { 2013a) } \end{gathered}$ |
| Human monocytoma | 5 |  | DCA-Hb-Hp* | (Zhang and Palmer, 2011) |
| Human leukemia | 0.1-50 |  | Cisplatin | (Heshe et al., 2011) |
|  | 1-30 |  | Nutlin-3 | $\begin{gathered} \text { (Agnoletto et al., } \\ 2014) \end{gathered}$ |
| Human myeloma | 5-25 | 200 | Bortezomib | $\begin{aligned} & \text { (Sanchez et al., } \\ & 2013) \end{aligned}$ |
|  | 10-40 |  | Bortezomib | (Fujiwara et al., 2013) |

$\left.\begin{array}{|c|c|c|c|c|}\hline \text { Human ovarian } & 0.16-0.62 & & & \text { (Saed et al., 2011) } \\ \hline \begin{array}{c}\text { Human } \\ \text { filbrosarcoma }\end{array} & 0.45-1.35 & & \begin{array}{c}\text { Omeprazole, } \\ \text { tamoxifen }\end{array} & \begin{array}{c}\text { (Ishiguro et al., } \\ \text { 2012) }\end{array} \\ \hline \begin{array}{c}\text { Human } \\ \text { osteosarcoma }\end{array} & 0.1-10 & & \text { Cisplatin } & \text { (Heshe et al., 2011) } \\ \hline \text { Human } & 0.02 & & & \text { Mitaplatin*, cisplatin }\end{array} \begin{array}{c}\text { (Dhar and Lippard, } \\ \text { 2009) }\end{array}\right]$

Recently DCA-derived new molecules; Mito-DCA and Mitaplatin are under investigation (Table 5). Mito-DCA has improved potency specificity toward tumor cells as compared to DCA without any toxic effects in normal cells. Mitaplatin has unique mechanism of action as it comprises of cisplatin to target nuclear DNA and DCA to regulate mitochondria (Dhar \& Lippard, 2009; Pathak, Marrache, Harn, \& Dhar, 2014; Saunier et al., 2016).

Table 5: PDK inhibitor and their biological effects. Adapted from (Saunier et al., 2016).

|  | PDK1 Inhibitory <br> potency (IC 50) | Biological effects (metabolism, proliferation/cell death, ROS production) |  |  |
| :---: | :---: | :---: | :---: | :---: |
|  |  | Cell model | Animal studies | Clinical trials |
| DCA | $1000 \mu \mathrm{M} \mathrm{PDK}$ activity assay <br> (Bao, Kasten, <br>  <br> Roche, 2004) | Multiple cancer cell lines <br> (Kankotia \& Stacpoole, 2014) | Human <br> Xenografts <br> (Kamarajugadda <br> et al., 2012; <br> Michelakis, <br>  <br> Mackey, 2008) | Metastatic breast cancer, brain tumor, non small cell lung cancer in human (Kankotia \& Stacpoole, 2014) |
| Mito-DCA | $30 \mu \mathrm{M}$ MTT assay (Pathak et al., 2014) | Prostate cancer cell lines (Pathak et al., 2014) |  |  |
| Mitaplatin | $0.05-18 \mu \mathrm{M}$ <br> MTT assay (Dhar \& Lippard, 2009) | Cervical, testis, lung and breast cancer cells, osteosarcoma (Dhar \& Lippard, 2009) |  |  |

### 2.2. DCA and Lipids homeostasis

Lipids are very energetic nutrients. They are naturally occurring in variety of molecules like fats (Fatty Acids), sterol, vitamins, glycerides etc. They also play an important role in cellular signaling (Mashaghi, Jadidi, Koenderink, \& Mashaghi, 2013; Subramaniam et al., 2011). Certain cells require energy from lipid metabolism. Major source of lipids are animal and plant triglycerides, sterols, and fats while excessive carbohydrates are also converted into triglycerides through lipogenesis. Lipids are metabolized in mitochondria via beta-oxidation where Acetyl-CoA is synthesized from FAs, which then incorporates in Krebs cycle (Berg, Tymoczko, \& Stryer, 2007).

Cholesterol is a sterol vital for cells as its part and parcel of cellular structure particularly membranous constructs and composes around $30 \%$ of all animal cell membranes. Other than structural constitutes they are also important in hormonal and bile acids synthesis (Hanukoglu, 1992). The vital and central location of PDH in the pathway from carbohydrates to fatty acids intimates DCA significance in lipids metabolism (Yount \& Harris, 1981), and suggests that DCA has a potential anti diabetic and lipid lowering properties (P. W. Stacpoole \& Felts, 1970; Peter W Stacpoole, George W Moore, \& David M Kornhauser, 1978). Effects of DCA on lipid metabolism are complex and appear to be tissue specific. DCA blocks endoplasmic reticulum enzyme HMG CoA reductase, thus inhibiting the rate limiting step of cholesterol biosynthesis in rat liver and human leukocytes (Harwood Jr, Bridge, \& Stacpoole, 1987; P. Stacpoole et al., 1983; P. W. Stacpoole \& Felts, 1970).

First evidence of DCA in relation to lipid metabolism was observed in 1970 when diabetic rats showed inhibition in fatty acid oxidation with DCA administration (P. W. Stacpoole \& Felts, 1970) while first clinical trail of DCA showed that oral dosing of DCA to diabetes mellitus or hyperlipoproteinemia patients resulting in significantly fall in hyperglycemia as well as plasma cholesterol and 19-67\% triglyceride reduction (Peter W Stacpoole et al., 1978). DCA was used to treat two cases of familial hypercholesterolemia (FH) where DCA reduced circulating cholesterol levels in both patients through a mechanism involving a reduction in LDL cholesterol (George W Moore et al., 1979). A decrease of cholesterol was also observed when normal dogs were given DCA (Gérard Ribes, Valette, \& LoubatièresMariani, 1979) similarly, DCA administration to healthy rats also lowers serum triglyceride
and when isolated rat hepatocytes were treated with DCA it decreases fatty acid synthesis along with up-regulation of oxidation in liver (Misbin, 1979). Whereas in muscles DCA has opposite effects where it tends to inhibit fatty oxidations (McAllister, Allison, \& Randle, 1973; P. W. Stacpoole \& Felts, 1971). Yet defined mechanisms of these effects are unidentified (M. O. James \& Stacpoole, 2016). A simple explanation could be stimulation of mitochondria functions would up-regulate beta-oxidation resulting in lower serum cholesterol levels.

Cancer cells have impaired cholesterol metabolism, which supports their progression. Cholesterol buildup within mitochondria directs the metabolic alterations in cells resulting in, in part, to the Warburg effect provoking aerobic glycolysis. Targeting mitochondrial cholesterol homeostasis/trafficking could prove a promising approach in cancer treatments (J. Montero et al., 2008; Ribas et al., 2016). Diisopropylamine dichloroacetate is a novel PDK 4 inhibitor (Yamane et al., 2014) that was used in patients suffering of fatty liver disease results in noteworthy suppression in alanine aminotransferase (ALT), total cholesterol (TC) and triglyceride (TG) (Y. SUN, ZHAO, LI, XIA, \& CHI, 2013).

## Le Dichloroacétate (DCA)

La majorité des cellules cancéreuses obtient de l'énergie à partir de la glycolyse anaérobie, connue comme «effet Warburg ». Malgré la présence d'oxygène, ces cellules préfèrent la glycolyse anaérobie par rapport à la phosphorylation oxydative (OXPHOS) afin d'éviter l'utilisation de la mitochondrie. Cette altération du métabolisme cellulaire est en faveur des cellules tumorales, car elle permet l'évasion immunitaire, la protection contre les antioxydants cellulaires et pourrait aussi servir comme cible pharmacologique intéressante pour inhiber la tumeur.

Le dichloroacétate (DCA) est une petite molécule qui cible l'activité mitochondriale en activant la pyruvate deshydrogénase (PDH) et en inhibant la pyruvate deshydrogénase Kinase (PDK). Étant donné que la phosphorylation oxydative (régulée par le complexe pyruvate déshydrogénase (PDC)) est sévèrement altérée dans les cellules cancéreuses, le DCA oblige les cellules à effectuer l'OXPHOS pour l'obtention de l'énergie. Le traitement par le DCA va donc diminuer la production du lactate dans les cellules tumorales permettant une exposition aux mécanismes de défense. Plusieurs maladies métaboliques sont traitées avec DCA. Cette molécule est incluse dans certains essais cliniques et utilisée dans le traitement de différents cancers d'origine ectodermique, mésodermique ou endodermique voir même dans les cas des tumeurs solides.

Les effets bénéfiques du DCA ne sont pas seulement spécifiques aux cancers, mais ils ont également des effets divers sur le métabolisme des glucides et des lipides. Le DCA a des propriétés anti-diabétiques et -lipidique révélées par son utilisation en clinique dans le traitement de patients atteints d'hypercholestérolémie (cas de familles souffrant d'un taux élevé de cholestérol plasmatique). Le DCA bloque également l'enzyme du réticulum endoplasmique (HMG CoA réductase), afin d'inhiber l'étape limitant la vitesse de la biosynthèse du cholestérol. Dans divers modèles animaux, l'administration du DCA a montré une diminution de la synthèse des acides gras et une augmentation de la régulation de la bêtaoxydation des acides gras mitochondriaux. Le DCA possède également un effet potentiel dans la récupération post-ischémique.

Malgré ces propriétés anticancéreuses, le DCA n'est pas approuvé par l'Agence américaine des produits alimentaires et médicamenteux (FDA), mais sous des investigations approfondies comme modulateur métabolique en raison de son exploit de stimulation de (PDC).

Récemment, deux nouvelles molécules dérivées de DCA; Mito-DCA et Mitaplatin sont également à l'étude.

## 3. Cellular Signaling

Cells respond to extracellular stimulus and environment through a complex and interlinked multichannel signaling. Surface receptors assist cells to receive stimuli and transfer the information to nucleus for proper and correct response through involvement of several proteins and biochemical changes that are part of signal transduction pathways resulting in appropriate physiological events. A single input signal can activate multiple pathways (Saucerman \& McCulloch, 2004). Approximately $12.2 \%$ of human genome comprise of these signal transduction proteins (Jordan, Landau, \& Iyengar, 2000). This signaling network is also vital for cellular development, proliferation, immunity and cellular homeostasis. Slightly deregulation in these transduction proteins and pathways can be harmful and results in lethal disease.

Mitogen activated protein kinases (MAPKs) are Ser/Thr kinases involved in diverse cellular effector response to extracellular stimuli. The MAPK family members regulate signal transduction cascades that are highly conserved among eukaryotes and are known to be involved in the control of several intracellular events, including proliferation, differentiation, migration, and apoptosis (A. E. Simões, Rodrigues, \& Borralho, 2016). Two main classes of MAPK in mammalian cells are; Conventional MAPKs- This group includes Extracellular signal-regulated kinases 1 \& 2 (ERK1/2), c-Jun amino (N) - terminal kinases 1/2/3 (JNK1/2/3), p38 isoforms ( $\alpha, \beta, \mathrm{d}$ and $\delta$ ) and ERK5. These members are activated by double phosphorylation of motif Thr-X-Tyr, residing in their activation loop. Differentiation among different kinases is based on presence of amino acids at the site of phosphorylation. For example: ERK with Thr-Glu-Tyr, JNK with Thr-Pro-Tyr and p38 with Thr-Gly-Tyr (Canagarajah, Khokhlatchev, Cobb, \& Goldsmith, 1997; Pearson et al., 2001). Atypical MAPKs- This group of MAPKs contains ERK3/4, ERK7 and Nemo-like kinase. They share various characteristics of the conventional group but the exact molecular mechanism of their activation is not well elucidated (Cargnello \& Roux, 2012).

Activation of conventional MAPK cascades is stimulated by several stimuli including internal metabolic stress as well as by external mitogens, hormones or neurotransmitters, cell-matrix, cell-cell interactions and cytokines by transmitting signals through tyrosine kinase, G-coupled protein (Barbara A Drew, Matthew E Burow, \& Barbara S Beckman, 2012; S.-H. Yang, Sharrocks, \& Whitmarsh, 2013). The canonical activation of MAPK involves a cascade of kinases with phosphorylation of three-tiered hierarchical module comprising a MAPK, a

MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK) (Fig. 15). The first kinase activated through phosphorylation in reaction to extracellular stimuli is MAPKKK that leads to phosphorylation and activation of MAPKK stimulating MPAK action through dual phosphorylation on Thr and Tyr residues within the conserved Thr-X-Tyr motif. MAPK activation ends in phosphorylation of large variety of target substrates for the appropriate function response (Barbara A Drew et al., 2012 \{Hoang, 2017 \#660; Hoang et al., 2017; Nithianandarajah-Jones, Wilm, Goldring, Müller, \& Cross, 2012).


Fig.15. MEK5 activation and downstream substrates. Adopted from (Hoang et al., 2017).
Majority of MAPK substrates comprise of transcription factors, transcriptional regulators and other protein kinases designated as MAPK activated protein kinases (MAPKAPKs) and are phosphorylated in the cytosol and nucleus (Keshet \& Seger, 2010). MEK5/ERK5 pathway can be activated by stress, mitogens or cytokines, leading to the regulation of various downstream targets including kinases and transcription factors.

### 3.1. MAPK Extra Regulated Kinase 5 (ERK5)

### 3.1.1. Structure of ERK5

Extra-regulated kinase 5, a protein encoded by MAPK7 (Mitogen-activated protein kinase 7) gene located on chromosome 17 in humans and chromosome 11 in mice. Two different groups identified this gene in 1995 simultaneously (J.-D. Lee, Ulevitch, \& Han, 1995; G. Zhou, Bao, \& Dixon, 1995). ERK5 is ubiquitously expressed in all tissues while more profuse in heart, lungs and kidney. Initially it was named big MAP kinase 1 (BMK-1) (G. Zhou et al., 1995). The MAPK7 gene encodes a protein of 816 amino acids with its N-terminal domain comprises of a big kinase domain containing approximately $66 \%$ of the sequence (Fig. 16), where it has binding region for MEK5 (MAPKK specific to ERK5) (Nithianandarajah-Jones et al., 2012; Yan, Luo, Lee, Abe, \& Berk, 2001).


Fig.16. Structure of ERK5. Adapted from (Honda et al., 2015; Nithianandarajah-Jones et al., 2012; A. E. Simões et al., 2016).
The ERK5 protein comprises 816 amino acid (a.a) residues with a N -terminal domain and a large and unique C-terminal domain. The N-terminal domain contains the cytoplasmic targeting region, a kinase domain with two MEK5 phosphorylation sites, a common docking (CD) domain, and the oligomerisation region. The unique C-terminal domain contains two proline-rich (PR) domains, a MEF2-interacting region, the nuclear localization signal (NLS) domain, and a transcriptional activation domain. The C-terminal domain also contains several different phosphorylation sites that are responsible for different physiological outcomes.

Resembling other MAPK, its CD domain can bind to definite docking (D)- domain containing substrates. Similarly its unique C-terminal domain makes it peculiar as it has interacting
region for myocyte enhancer factor-2 (MEF2), proline rich binding regions for Src-homology 3 (SH3) and a nuclear localization signal (NLS) domain. Prominently, a transcriptional activation domain has also observed on C-terminal enabling ERK5 to regulate transcriptional activation of certain genes (Buschbeck \& Ullrich, 2005; Nithianandarajah-Jones et al., 2012) Interaction between N and C -terminals results in folded conformation under inactive phase following a decrease in NLS which generates a nuclear export signal (NES) for cytoplasmic localization of ERK5. However, MEK5 phosphorylation alters this N and C-terminal interaction aggregating NLS resulting in ERK5 nuclear translocation. The ERK5 returns in resting folded condition in cytoplasm upon increase in NES due to dephosphoryaltion (Kondoh, Terasawa, Morimoto, \& Nishida, 2006; Plotnikov, Zehorai, Procaccia, \& Seger, 2011). Diverse localization of ERK5 is observed in different cell types; cytoplasm localization in the breast cancer MCF7 cell line (Esparís-Ogando et al., 2002) and nuclear localization in HeLa and ratl cells (Raviv, Kalie, \& Seger, 2004).

### 3.1.2. Activation of ERK5

Numerous intra as well as extra cellular stimuli comprising stress (oxidative, osmotic), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) result in ERK5 activation. ERK5 is also activated in certain physiological and pathological conditions like nerve growth factor, IL-6 during inflammation, ischemia and hypoxia (Barbara A Drew et al., 2012). This activation is a complex phenomenon involving a cascade of kinases with phosphorylation. Initially MEKK2 or MEKK3 are activated in result of extracellular stimuli. They share almost $94 \%$ sequence but differ in regulatory domain of N terminal for differential regulation of ERK5 signaling cascade. Activated MEKK2/3 phosphorylate Ser311/Thr315 residue of MEK5. Both can activate MEK5 independently in leukemia inhibitory factor (LIF) signaling pathway (Nakaoka et al., 2003) however MEKK2 has a higher binding affinity for MEK5 (Nithianandarajah-Jones et al., 2012). The only kinase that can directly activate ERK5 by dual phosphorylation is MEK5. This phosphorylation of Thr218 and Tyr220 residues of the TEY motif occurs within the activation loop of ERK5 kinase domain (Fig. 17). During mitosis, cyclin dependent kinases can also phosphorylate ERK5 particularly during the G2-M phase transition (Álvarez-Fernández et al., 2013; IñestaVaquera et al., 2010). Recently, a new cross-talk mechanism within MAPK family members has been proposed where activated ERK1/2 can phosphorylates ERK5 at the Thr732 residue in the C-terminal domain without the typical activation of the N -terminal kinase domain resulting in ERK5 nuclear translocation (Honda et al., 2015). Due to mutation in TEY motif a
mutant ERK5-AEF is produced which loose activation by MEK5 (Nimesh, Campbell, Morrice, Peggie, \& Cohen, 2003). Once ERK5 is activated it has the ability of autophosphorylation of several residues in the C-terminal domain for enriched transcriptional activity (Morimoto, Kondoh, Nishimoto, Terasawa, \& Nishida, 2007). Several transcription factors (TF) are downstream targets of activated ERK5 predominantly MEF2 A, B, C, D and regulate cellular differention (Kato et al., 1997; Kato et al., 2000). MEF2 activity is regulated by C-terminal of ERK5 containing MEF2 interacting region and transactivation domain (Fig. 17). ERK5 looses its MEF2 activation ability if mutation occurs at this region (Yan et al., 2001). ERK5 also regulates many other transcription factors like SAP1, CREB, SGK, c-FOS, FRA1 and c-MYC (Nithianandarajah-Jones et al., 2012; Terasawa, Okazaki, \& Nishida, 2003).


Fig.17. ERK5 activation Pathway. Adapted from (Barbara A Drew et al., 2012).
The stimulation of MEKK2 or MEKK3 by cell stressors, mitogens or cytokines results in phosphorylation of MEK5 on its serine 311 and threonine 315 residues. MEK5 phosphorylates the threonine 218 and tyrosine 220 residues of ERK5. ERK5 can phosphorylate its target molecules or autophosphorylates its carboxyl-terminal region, which contains a NLS region allowing ERK5 to shuttle from the cytosol to the nucleus.

### 3.2. Biological Roles of ERK5

Many external stimuli like osmotic stress, oxidative stress and growth factors can activate ERK5 suggesting the vital role of ERK5 in diverse biological processes. Erk5 -/- mice are embryonically lethal with obvious cardiac imperfections and defective angiogenesis (Hayashi \& Lee, 2004; Kesavan et al., 2004; Sohn, Sarvis, Cado, \& Winoto, 2002; Watson et al., 2001) and lack a protective mechanism against apoptosis (Hayashi \& Lee, 2004). Mice with Mek5-/and Mekk3-/- showed various phenotypic anomalies (Nithianandarajah-Jones et al., 2012). The endothelial cells (EC) from these mice have defective morphology along with immature vasculature resulting in loss of vascular integrity, vessel leakiness and ultimate death due to hemorrhages (Sohn et al., 2002). ECs requires VEGF-induced activation of AKT signaling by ERK5 to protect themselves from apoptosis thus knockout mice leads to death in 2-3 weeks due to leaky vessels (Roberts, Holmes, Müller, Cross, \& Cross, 2010). ERK5 enhances degradation of hypoxia inducible factor $1 \alpha$ (HIF-1 $\alpha$ ) henceforth negatively regulate HIF-1 $\alpha$ activity in ECs (Pi et al., 2005). Thus ERK5 signaling is crucial for cardiovascular system development and vascular integrity maintenance along with ECs function (A. E. Simões et al., 2016).

Under stress conditions, ERK5 induced MEF2 pathway and this is vital in survival of neurons (L. Liu et al., 2003; Suzaki et al., 2002). Nerve growth factor (NGF) activates ERK5 for neuron survival. Erk5-/- mice showed retard growth of head region and died in early embryonic age as previously described due vascular abnormalities (Barbara A Drew et al., 2012).

Phosphorylation of p90 ribosomal S6 kinase (RSK) by ERK5 leads to activation of cAMP response element binding protein (CREB) regulating pro-apoptotic and survival proteins. Activation of AKT pathway leading to cytosolic sequestration of forkhead box 3a (FoxO3a) (TF regulating BIM expression) is also mediated by ERK5 (Finegan, Wang, Lee, Robinson, \& Tournier, 2009).

Activation of ERK5 also plays a key role in inflammatory responses by the activation of KLF2 under laminar shear stress that triggers expression of nitric oxide synthase (eNOS) to initiate an anti-inflammatory response (Finegan et al., 2015; Kinderlerer et al., 2008; Woo et al., 2008). Similarly ERK5 is also important in cell proliferation becuase Granulocyte ColonyStimulating Factor (G-CSF) and Epithelium Growth Factor (EGF) can stimulate ERK5
activity regulating the transcriptional activity of $c-J U N$ via MEF2 activation thus controlling cellular proliferation (Barbara A Drew et al., 2012).

### 3.3. ERK5 in Cancer

The involvement of MEK5/ERK5 signaling in cell survival, proliferation, angiogenesis, motility, differentiation and in repressing the apoptosis of normal cells is very well established. Due to its involvement on these mechanisms this pathway can also support tumor development and progression. Conversely, there are an increasing body of evidences implicating MEK5/ERK5 signaling pathways in onset, progression and therapy response in various types of cancers (Table 6), and ERK5 is overexpressed in various types of cancers (Al-Ejeh et al., 2014; Miranda, Rozali, Khanna, \& Al-Ejeh, 2015; Ortiz-Ruiz et al., 2014; A. E. Simões et al., 2016; X. Wang \& Tournier, 2006).

### 3.3.1. Oncogenes and ERK5

Tumor cell have high proliferation rate and survival and so these properties can be supported by ERK5 via regulating many oncogenes. Most tumor cells have mutated or up-regulated expression of oncogenes. Tumor progression locus 2 (TPL2)/Cancer Osaka thyroid (COT) is associated with the ERK5 activity. This protein triggers transcription of $c-J U N$ promoter through JNK, p38 and ERK5 and is critical for the oncogenic transformation. ERK5 is activated by COT through direct phosphorylation of MEK5 where as COT looses its transforming capacity in presence of dominant negative MEK5 (Chiariello, Marinissen, \& Gutkind, 2000; Barbara A Drew et al., 2012). In neuroblastoma ERK5 regulates ALK (Anaplastic lymphoma kinase) induced transcription of oncogene MYCN stimulating tumor development (Umapathy et al., 2014). ERK5 is also associated to regulate oncogenic effect of RAS and in several cancer types such as pancreatic, lung, colon, breast and skin its mutation has been described. During oncogenesis, ERK5 induced RAS is also associated in morphological changes and survival of tumor cells (Barbara A Drew et al., 2012; Pekow et al., 2015; X. Wang \& Tournier, 2006). ERK5 activation has also been reported by SRC (proto-oncogene) under stress conditions, which is critical in viruses induced tumorogenisis. ERK5 along with ERK1/2 contributes in $S R C$-induced proliferation. $S R C$ mediated ERK5 stimulation results in loss of actin stress fibers leading to defective actin cytoskeleton. Inhibition of only ERK1/2 fails to restore cytoskeleton in fibroblasts (Barros \& Marshall, 2005; Scapoli, Ramos-Nino, Martinelli, \& Mossman, 2004).

### 3.3.2. ERK5 and Tumorigenesis

Normal cells under neoplastic conditions acquire diverse features; intensive proliferation via sustaining proliferative signaling, immune evasion and metastasis, high angiogenesis and metabolic modification. Since ERK5 regulates several pathways involved in these processes hence numerous investigations establishing the role of ERK5 in tumorigenesis and cancer progression (Keshet \& Seger, 2010; Pearson et al., 2001; A. E. Simões et al., 2016). Through phosphorylation of MEF2 transcription factors, MEK5 has been shown to regulate the expression of $c-J U N$, a proto-oncogene vital to cell growth (Kato et al., 1997; Rovida et al., 2015). Interestingly recently it was reported that proliferation in colon cancer cells harboring KRAS and BRAF mutations was not the result of ERK5 signaling, because its inhibition abrogated proliferation possibly due to feed forward mechanism of ERK5 activation by ERK1/2 (Lochhead et al., 2016). ERK5 knockdown studies using 56 RNA interference (RNAi) or pharmacological inhibition by XMD8-92 treatment delayed cell cycle progression and decreased proliferation in various cancer types (Hoang et al., 2017).

### 3.3.3. ERK5 and leukemia

Leukemia is characterized by the abnormal and augmented proliferation of transformed progenitor white blood cells. These progenitor cells fail to differentiate into mature cells and as a result accumulate inside bone marrow. It is allotted two forms according to pathophysiology: acute and chronic leukemia and it could be myeloid or lymphoid in accordance to origin (Manzotti et al., 2015; Yendamuri \& Calin, 2009). The role of ERK5 in survival of leukemic cells is very significant but still requires additional investigation because of the unclear role of ERK5 in differentiation therapies (A. E. Simões et al., 2016). Majority of patients with acute myeloid leukemia (AML) carry mutation in Fms-like tyrosine kinase-3 (FLT3), which has a vital role in proliferation and survival of leukemic cells. Activation of AKT by FLT3 is ERK5 dependent and inhibition of MEK5/ERK5 in leukemic cell lines (MOLM-13 and MV4-11) carrying FLT3 mutation provokes apoptosis (Razumovskaya, Sun, \& Rönnstrand, 2011). ERK5 promotes survival of leukemic cells by regulating the expression of oncogenic variants of the tyrosine kinase c-Abl (v-Abl and Brc/Abl) (Buschbeck, Hofbauer, Di Croce, Keri, \& Ullrich, 2005). On the other hand, ERK5 was observed to be involved in the monocytic differentiation of human AML cells upon cell treatment with 1,25D vitamin D3 (Laszlo et al., 2015).

ERK5 is essential for the protection of ROS induced apoptosis in all leukemic cells (Khan et al., 2016; Nuria Lopez-Royuela et al., 2014). Leukemic cells bearing small hairpin RNA for ERK5 (shERK5) spotted sensitive to apoptosis and failed to produce tumor in vivo (Johan Garaude et al., 2006). This suppression of ERK5 represses the expression of MHC class I at plasma membrane in leukemic cells and injecting shERK5 expressing cells triggers NK cells resulting in tumor elimination and slabbing tumorigenesis (Charni et al., 2009). OXPHOSmediated expression of MHC-I requires ERK5 as shERK5 expressing cells were unable to upregulate MHC-I expression and compromised survival (Seyma Charni et al., 2010).

### 3.3.4. ERK5 and Tumor progression

Since ERK5 is vital for cell survival and proliferation so is observed in the promotion of tumor. Firstly, it was demonstrated that in HeLa cells EGF-induced proliferation is ERK5 dependent. EGF activation leads to direct phosphorylation of the Serine/threonine-protein kinases (SGK) by ERK5 allowing cells to enter into S-phase, which was blocked by dominant negative mutant form of ERK5 (Hayashi et al., 2001). It was demonstrated that ERK5 favors cell cycle progression from G1- to S-phase and similarly controls the expression of Cyclin D1 (Mulloy, Salinas, Philips, \& Hipskind, 2003). ERK5 was also involved in cell cycle progression in many other cell lines like, (MCF7 and BT474) and multiple myeloma MM1S (Barbara A Drew et al., 2012; Esparís-Ogando et al., 2002). Similarly, MEK5 promote the proliferation of HEK-293 and LNCaP cell lines (Mehta et al., 2003). The phosphorylation of ser403 and Thr 409 in promyelocytic leukemia protein (PML) nuclear body is mediated by ERK5, hence suppressing its activity resulting in p21 repression. This permits cells to pass G1- S checkpoint in cell cycle progression resulting in tumor proliferation (Q. Yang et al., 2010). siERK5 reduced cell propagation in prostate bladder cells but interestingly RNAi knockdown fails to decrease proliferation in PC3 cells showing ERK5 supported cell proliferation is cancer dependent (Lochhead, Gilley, \& Cook, 2012). Cell growth is inhibited by knockdown of ERK5 in the ERK5-dysregulated hepatocellular and esophageal cancer cells (Gavine et al., 2015; Zen et al., 2009). However contradictory findings by some other groups delineate between kinase and transcriptional activity of ERK5 resulting in discrepancies in defining ERK5 role in regulation of cellular proliferative responses (Gomez, Erazo, \& Lizcano, 2016). Further investigation will elaborate the clear function of ERK5 impartial of its catalytic status (Hoang et al., 2017).

### 3.3.5. ERK5 and tumor metastasis

Tumor cells alter their morphology and reduced attachment to extra cellular matrix (ECM) promoting metastasis and invasion. MEK5-ERK5 signaling is demonstrated to be involved in bone and lymph node metastasis in advanced prostate, breast, colon, kidney and oral squamous cell carcinoma (Antoon et al., 2013; Mehta et al., 2003; A. Simões et al., 2015; Sticht et al., 2008b). It breast cancer cells ERK5 forms a complex with integrin and FAK regulating their adhesion and facilitate migration (Sawhney, Liu, \& Brattain, 2009). In prostate cancer cell, transcription of activator protein 1 (AP-1) is regulated by MEK5, which provokes expression of matrix metalloproteinase (MMP-2) and MMP-9 degrading synthetic ECM in vitro hence enhancing metastasis of cancer. A critical role is played in metastasis by pertusions (actin-base) of the plasma membrane called as invadopodia and podosomes, which are involved in cell attachment to the ECM and ERK5 was associated in the formation of invadopodia in PC3 whereas podosomes in MEFs (Mehta et al., 2003; Ramsay et al., 2011; Schramp, Ying, Kim, \& Martin, 2008). Molecular inhibition of ERK5 in vitro suppressed cell motility and invasion of liver, breast, and prostate cancer cells (Cronan et al., 2012; Javaid et al., 2015). Similarly in ERK5 negative cell lines or chemically ERK5 inhibition through XMD8-92 impaired cell motility and invasiveness (Madak-Erdogan, Ventrella, Petry, \& Katzenellenbogen, 2014).

### 3.3.6. ERK5 and tumor resistance to cell death

Apoptosis in tumor cells can occur due to two main reasons (1) Amplified proliferation of tumor cells resulting in DNA damage (2) oncogenic signaling stress. To evade cellular apoptosis tumor cells have formulated mechanisms of upregulation of pro-survival proteins (BCL-2) and down regulation of pro-apoptotic proteins (BAX and BIM). Role of ERK5 in resistance to cell death was noticed in MCF7 cell line where ERK5 is required for resistance to etoposide, TNF- $\alpha$ - and TRAIL (TNFA-related apoptosis-inducing ligand) induced apoptosis (Weldon et al., 2002). Mek-/- MEFs are sensitive to oxidative stress display the critical role of ERK5/MEK5 pathway in protection against stress induces apoptosis (X. Wang et al., 2005). Leukemic T cells bearing shERK were observed sensitive to death receptor induced apoptosis with decreased NF-kB nuclear accumulation denoting that ERK5 may facilitate tumor survival via NF-kB nuclear localization and activation (Johan Garaude et al., 2006). ERK5 is activated with IL-6 secretion by T cells and macrophages in multiple myeloma cells isolated from patients. Mutant ERK5 (ERK5-AEF) transfection sensitizes
these cells to PS431 (proteasome inhibitor) and dexamethasone induced apoptosis whereas overexpression of ERK5 in these cells facilitates resistance (Lochhead et al., 2012). Moreover, MEK5-ERK5 signaling promotes cellular evasion of apoptosis and suppressing ERK5 promotes cell death in apoptotic resistance cells (Housman et al., 2014; Shukla et al., 2013; Weldon et al., 2002).

Table 6: Role of ERK in tumor progression and resistance. Adopted from (A. E. Simões et al., 2016).

| Clinical and molecular supporting evidence for ERK5 involvement in human tumor development and resistance |  |  |
| :---: | :---: | :---: |
| Evidence | Tumor | References |
| Clinical evidence |  |  |
| Up-regulation of ERK5 signaling in human carcinogenesis | Breast | (Al-Ejeh et al., 2014; J. C. Montero et al., 2009; Ortiz-Ruiz et al., 2014) |
|  | Prostate | (McCracken et al., 2008 \{Clapé, 2009 \#261; Mehta et al., 2003)\} |
|  | Colon | (B. Hu et al., 2012; A. Simões et al., 2015; Tian, Sun, Zhao, Xiong, \& Fang, 2015) |
|  | Osteosarcoma | (Tesser-Gamba et al., 2012; van Dartel et al., 2002) |
|  | Hepatocellular carcinoma | (L.-X. Liu et al., 2003; Rovida et al., 2015; Zen et al., 2009) |
|  | Lung | (Gavine et al., 2015; Qiu et al., 2013) |
| Up-regulation of ERK5 signaling correlates with poorer prognosis and/or acquired resistance to therapy | Breast | (Al-Ejeh et al., 2014; Antoon et al., 2013; M. Miranda et al., 2015; Ortiz-Ruiz et al., 2014) |
|  | Prostate | (McCracken et al., 2008; Mehta et al., 2003; Ramsay et al., 2011) |
|  | Colon | (B. Hu et al., 2012) |
|  | Osteosarcoma | (Tesser-Gamba et al., 2012) |
|  | Mesothelioma | (Shukla et al., 2013) |
| Up-regulation of ERK5 signaling increases tumor metastasis incidence | Breast | (Antoon et al., 2013; Mulloy et al., 2003) |
|  | Prostate | (McCracken et al., 2008; Mehta et al., 2003; Ramsay et al., 2011) |
|  | Colon | (B. Hu et al., 2012; A. Simões et al., 2015) |
|  | Osteosarcoma | (Tesser-Gamba et al., 2012) |
|  | Mesothelioma | (Shukla et al., 2013) |
|  | HNSCC | (Sticht et al., 2008a) |
| Molecular evidence |  |  |
|  | Breast | (Cronan et al., 2012; Javaid et al., 2015; Mulloy et al., 2003; Perez-Madrigal, Finegan, Paramo, \& Tournier, 2012) |
|  | Prostate | (Dudderidge et al., 2007; McCracken et al., 2008; <br> Mehta et al., 2003) |
|  | Colon | (Borralho et al., 2011; A. Simões et al., 2015) |


| ERK5 signaling promotes tumor cell proliferation | Hepatocellular carcinoma | (Rovida et al., 2015; Zen et al., 2009) |
| :---: | :---: | :---: |
|  | Pancreatic | (Sureban et al., 2014) |
|  | Neuroblastoma | (Umapathy et al., 2014) |
|  | Mesothelioma | (Shukla et al., 2013) |
|  | Lung | (Q. Yang et al., 2013) |
|  | Leukemia | (Johan Garaude et al., 2006; Razumovskaya et al., 2011; T. Suzuki \& Yang, 2014) (Nuria LopezRoyuela et al., 2014) |
|  | Bladder | (Noguchi et al., 2011) |
| ERK5 signaling promotes tumor cell migration and metastasis | Breast | (Antoon et al., 2013; Javaid et al., 2015; MadakErdogan et al., 2014) |
|  | Prostate | (Mehta et al., 2003; Ramsay et al., 2011; Sawhney et al., 2009; Y. Zheng, Asara, \& Tyner, 2012) |
|  | Colon | (Mansour et al., 2015; A. Simões et al., 2015) |
|  | Osteosarcoma | (Yue, Ren, Su, Wang, \& Zhang, 2014) |
|  | Hepatocellular carcinoma | (Rovida et al., 2015) |
|  | Mesothelioma | (Shukla et al., 2013) |

### 3.3.7. ERK5 and Tumor associated angiogenesis

Proliferating cells have higher need of energy compared to non-proliferating cells hence angiogenesis in tumor is different in many ways like; intermittent and static blood flow from leaky blood vessels. ERK5 pathway is crucial for tumor-associated angiogenesis (Nithianandarajah-Jones, Wilm, Goldring, Müller, \& Cross, 2014; A. Simões et al., 2015). In EC, ERK5 mediates VEGF-induced tubular morphogenesis (Roberts et al., 2010). The tumor vascular density in mice is restricted through targeted Erk5 deletion while in xenograft models this deletion leads to inhibited vasculature development (Hayashi, Fearns, Eliceiri, Yang, \& Lee, 2005).

### 3.4. ERK5 and Tumor suppressive miRNAs

The relationship of microRNAs and tumor proliferation is comprehensively investigated. The association of ERK5 and miRNAs in relation with cancer appeared with the study of miR-143 and miR-145. In B-cells malignancies, bladder cancer, prostate cancer and in colon cancers expression of miR-143 and miR-145 is down regulated on the other hand ERK5 is up regulated (Akao, Nakagawa, Kitade, Kinoshita, \& Naoe, 2007; Nithianandarajah-Jones et al., 2012; Pekow et al., 2015) and overexpression of miR-143 precursors by transfection target ERK5 resulting in decrease proliferation and increase apoptosis of leukemic cells (Akao,

Nakagawa, Iio, \& Naoe, 2009; Akao et al., 2007). In fact, studies demonstrated that miRNA143 up-regulation led to the inhibition of cell migration and invasion possibly related to ERK5 targeting (Ni, Lin, Liu, \& Xiao, 2015; Pekow et al., 2015). While ERK5 is a target of miR-143 so cell proliferation was strongly inhibited upon transfection of miR-143 and miR145 precursors likewise when ERK5 is knock downed. Cancer cells expressing reduced miR143 could regulate tumor genesis through ERK5 activation (Clapé et al., 2009; Noguchi et al., 2011; Pekow et al., 2015).

### 3.5. Inhibitors of MEK5/ERK5

ERK5 is a promising target for cancer therapy and different drugs are being used to investigate the role of MEK5/ERK5 inhibition in various physiological and pathological phenomenons (Myers et al., 2016). Inhibition of MEK5/ERK5 has been revealed to have a significant influence on the sensitization of cancer cells to chemotherapy agents, justifying the potential of MEK5/ERK5 inhibitors for future clinical use (Table-7)(A. E. Simões et al., 2016). U1026 is dose dependent inhibitor of MEK5, whereas this activity is partially inhibited by PD98059. Recently, BIX02188 and BIX02189 are identified as particular inhibitors for MEK5. They block MEF2-driven gene expression through ERK5 inhibition without inhibiting other related MAP kinases. Stress induced ERK5-MEF2 pathway activation in HeLA and HEK293 cells is blocked by these inhibitors without any cytotoxic effect (Tatake et al., 2008). Inhibitor for CDK5, benzimidazole compounds inhibit ERK5 phosphorylation in human embryonic kidney cells but still this MEK5 mediated inhibition of ERK5 is not fully scrutinized (Flaherty et al., 2010). Another effective compound, XMD8-92, which inhibits directly ERK5 activity has been developed after modification in ATP-competitive polo kinase inhibitor BI-2356. It is highly selective in ERK5 inhibition compared with other kinases without hindering growth factor mediated ERK1/2 activation. ERK5 inhibition with XMD892 stimulates promyelocytic leukemia protein (PML) obstructing proliferation of tumor cell (Q. Yang et al., 2010). Recently XMD8-92 is reported to inhibit bromodomains that is a conserved protein motifs that are responsible for the recognition of acetyl-lysine residues during transcriptional processes (E. C. Lin et al., 2016). Conceivably due to their late discovery, the development of MEK5 or ERK5 inhibitors and the translation of these inhibitors to the clinics have yet to be explored (A. E. Simões et al., 2016).

Table 7: ERK inhibition in tumor treatment. Adopted from (A. E. Simões et al., 2016).

| ERK5 inhibition promotes chemotherapy sensitization in several human tumors |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: |
| Chemotherapy Sensitization | Tumor | In vivo/ in vitro | ERK5 Inhibition | Reference |
| Etoposide | Breast | In vitro | DN-MEK5 | (Weldon et al., 2002) |
| Trastuzumab | Breast | In vitro | shERK5; ERK5 ${ }^{\text {AEF }}$ | (J. C. Montero et al., 2009) |
| Fulvestrant | Breast | In vitro | shERK5 | (Antoon et al., 2013) |
| Tamoxifen | Breast | In vitro | shERK5 | (Antoon et al., 2013) |
| Docetaxel | Breast | In vivo + In vitro | XMD8-92 | (Al-Ejeh et al., 2014) |
| Doxorubicin | Breast <br> Cervical carcinoma Lung Mesothelioma Skin carcinoma | In vivo <br> In vitro | XMD8-92 <br> XMD8-92 <br> XMD8-92 <br> shERK5 <br> ERK5 $\Delta$ Depidermis; <br> XMD8-92 | $\begin{aligned} & \text { (Al-Ejeh et al., 2014; } \\ & \text { Finegan et al., 2015; } \\ & \text { Shukla et al., 2013; Q. } \\ & \text { Yang et al., 2013) } \end{aligned}$ |
| Cisplatin | Breast <br> Mesothelioma | In vitro In vivo $+$ In vitro | shERK5; TG02 shERK5 | (Ortiz-Ruiz et al., 2014; Shukla et al., 2013) |
| Vinorelbine | Breast | In vitro | shERK5; TG02 | (Ortiz-Ruiz et al., 2014) |
| Imatinib | CML (leukemia) | In vitro | ERK5 ${ }^{\text {AEF }}$ | (Buschbeck et al., 2005) |
| Dexamethasone | Multiple myeloma | In vitro | ERK5 ${ }^{\text {AEF }}$ | (Carvajal-Vergara et al., 2005) |
| Bortezomib | Multiple myeloma | In vitro | ERK5 ${ }^{\text {AEF }}$ | (Carvajal-Vergara et al., 2005) |
| Cytarabine | AML (leukemia) | In vitro | siERK5 | (Y. Xu, Cao, Gong, \& Rong, 2015) |
| Crizotinib | Neuroblastoma | In vivo <br> In vitro | siERK5; XMD8-92 | (Umapathy et al., 2014) |
| 5-FU | Colon | In vivo <br> In vitro | $\begin{aligned} & \text { siERK5; miR143; } \\ & \text { XMD8-92 } \end{aligned}$ | (Borralho et al., 2009; Pereira et al., 2016) |

DN, dominant negative; ERK5AEF, dominant negative form of ERK5; siERK5, ERK5 small interference RNA; $\Delta$, knockout.

Several investigations have demonstrated that targeting MEK5-ERK5 cascade decreased intravascular invasion consequently decreased circulating tumor cells and formation of metastatic lesions thus implicating the importance of this pathway in tumor progression and metastasis. Furthermore, this pathway is strongly linked to chemoresistance since the importance of this MEK5-ERK5 in cancer biology is well established, yet further investigations are warranted in determining the potential role of MEK5-ERK5 inhibition in targeting aggressive cancer types to delay the onset of drug resistance and maximize patient
response to therapy. Similarly further detailed understanding of this pathway will provide a decisive platform to expand the current spectrum of MEK5-ERK5 inhibitor therapies at wider level.

## La MAP kinase ERK5 (Extracellular signal regulated kinase 5)

Les cellules réagissent au stimulus extracellulaire par une signalisation complexe et interconnectée. Les récepteurs de surface aident les cellules à recevoir des stimuli et à transmettre des signaux au noyau via des voies de signalisation impliquant plusieurs protéines. La protéine ERK5 (Extracellular signal regulated kinase 5) est omniprésente dans tous les tissus et abondante dans le cœur. Les souris déficientes en ERK5 montrent une croissance retardée ainsi que diverses déformations cardiovasculaires conduisant à la mort. Plusieurs stimuli intra et extra cellulaire peuvent activer ERK5 par un mécanisme complexe.

L'activation d'ERK5 est régulée par la phosphorylation de plusieurs gènes en amont mais une fois activé, ERK5 peut réguler son propre destin. ERK5 a un rôle vital dans la survie cellulaire, la prolifération et d'autres fonctions physiologiques comme le développement de divers organites, en particulier le développement du système cardiovasculaire. De même ERK5 est impliqué dans la maintenance de l'intégrité vasculaire. L'activation d'ERK5 a montrée un rôle clé dans les réponses inflammatoires puisqu'elle régule plusieurs gènes impliqués dans le mécanisme de défense cellulaire durant l'inflammation. Dans divers conditions de stress, la faible expression d'ERK5 sensibilise les cellules à l'apoptose.

La prolifération cellulaire est également influencée par l'activité ERK5 qui est impliqué dans la régulation de la division cellulaire. Nombreuses études cliniques démontrent l'implication de la voie de signalisation MEK5/ERK5 dans le développement et la progression de la tumeur. La surexpression d'ERK5 a été rapportée dans différent types de cancer. ERK5 régule la transcription de plusieurs oncogènes favorisant ainsi l'apparition et le développement de la tumeur. La surexpression d'ERK5 est impliquée aussi dans les métastases tumorales et dans la résistance aux traitements contre le cancer. La voie ERK 5 devienne donc une cible prometteuse pour le traitement des cancers et divers inhibiteurs ERK5-MEK5 sont en cours d'exploitation.

Des niveaux élevés d'expression et d'activité d'ERK5 sont corrélés à un mauvais pronostique chez des patients souffrant de cancers du sein. L'inhibition de cette voie a été révélée pour avoir une influence significative sur la sensibilisation des cellules cancéreuses aux agents de la chimiothérapie, justifiant une utilisation future des inhibiteurs de la voie MEK5/ERK5 en clinique. En raison de la découverte tardive de l'intérêt de cette voie, le développement d'inhibiteurs de MEK5 ou ERK5 et leurs tests en cliniques n'ont pas été encore explorés.

## 4. Cellular Redox Homeostasis

Cultivating cells demand energy for all cellular processes and construction of macro and micro molecules. Glucose is primarily used to fulfill this demand of energy and aerobic metabolism of a single molecule of glucose yields 36 molecules of ATP, while only 2 molecules of ATP are produced during anaerobic metabolism. But this high amount of energy during aerobic metabolism results in incomplete oxidation of $\mathrm{O}^{2}$ and consequently production of potentially damaging reactive oxygen species (ROS) like; superoxide anion radical ( $\mathrm{O}^{2-}$ ), hydrogen peroxide $\left(\mathrm{H}_{2} \mathrm{O}_{2}\right)$ and the hydroxyl radical $\left(\mathrm{HO}^{-}\right)$which can damage proteins, lipids and DNA. Since mitochondria are main producer of ATP through OXPHOS thus these ROS are mainly produced in mitochondria during enzymatic reactions in electron transport and when ROS exceeds than the limit competency of cells a state of oxidative stress is considered to exist since during this state balance between ROS and antioxidant defense mechanism is disturbed in favor of oxidants (Halliwell, 2007; Y. Lu \& Cederbaum, 2008; Murphy, 2009; Sies, 1991; Tebay et al., 2015).

Cells activate their antioxidant defense mechanism, which directly or indirectly cope this situation through complex enzymatic reactions to inactivate such species like superoxide dismutase (SOD) and catalase reduce $\mathrm{O}^{2-}$ to $\mathrm{H}_{2} \mathrm{O}_{2}$ and further in $\mathrm{H}_{2} \mathrm{O}$ and $\mathrm{O}_{2}$. Cells under stress conditions triggers inductions of several cytoprotective genes, which can generate a long lasting antioxidant response. Cellular defense mechanism also involved the removal of potentially harmful xenobiotics through activation of drug-metabolizing enzymes and drugefflux transporters. This results in removal of molecules that can generate ROS by redox cycling or by removing electrophiles that deplete endogenous antioxidants (Bauer, 2014; Dinkova - Kostova \& Talalay, 2008; Tebay et al., 2015).

### 4.1. NFE2L2 (NRF2)

Nuclear factor (erythroid-derived 2)-like 2 also named, as NFE2L2 or NRF2, is a transcription factor encoded by the NFE2L2 gene. Firstly NRF2 is identified in 1994 a 66kDa protein ubiquitously expressed in all organs with the highest concentrations in the kidney, muscle, lung, heart, liver, and brain. In human NRF2 protein comprises of 605 amino acids while 597 amino acids in mouse and rat. Anatomically, NRF2 composes of a basic leucine zipper (bZip) with a Cap "n" Collar (CNC) (J. D. Hayes \& Dinkova-Kostova, 2014; Moi, Chan, Asunis, Cao, \& Kan, 1994) and can be divided into a number of regions stated as

NRF2-ECH homology (Neh) domains. Up-till now seven Neh domains have been identified as shown in Figure 18. Every Neh domains is functionally different. The Neh1 domain contains the conserved CNC-bZIP region essential for its activity as a transcription factor and allowing it to heterodimerize with MAF a small bZIP proteins while NRF2 cytosolic repressor protein KEAP1 binds at Neh2 (Fig. 18-19) (Itoh, Igarashi, Hayashi, Nishizawa, \& Yamamoto, 1995; Mohler, Vani, Leung, \& Epstein, 1991; Motohashi, Katsuoka, Engel, \& Yamamoto, 2004; Motohashi \& Yamamoto, 2004).


Fig. 18. Domain structure of human NRF2. The relative positions of the Neh domains of transcription factor Nrf2 are shown. Adopted from (Tebay et al., 2015).

### 4.2. KEAP1

Kelch-like ECH-associated protein 1 is a cysteine rich protein encoded by the KEAP1 gene. These cysteine residues are important sensor of stress and are susceptible to diverse range of chemicals. This protein is located in cytoplasm as a negative regulator of NRF2 since it targets NRF2 for proteasomal degradation in cytoplasm (Itoh et al., 2003; Tebay et al., 2015; Watai et al., 2007). KEAP1 possesses approximately $4 \%$ cysteine content compared to other proteins where cysteine contents are about 2\% (Hansen, Roth, \& Winther, 2009). Under normal cellular homeostatic environments it serves as substrate adaptor facilitating the CUL3 ubiquitin ligase complex (CRL ${ }^{\text {Keap1 }}$ ) to ubiquitylate NRF2 in cytoplasm (Fig. 20) (Cullinan, Gordan, Jin, Harper, \& Diehl, 2004; A. Kobayashi et al., 2004; D. D. Zhang, Lo, Cross, Templeton, \& Hannink, 2004).


Fig. 19. A cytoskeleton protein structure of NRF2 and KEAP1. Adopted from (Aboonabi \& Singh, 2015).
NRF2 (black) interacts with two molecules of KEAP1 (red chain) through its Neh2 ETGE and DLG motifs. Both ETGE and DLG bind to similar sites on the bottom surface of the KEAP1 Kelch.

### 4.3. Regulation of NRF2/KEAP1 Pathway

Numerous stimuli can trigger NRF2 however oxidative stressors and metabolic stimuli requiring NADPH and ATP production regulate mainly its activity. On the other hand stress response protein kinases also can trigger NRF2 activity. It is therefore possesses a complex regulatory mechanisms at both transcriptional and posttranslational levels. The transcriptional activity of NRF2 is uppermost among all the CNC-bZIP family of transcription factors (J. D. Hayes \& Dinkova-Kostova, 2014; Katoh et al., 2001; Qaisiya, Zabetta, Bellarosa, \& Tiribelli, 2014). The gene of NRF2 encloses a xenobiotic response element (XRE) and two XRE-like sequences where the arylhydrocarbon receptor (AhR) can binds and consequently initiating transcriptional activity due to polycyclic aromatic hydrocarbons (PAHs). Interestingly NRF2 also regulate AhR, thus a feedback loop exists (Miao, Hu, Scrivens, \& Batist, 2005; Qiang, Kinneer, Yongyi, \& KAN, 2004; Shin et al., 2007). Two (antioxidant responsive element) ARE-like sequences are also present in the upstream region of the transcription start site (TSS) in NFE2L2 promoter that enabled self-regulated expression after initial transcription (Kwak, Itoh, Yamamoto, \& Kensler, 2002). The gene also has an NF-kB binding site thus
initiating transcription during inflammatory conditions while during fasting condition expression of peroxisome proliferator-activated receptor $\alpha$ (PPAR $\alpha$ ) also regulate NRF2 (Stuart A Rushworth et al., 2012; Sanderson, Boekschoten, Desvergne, Müller, \& Kersten, 2010).

Expression of NRF2 is principally regulated at the post-translational level. Under normal condition protein is constantly degraded by the 26 S proteasome along with several E3 ubiquitin ligases via KEAP1 (Tebay et al., 2015). Neh2 domain of NRF2 contains two conserved KEAP1 binding cites 1) high-affinity ETGE motif and 2) low-affinity DLG motif. The binding affinity of Keap1 for the ETGE motif is approximately 100 -fold higher than the DLG motif (Fukutomi, Takagi, Mizushima, Ohuchi, \& Yamamoto, 2014; Tong et al., 2007). Exposure to oxidative stress or electrophilic stress, results in conformational changes in KEAP1 by disturbing critical cysteine residues mainly Cys151, Cys273, and Cys288 disturbs the weak binding at DLG motif which results in impairment of KEAP1-CUL3 ubiquitination system and consequently degradation of NRF2 is disturbed and it starts building up in cytoplasm. This leads to NRF2 translocation to the nucleus, where it forms a heterodimer with its partner sMAF (v-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog) and binds to ARE sequences to regulate the transcription of several genes (Fig. 20) (Itoh et al., 1997; Menegon, Columbano, \& Giordano, 2016; Qaisiya et al., 2014; Sekhar, Rachakonda, \& Freeman, 2010; Tebay et al., 2015; Tong et al., 2007; Yamamoto et al., 2008; D. D. Zhang, 2006). The exact regulation of NRF2 translocation from cytoplasm to the nucleus is still unclear (Tebay et al., 2015). The antioxidant response element is the cis-acting DNA sequence residing in the promoter regions of genes that can induce antioxidants response and to which small NRF2-MAF heterodimers are engaged (J. Hayes et al., 2000).

Indirect activation of NRF2 pathway is also regulated by several stress-response protein kinases such as protein kinase C (PKC), mitogen activated protein kinase (MAPK) cascade, phosphatidylinositide 3-kinases (PI3K) and protein kinase like endoplasmic reticulum kinase (PERK). NRF2 mediated activation of multiple antioxidant genes was also observed by unconjugated bilirubin (UCB) mediated oxidative stress (Cullinan et al., 2004; Itoh, Tong, \& Yamamoto, 2004; Kwak, Wakabayashi, \& Kensler, 2004; Qaisiya et al., 2014; D. D. Zhang, 2006). ERK5 mediated NRF2 activation was also observed to play a protective role in laminar flow induced inflammation in endothelial cells (Miso Kim et al., 2012).


Fig. 20. The KEAP1-NRF2-ARE signaling pathway. Adopted from (Menegon et al., 2016). Binding of KEAP1 to CUL3 leads to KEAP1 homodimerization. The high-affinity ETGE motif of NRF2 initially binds to the KELCH domain of KEAP1 and the lower-affinity DLG motif binds to the second KEAP1, closing the conformation of the complex. Under homeostatic conditions (right), NRF2 is polyubiquitinated at its lysine-rich (KKKKKKK) region and is then targeted to proteasome for degradation. Under increasing ROS levels conditions (left), the modification of cysteine residues on KEAP1 (red stars) imposes a conforma- tional change that disrupts the weak KELCH-DLG binding, resulting in diminished NRF2 ubiquitination, without dissociation of NRF2 from KEAP1. NRF2 protein levels are thus increased and NRF2 translocates into the nucleus where it associates with sMAF (musculo-apo- neurotic fibrosarcoma), subsequently binding ARE (antioxidant responsive element) sequences on target genes, regulating their transcription.

Repression of KEAP1 protein also leads to NRF2 nuclear translocation thus triggering its activity. Loss-of-function mutations of KEAP1 have been found in several human cancers majority in lung cancer leading to overexpression or constitutive activation of the pathway. Mutation in the KEAP1 binding cites (either ETGE or DLG motif) impair its binding to NRF2 which consequently abolish KEAP1-mediated NRF2 degradation (Katoh et al., 2005;
M. Kobayashi et al., 2002; McMahon, Thomas, Itoh, Yamamoto, \& Hayes, 2006; Menegon et al., 2016). Within KEAP1, Cys-151 is vital for sensing the electrophiles thus it is demonstrated that mutation of Cys-151 to serine or alanine leads to loss of NRF2 activation. Similarly, replacement of Lys-131, Arg-135, and Lys-150, that resides around Cys-151 and increase its reactivity, to Met residues has been demonstrated to reduce NRF2 activity against different electrophiles (McMahon, Lamont, Beattie, \& Hayes, 2010; D. D. Zhang \& Hannink, 2003). Interestingly it was also observed that KEAP1-inactivating compounds such as sulforaphane (SFN) results in more strong binding of KEAP1 to NRF2 at ETGE and DLG motifs leads to conformational change of KEAP1 and thus prevents ubiquitylation of bound NRF2 due to trapping the CUL3 complex and further preventing sequestration of newly synthesized NRF2. Consequently newly translated NRF2 bypasses the CRL ${ }^{\text {Keap1 }}$ complex and translocate to nucleus (Baird, Llères, Swift, \& Dinkova-Kostova, 2013; Tebay et al., 2015).

Gain-of-function mutations also result in hyperactivity of NRF2 regulated pathways thus playing a survival role in cancer. Mutations in the NRF2 coding region, Neh2 a clustered in the KEAP1-binding domain, were first described in lung cancer (Menegon et al., 2016; Shibata et al., 2008).

The expression of p 21 (or CIP1/WAF1) is upregulated in elevated ROS and directly competes with KEAP1 for NRF2 binding to the DLG motif thus impairing the ubiquitination of NRF2 and further stimulates its action (W. Chen et al., 2009). Similarly, sequestosome 1 protein (p62/SQSTM) contains a STGE-binding motif (similar to the NRF2-ETGE motif) and compete with NRF2 binding to KEAP1, hence disrupting the NRF2-KEAP1 complex and promoting NRF2 activity (Copple, 2011; Komatsu et al., 2010). These finding suggests that accumulation of p 21 and p62 consequently triggers the sustained activation of NRF2 and can contribute in tumor growth.

Certain miRNAs also targets KEAP1 protein thus hindering NRF2 degradation. The microRNA-141 was observed to induce cisplatin resistance through targeting KEAP1 in ovarian cells (Van Jaarsveld et al., 2013). Similarly microRNA-200a (miR- 200a) activates NRF2 transcriptional activity by degrading KEAP1 mRNA (Eades, Yang, Yao, Zhang, \& Zhou, 2011; J.-J. Yang et al., 2014). The activity of KEAP1 is also repressed by microRNA-7 (miR-7), which is highly expressed in the brain, by targeting the 3'UTR of its mRNA in human neuroblastoma cells (Kabaria et al., 2015). Recently we demonstrated that ERK5 activates the transcription factor MEF2, which binds to the promoter of the miR-23a-27a-24-

2 cluster. Newly generated miR-23a disrupts KEAP1 mRNA by targeting to its 3'UTR and this lower KEAP1 levels increase the basal expression of the NRF2-dependent genes NQO-1 and HO-1 (Khan et al., 2016). On the other hand, miR-28 expression has been shown to decrease NRF2 mRNA and protein levels while miR-144 was observed to regulate NRF2 negativly targeting two distinct sites in the NRF2 untranslated region thus down regulating its activity in patients suffering from sickle cell disease (Sangokoya, Telen, \& Chi, 2010; M. Yang, Yao, Eades, Zhang, \& Zhou, 2011).

### 4.4. NRF2 Response mechanism

Approximately, 250 genes are regulated by NRF2 (Table 8) that are directly and indirectly involved in a range of biological functions ranging from cytoprotection under stress conditions to metabolic pathways resulting in NADPH and ATP production (J. D. Hayes \& Dinkova-Kostova, 2014; Holmström et al., 2013; Thimmulappa et al., 2002). NRF2 activation leads to regulation of various process such as; Glutathione (GSH) homeostasis through cysteine uptake transporter ( xCT ), glycine uptake transporter (Gly1) and regulating the rate limiting enzyme of GSH synthesis; $\gamma$-glutamylcysteine ligase catalytic and modulatory subunits ( $\gamma$-GCL-c and $\gamma$-GCL-m, respectively) (J.-M. Lee, Calkins, Chan, Kan, \& Johnson, 2003; Moinova \& Mulcahy, 1998; Sasaki et al., 2002; Wild, Moinova, \& Mulcahy, 1999). Likewise NRF2 mediated antioxidant and detoxification is regulated through heme oxygenase 1 (HO-1), ferritin heavy chain (FTH) and NADPH-Quinone oxidoreductase 1 (NQOI) (Alam et al., 1999; Pietsch, Chan, Torti, \& Torti, 2003; Rushmore, Morton, \& Pickett, 1991; J. Sun et al., 2002). Similarly NADPH homeostasis is intervened through malic enzyme (ME1) while cellular stress response is secured by transcription factor 3 (ATF3) activation (K.-H. Kim, Jeong, Surh, \& Kim, 2009; J.-M. Lee et al., 2003; Qaisiya et al., 2014; K. C. Wu, Cui, \& Klaassen, 2011).

Table 8: Recapitulates all the target genes of Nrf2 and their role in various biological processes. Adopted from (Tebay et al., 2015).

| Primary Role | Gene | Protein | Function | Cellular <br> processes | References |
| :---: | :---: | :---: | :---: | :---: | :---: |
| Redox homeostasis | GCLC | Glutamatecysteine ligase catalytic subunit | Catalytic subunit of the enzyme responsible for the rate-limiting step in synthesis of the cellular antioxidant glutathione | Maintenance of cellular redox homeostasis | (Agyeman et al., 2012; Chorley et al., 2012; Malhotra et al., 2010) |
| Redox homeostasis | GLCM | Glutamatecysteine ligase modifier subunit | Modifier subunit of the enzyme responsible for the rate-limiting step in synthesis of the cellular | Maintenance of cellular redox homeostasis | (Agyeman et al., 2012; Chorley et al., 2012; MacLeod et al., 2009; Paek et al., |


|  |  |  | antioxidant glutathion |  | 2012) |
| :---: | :---: | :---: | :---: | :---: | :---: |
| Redox homeostasis | GPX2 | Glutathione peroxidase 2 | Detoxification of $\mathrm{H}_{2} \mathrm{O}_{2}$ and an important cellular antioxidant | Maintenance of cellular redox homeostasis | (Banning, <br> Deubel, Kluth, <br>  <br> Brigelius-Flohé, 2005; Singh et al., 2006) |
| Redox homeostasis | PRDX1 | Peroxiredoxin 1 | Reduces peroxides, <br> regulates cellular  <br> concentrations of $\mathrm{H}_{2} \mathrm{O}_{2}$  | Maintenance of cellular redox homeostasis | (Agyeman et al., 2012; Chorley et al., 2012; <br> Hawkes, <br>  <br> Tonissen, 2014) |
| Redox homeostasis | SRXN1 | Sulfiredoxin | Contributes to the thioredoxin-based antioxidant system that reduces oxidized protein thiols; reduces sulfinic acid in proteins | Thiol maintenance, maintenance of cellular redox homeostasis | (Agyeman et al., 2012; Chorley et al., 2012; Malhotra et al., 2010) |
| Redox homeostasis | TXN1 | Thioredoxin 1 | Reversible oxidation of active center allows participation in dithiol-disul fide exchange reactions; reduces sulfenic acid in proteins | Thiol maintenance, maintenance of cellular redox homeostasis | (Agyeman et al., 2012; Chorley et al., 2012; Hawkes et al., 2014; Malhotra et al., 2010) |
| Detoxification | ABCB6 | $\begin{aligned} & \text { ATP-binding } \\ & \text { cassette B6 } \\ & \text { (MDR/TAP) } \end{aligned}$ | Mitochondrial transporter; transports heme and porphyrin in an ATP dependent manner, crucial for heme metabolism | Phase III drug metabolism, heme metabolism | (Agyeman et al., 2012; <br> Malhotra et al., 2010) |
| Detoxification | ABCC2 | $\begin{aligned} & \text { ATP-binding } \\ & \text { cassette C2 } \\ & \text { (MRP2) } \end{aligned}$ | Biliary transport/hepatic excretion; involved in the extrusion of certain anticancer drugs and implicated in multidrug resistance | Phase III drug metabolism | (Malhotra etal., 2010)a(Agyeman etal., 2012; Junget al., 2013;Maher et al., <br> 2007) |
| Detoxification | AKR1B10 | Aldo-keto reductase 1B10 | Converts retinal to retinol; reduces aromatic and aliphatic aldehydes | Phase I drug metabolism, heme metabolism | (Agyeman et al., 2012; Hirotsu et al., 2012; Jung et al., 2013) |
| Detoxification | AKR1C1 | Aldo-keto reductase 1C1 | Converts4-hydroxy-2- <br> nonenal to 1,2-dihydroxy <br> nonene; inactivates <br> progesteron l${ }^{2}$ | Phase I drug metabolism | (Agyeman et al., 2012; <br> Burczynski, Sridhar, <br>  <br> Penning, 2001; <br> Jung et al., 2013; <br> MacLeod et al., 2009) |
| Detoxification | AKR1C3 | Aldo-keto reductase 1C3 | Type $517 \beta$-hydroxysteroid dehydrogenase and prostaglandin F2 $\alpha$ synthase | Phase I drug metabolism | (H.-K. Lin et al., $\quad 1997$; Penning et al., 2000 ) |
| Detoxification | CES1G | Carboxyl esterase 1G | Catalyzes the transesterification of xenobiotics, hydrolysis of long-chain fatty acid esters | Phase I drug metabolism, fatty acid oxidation, fatty acid degradation | $\begin{aligned} & \text { (Paek et al., } \\ & 2012) \end{aligned}$ |
| Detoxification | CES1H | Carboxyl esterase 1H | Catalyzes the transesterification of xenobiotics, hydrolysis of long-chain fatty acid esters | Phase I drug metabolism, <br> fatty acid oxidation, fatty acid degradation | $\begin{aligned} & \text { (Paek et al., } \\ & 2012) \end{aligned}$ |
| Detoxification | GSTA1 | Glutathione Stransferase A1 | Detoxification and metabolism of electrophilic | Phase II drug metabolism, | (Chanas et al., 2002; Hirotsu |


|  |  |  | compounds; metabolism of bilirubin and certain anticancer drugs; also displays glutathione peroxidase activity | cytoprotection | et al., 2012; <br> Malhotra et al., 2010) |
| :---: | :---: | :---: | :---: | :---: | :---: |
| Detoxification | GSTM1 | Glutathione Stransferase M1 | Detoxi!cation and metabolism of electrophilic compounds | Phase II drug metabolism | (Chanas et al., 2002; Hirotsu et al., 2012; Malhotra et al., 2010) |
| Detoxification | NQO1 | $\begin{aligned} & \text { NAD }(\mathrm{P}) \mathrm{H} \\ & \text { quinone } \\ & \text { oxidoreductase } \\ & 1 \end{aligned}$ | Reduces quinones to hydroquinones and prevents the one-electron reduction of quinones that would otherwise produce free radicals | Phase II drug metabolism | (Agyeman et al., 2012; Chorley et al., 2012; <br> MacLeod al., 2009) |
| Heme metabolism | FECH | Ferrochelatase | Catalyzes the insertion of $\mathrm{Fe}^{2+}$ into protoporphyrin IX during heme synthesis; localized in mitochondria | Heme metabolism | (Chorley et al., 2012; <br> MacLeod et al., 2009) |
| Heme metabolism | HMOX1 | Heme oxygenase 1 | Cleaves heme to produce biliverdin during heme catabolism | Heme metabolism | (Agyeman et al., 2012; Chorley et al., 2012; Malhotra et al., 2010) |
| Lipid metabolism | AWAT1 | $\begin{aligned} & \text { Acyl-CoA wax } \\ & \text { alcohol } \\ & \text { acyltransferase } \\ & 1 \end{aligned}$ | Catalyzes the production of wax esters from long-chain alcohols and acyl- <br> CoA-derived fatty acids; enriched in skin | Lipid metabolism | $\begin{array}{lll} \hline(\text { Paek } \\ 2012) & \text { et al., } \end{array}$ |
| Lipid metabolism | FABP1 | Fatty acid binding protein 1 | Binds long-chain fatty acids, their CoA derivatives, and bile acids in the cytoplasm for intracellular transport | Lipid uptake and intracellular transport | $\begin{aligned} & \text { (Yates et al., } \\ & 2009) \end{aligned}$ |
| Lipid metabolism | LIPH | Lipase H | Membrane-bound triglyceride lipase that hydrolyzes phosphatidic acid to produce 2-acyl lysophosphatidic acid, which is a potent bioactive lipid mediator | Platelet aggregation, smooth muscle contraction, cell proliferation and motility | $\begin{aligned} & \text { (Paek et al., } \\ & 2012) \end{aligned}$ |
| Lipid metabolism | PPAR $\gamma$ | Peroxisome proliferatoractivated receptor $\gamma$ | Transcription factor that orchestrates lipid metabolism; key regulator of adipocyte differentiation and glucose homeostasis | Lipid <br> mobilization, $\beta$ <br> -oxidation of <br> fatty acids, <br> adipocyte <br> differentiation, <br> glucose <br> metabolism | (Chorley et al., 2012; Yates et al., 2009) |
| Lipolysis | ACOT7 | Acyl-CoA thioesterase 7 | Catalyzes the hydrolysis of palmitoyl-CoA and other long-chain fatty acids to form free fatty acid and CoA | Fatty acid oxidation, fatty acid degradation | (K. C. Wu et al., 2011) |
| Lipolysis | ACOX2 | Acyl-CoA oxidase 2 | Catalyzes peroxisomal degradation of long branched-chain fatty acids and bile acid intermediates | Fatty acid oxidation, fatty acid degradation | $\begin{aligned} & \text { (Paek et al., } \\ & 2012) \end{aligned}$ |
| Lipogenesis | ACLY $\downarrow$ | ATP citrate lyase | Catalyzes the production of acetyl-CoA and oxaloacetate from CoA and citrate using ATP | Lipogenesis, cholesterol synthesis, gluconeogenesis | (Kitteringham et al., 2010; K. C. Wu et al., 2011; Yates et al., 2009) |
| Lipogenesis | FASN $\downarrow$ | $\begin{array}{lc} \hline \text { Fatty } \\ \text { synthase } \end{array} \quad \text { acid }$ | Generation of long-chain fatty acids such as palmitate from malonyl-CoA and | Lipogenesis | (Kitteringham et al., 2010; K. C. Wu et al., |


|  |  |  | acetyl-CoA using NADPH |  | 2011; Yates et al., 2009) |
| :---: | :---: | :---: | :---: | :---: | :---: |
| Lipogenesis | SCD1 $\downarrow$ | Stearoyl-CoA desaturase 1 | Catalyzes the introduction of a double bond into stearoyl-CoA to create the mono-unsaturated fatty acid oleic acid | Lipogenesis | (Okada et al., 2013; K. C. Wu et al., 2011) |
| Lipogenesis | SREBF1 | Sterolregulatory element binding transcription factor 1 | Transcription factor that controls expression of the LDL receptor, and genes involved in glucose metabolism and lipid synthesis | Lipogenesis, glucose metabolism | $\begin{aligned} & \text { (Yates et al., } \\ & 2009) \end{aligned}$ |
| Glucose metabolism | FGF21 $\downarrow$ | Fibroblast growth factor 21 | Stimulates glucose uptake in adipocytes, involved in regulation of insulin sensitivity | Glucose uptake and clearance, insulin signaling | (Chartoumpeki s et al., 2011) |
| Cholesterol synthesis | HMGCS1 | 3-Hydroxy-3-methylglutarylCoA synthase 1 | Catalyzes the condensation of acetyl-CoA and acetoacetyl-CoA to produce HMG-CoA, the substrate for HMG-CoA reductase | Cholesterol synthesis | (K. C. Wu et al., 2011; <br> Yates et al., 2009) |
| NADPH generation | G6PD | Glucose-6phosphate dehydrogenase | Generates NADPH in the pentose phosphate pathway; maintains cellular glutathione redox status | Pentose phosphate pathway, NADPH generation, maintenance of cellular redox homeostasis | (Agyeman et al., 2012; Hirotsu et al., 2012; Malhotra et al., 2010; K. C. Wu et al., 2011) |
| NADPH generation | IDH1 | Isocitrate dehydrogenase 1 | Catalyzes the oxidative decarboxylation of isocitrate to $\alpha$-ketoglutarate, using $\mathrm{NADP}^{+}$as a cofactor, outside the context of the citric acid cycle in the cytoplasm or in peroxisomes | NADPH generation | (Hirotsu et al., 2012; Malhotra et al., 2010; K. C. Wu et al., 2011) |
| Pentose synthesis | TALDO1 | Transaldolase 1 | Involved in production of ribose 5'-phosphate required for nucleic acid synthesis | Pentose phosphate pathway | (Agyeman et al., 2012; Jung et al., 2013; K. C. Wu et al., 2011) |
| Pentose synthesis | TKT | Transketolase | Channels excess sugars from the pentose phosphate pathway to glycolysis by the creation of glyceraldehyde 3-phosphate | Pentose phosphate pathway, glycolysis | (Agyeman et al., 2012) |
| Scavenger receptor | CD36 | CD36 molecule/fatty acid translocase | Major platelet glycoprotein that binds long-chain fatty acids and functions in the transport and regulation of transport of fatty acids | Fatty acid  <br> transport,  <br> adhesion  | $\begin{aligned} & \text { (Ishii et al., } \\ & 2004 \text { ) } \end{aligned}$ |
| Autophagy | $\begin{aligned} & \hline \text { p62/ } \\ & \text { SQSTM1 } \end{aligned}$ | Sequestosome 1 | Required for formation and autophagic degradation of polyubiquitin containing bodies; used as a scaffold protein | Autophagy, inflammation | $\begin{array}{lll} \hline \text { (Jain } & \text { et al., } \\ 2010) & & \end{array}$ |
| Tissue regeneration | NOTCH1 | Notch 1 | Transmembrane protein containing multiple epidermal growth factor-like repeats that is involved in signaling processes during development and tissue regeneration | Cell fate determination, developmental signaling | (Wakabayashi et al., 2010) |
| Regulation of xenobiotic | AHR | Aryl <br> hydrocarbon | Transcription factor that binds planar aromatic | Xenobiotic metabolism | (Shin et al., 2007 ) |


| response |  | receptor | compounds and upregulates genes involved in xenobiotic metabolism, including CYP family members |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: |
| E3 ligase substrate adaptor | Keap1 | Kelch-like ECH associated protein 1 | E3 ubiquitin ligase substrate adaptor that targets proteins for degradation by the 26 S proteasome; known negative regulator of Nrf 2 | Targeting for ubiquitination, Nrf2 repression, regulation of antioxidant response | (Chorley et al., 2012) |
| Heterodimeri c binding partner | MAFG | Musculoapone urotic <br> "brosarcoma G | Transcriptional regulator; forms heterodimers with a number of transcription factors, including Nrf2, allowing their activation; also forms homodimers restricting the activation of obligate heterodimeric partner molecules | Transcriptional activation, regulation of antioxidant response | (Chorley et al., 2012; <br> MacLeod et al., 2009; Malhotra et al., 2010; Paek et al., 2012) |
| Regulation of antioxidant response | NFE2L2 | $\begin{aligned} & \text { NF-E2 p45- } \\ & \text { like 2 } \end{aligned}$ | Transcription factor that regulates genes involved in the oxidative stress response; maintains cellular redox homeostasis and detoxification and contributes to lipid and carbohydrate metabolism | Drug metabolism, xenobiotic metabolism, maintenance of cellular redox homeostasis | (Kwak et al., 2002) |
| Regulation of lipid metabolism | $\text { PPAR } \alpha$ | Peroxisome proliferatoractivated receptor $\alpha$ | Transcription factor, key  <br> regulator of lipid <br> metabolism; activator of  | Lipid mobilization, fatty acid oxidation, lipogenesis, gluconeogenesis, ketogenesis | (Yuji Tanaka, Ikeda, <br> Yamamoto, Ogawa, \& Kamisako, 2012; Yates et al., 2009) |
| Transcription al regulator | RXR $\alpha$ | Retinoid X receptor $\alpha$ | Transcriptional regulator; mediates the biological effect of retinoids by Forming homo- or heterodimers and binds to target gene sequences; known negative regulator of Nrf2; known binding partner and activator of PPAR $\alpha$ | Transcriptional activation, Nrf2 repression, regulation of antioxidant response, PPAR $\alpha$ activation | (Chorley et al., 2012; Malhotra et al., 2010) |

Genes that are with an adjacent down arrow are those that have been reported to be down regulated by Nrf2.

### 4.5. NRF2 and Cancer

NRF2 has a cytoprotective role in cellular defense system thus it is considered to have a tumor suppressor effects (Iida et al., 2004; Ramos-Gomez, Dolan, Itoh, Yamamoto, \& Kensler, 2003) since it has numerous beneficial effects in cancer chemoprevention as oxidative stress can initiate cancer and anti-oxidative role of NRF2 might has an anticancer role. Furthermore loss of NRF2 supports metastasis and Nrf2 null mice are more prone to carcinogenic (J. D. Hayes, McMahon, Chowdhry, \& Dinkova-Kostova, 2010; Ramos-Gomez et al., 2001; Satoh et al., 2010).

However some reports demonstrates the dark side of the picture elaborating the oncogenic potential of NRF2 with evidence that NRF2 has a survival role in tumor and its activation would support the disease as over activation of NRF2 in tumors generates an environment which favor cancer survival avoiding from excessive oxidative stress, chemotherapeutic agents or radiotherapy. Thus it makes it unclear whether NRF2 has tumor suppressor or oncogenic character (Fig. 21) (J. D. Hayes et al., 2010; A. Lau, Villeneuve, Sun, Wong, \& Zhang, 2008; X.-J. Wang et al., 2008; P. Zhang et al., 2010). Activation of NRF2 results in up-regulation of genes involved in drug metabolism and consequently triggers resistance to chemotherapeutic drugs. Over activation also induces metabolic reprogramming towards anabolic pathways parallel to glycolysis promoting tumor growth (Mitsuishi et al., 2012). Anti apoptotic proteins include ARE regions in their promoters thus are regulated by NRF2 and up-regulation of BCL2 and BCL-xL proteins by NRF2 can protect tumor cells from apoptosis (Niture \& Jaiswal, 2012, 2013). It is also observed that the NRF2/KEAP1 pathway is dysregulated during very early steps of the carcinogenesis in the liver of rats leading to metabolic reprogramming and the onset of the Warburg effect (Petrelli et al., 2014).

Schematic Representation of the Dual Role of NRF2 in Cancer Onset and Progression

## Tumor Suppressor

NRF2 activation is important in chemoprevention

Many dietary and chemical chemopreventive compounds are NRF2 activators

> NRF2 activates the main cellular defense mechanism against metabolic, xenobiotic, and oxidative stress
> Nrf2-deficient mice are more sensitive to carcinogenesis

Nrf2 loss has been related to enhanced metastasis

Oncogene

NRF2 constitutive activation is present in many tumor types

NRF2 hyperactivation favors cancer cell survival and promotes cell growth

NRF2 hyperactivation protects tumor cells from oxidative stress, chemotherapy, and radiotherapy

NRF2 activation is due to genetic, transcriptional, post-transcriptional, and post-translational mechanisms

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Fig. 21. Role of NRF2 in Cancer. Adopted from (Menegon et al., 2016).
The left side of the figure lists some of the main evidence supporting the tumor-suppressive role of NRF2; the right side of the figure summarizes the evidence to support the oncogenic role of NRF2. The bifrontal figure represents Janus, the Roman god with two faces.

The transcriptional activity of NRF2 is triggered by activated oncogenes, such as $K R A S$ (G12D), BRAF (V619E) and c-MYC (ERT2) stimulating its activity to regulate reduced intracellular redox environment for tumor growth and $K R A S$ (G12D)-induced tumorigenesis was inhibited by targeting NRF2 since it regulate its transcription through MEK-ERK signaling (DeNicola et al., 2011; Tao et al., 2014). Similarly, inhibition of the NRF2 oxidative stress pathway in leukaemia cells renders them more sensitive to cytotoxic chemotherapy (Abdul-Aziz, MacEwan, Bowles, \& Rushworth, 2015).

Based on these tumors promoting effects of NRF2 activation it could be an important pharmacological target and sadly, so far specific and effective NRF2 inhibitors are unavailable (Menegon et al., 2016). But since NRF2 has dual role in cancer the therapeutic utility of NRF2 inhibition depends on the molecular and clinical context and the type and
stage of cancer. Moreover it will be hard to define the boundary between health benefits and side effects of a diet rich in antioxidants.

Commentary

# Keep Harm at Bay: Oxidative Phosphorylation Induces Nrf2-Driven Antioxidant Response Via ERK5/MEF2/miR-23a Signaling to Keap-1 

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Nuclear factor E2-related factor 2 ( $\mathrm{Nrf2}$ ) is a key transcription factor which induces the expression of various cellular antioxidant and detoxifying enzymes through the binding and transcriptional activation of antioxidant response elements (ARE) in the promoters of their genes. The Nrf2/ARE pathway is known to protect cells against various stress stimuli, primarily oxidative stress associated with increased production of reactive oxygen species (ROS). Nrf2 activity is tightly regulated by a cytoplasmic inhibitory protein Kelch-like ECH-associated protein-1 (Keap-1) which acts as an adaptor between Nrf2 and cullin-3 ubiquitin ligase and promotes rapid proteasomal degradation of Nrf2. Being a sensor of various exogenous and endogenous electrophilic compounds and ROS, Keap-1 undergoes conformational changes upon interaction with such agents and this causes Nrf2 release from the complex, thus allowing it to translocate to the nucleus and transactivate Nrf2responsive genes [see (Harder et al., 2015; Tebay et al., 2015) for recent reviews].

In their study, Khan et al. (this issue) demonstrate that there is also an alternative mechanism whereby the Nrf2/ARE pathway can be activated in human leukemia cells performing oxidative phosphorylation (OXPHOS). The authors found that while OXPHOS results in increased generation of ROS, the induction of the "classical" Nrf2-responsive genes encoding heme oxygenase-1 (HO-1) and $\mathrm{NAD}(\mathrm{P}) \mathrm{H}$ :quinone oxidoreductase (NQO1) can also occur in a ROS-independent manner. This was associated with a decrease in Keap- 1 mRNA levels, implying that lower protein levels of Keap-1 may facilitate stabilization of the de novo synthesized Nrf2 protein, thus increasing the functional activity of the Nrf2/ARE pathway. The data also show that the MAPK ERK5 is upregulated in this system. This suggests that ERK5 kinase is responsible for the downregulation of Keap-1, which may be mediated by the downstream target of ERK5, the transcription factor MEF2C. Khan et al. show that MEF2C

[^0]binds to the promoter of the microRNA miR-23a-27a-24-2 cluster and that miR-23a destabilizes Keap-1 mRNA by interacting with its $3^{\prime}$-untranslated region (3'UTR). Taken together, the results suggest that downregulation of Keap-1 in leukemic cells performing OXPHOS is mediated by the ERK5/MEF2/miR-23a signaling and that the resulting stabilization of Nrf2 leads to the activation of the Nrf2/ARE pathway, thus protecting the cells from the deleterious effects of ROS.

The findings by Khan et al. are in line with the previously reported ability of microRNAs to downregulate Keap-1 expression by targeting the $3^{\prime}$-UTR of its mRNA in several types of cancer cells (Eades et al., 2011; Kabaria et al., 2015; van Jaarsveld et al., 2013). Interestingly, MEF2A and MEF2C were shown to positively regulate the expression of different microRNAs, including miR-23a, in human vascular smooth muscle cells undergoing oxidative stress-induced senescence (Zhao et al., 2015) and in cardiac myocytes from mice with myotonic dystrophy (Kalsotra et al., 2014), respectively. Therefore, the ERK5/MEF2/miR-23a/Keap-1 axis may represent a key regulatory pathway in a broad range of cell types under various pathological conditions.

The importance of microRNAs in regulation of hematopoiesis and its aberrations has been known for several years [e.g., (Schotte et al., 2012)]. For instance Gocek et al. (2011) found that the upregulation of miR-32 by 1,25-dihydroxyvitamin $D_{3}$ in human myeloid leukemia cells leads to the targeting of the pro-apoptotic protein Bim, and inhibition of cytarabine-induced apoptosis, the latter frequently the result of excess generation of intracellular ROS. The report by Khan et al. demonstrates the important connection between microRNAs, Keap-1 and the alleviation of cellular oxidative stress. As such, it may indicate one basis for the emergence of resistance to cytotoxic chemotherapy of human neoplastic diseases. The novel, ERK5/ microRNA-dependent, mode of Keap-1 downregulation suggested by this study adds to the reported mechanisms related to Keap-1 promoter hypermethylation and inactivating mutations that lead to hyperactivation of Nrf2/ARE, and thus to tumorigenesis and chemoresistance [e.g., (Zhang et al., 2010)]. In this scenario, antisense oligonucleotides which block miR-23a expression can be developed as therapeutic agents to fight consequences of the loss of function of Keap-1.

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## L'homeostasie Redox Cellulaire

Les cellules en prolifération ont besoin d'énergie et la phosphorylation oxydative (OXPHOS) est la voie métabolique générant la plus grande quantité d'énergie par gramme de glucose à travers l'oxydation de l'oxygène au niveau mitochondrial. Mais ce processus en contrepartie génère de nombreuses espèces oxygénées réactives (EOR) qui peuvent endommager les protéines, les lipides et l'ADN. En réponse, les cellules activent leur système de défense antioxydante qui permet de façon directe ou indirecte et à travers des réactions enzymatiques complexes de neutraliser les EOR. En condition de stress un certain nombre de gènes cytoprotecteurs sont activés ce qui génère une réponse antioxydante stable dans la durée.

La voie de signalisation Keap1-Nrf2 est le principal régulateur des mécanismes de défense antioxydante au niveau cellulaire. Keap1 est une protéine riche en résidus cystéine et a un rôle inhibiteur sur Nrf2 (ou Nuclear Factor Erythroid 2 Related Factor 2). Nrf2 est aussi appelé NFE2L2 et son expression est principalement régulée au niveau post-traductionnel.

En conditions normales, Nrf2 est séquestré dans le cytoplasme et est constamment dégradé grâce à un système ubiquitine dépendant pour lequel Keap1 sert de régulateur. L'exposition au stress oxydant de Keapl modifie les résidus cystéines de ce dernier ce qui empêche sa liaison à Nrf2 et aboutit à une impossibilité de recruter le complexe d'ubiquitination associé à Keap1 stabilisant ainsi les protéines Nrf2 libres et permettant leur migration vers le noyau. Dans le noyau Nrf2 forme un hétérodimère avec la protéine MAF et ce complexe se lie sur les séquences élément de réponse aux antioxydants (ERA) présentes dans les séquences promotrices de nombreux gènes. Ceci induit l'initiation de la transcription de gènes impliqués dans la réponse antioxydante. Certaines protéines comme p21 et p65 peuvent aussi perturber l'interaction entre Keap1 et Nrf2 aboutissant à sa translocation nucléaire.

Nrf2 régule l'expression d'approximativement 250 gènes qui sont impliqués directement ou indirectement dans une grande variété de fonctions biologiques. De part son rôle cytoprotecteur dans les mécanismes de défense cellulaire, Nrf2 est considéré comme un suppresseur de tumeur par contre en cas de mutation perte de fonction de Keaplou de mutations gain de fonction de Nrf2, ce dernier devient hyperactif ce qui induit la croissance cellulaire, la survie cellulaire et l'échappement au système immunitaire plus particulièrement en cas de cancer. De plus l'hyperactivation de Nrf2 conduit à l'augmentation de l'expression de gènes impliqués dans le métabolisme des xénobiotiques et par conséquent confère une
résistance aux agents de chimiothérapie. En parallèle cela induit une reprogrammation métabolique vers l'anabolisme ce qui favorise la croissance tumorale.

Ainsi Nrf2 présente à la fois les caractéristiques d'un suppresseur de tumeur et/ou d'un oncogène. Des inhibiteurs spécifiques et efficaces de Nrf2 ne sont pas encore disponibles sur le marché et compte tenu des deux aspects opposés de son rôle l'utilité d'inhiber Nrf2 dépendra du contexte moléculaire et clinique, de plus il sera difficile de définir la frontière entre les effets bénéfiques d'une telle thérapie et les potentiels effets secondaires liés à l'inhibition des mécanismes antioxydants.

## 5. MicroRNA

MicroRNAs (miRNAs) are small non-messenger or non-coding RNAs found in animals, plants and some viruses. They usually comprise of 18-25 nucleotides produced from hairpin shape precursors (Ambros, 2004; Bertoli, Cava, \& Castiglioni, 2015). The function of miRNA was first discovered in 1993 when it was observed that lin-4, a gene in C. elegans responsible for larval development, produce a small RNA repressing the lin-14 gene function (R. C. Lee, Feinbaum, \& Ambros, 1993; Wightman, Ha, \& Ruvkun, 1993). This small RNA, which causes translational repression of lin-14 gene function, is the founding member of a group of tiny RNAs called microRNAs or miRNAs (Bartel, 2004). They repress the production of target protein by acting post-transcriptionally on messenger RNA (mRNA) and control the gene activity as post-transcriptional regulators (Ambros, 2011). They do not translate into proteins and regulate a variety of biological process including; metabolism, immunity, cell proliferation, apoptosis and many others (Bartel, 2004). About 30\% of total human genome is controlled by miRNAs and till now more than 28000 miRNAs have been identified in human, mammals and plants and half of them are in the form of cluster. Majority of miRNAs are located within the cell, some miRNAs, commonly known as circulating miRNAs or extracellular miRNAs, have also been found in extracellular environment, including various biological fluids and cell culture media (N. C. Lau, Lim, Weinstein, \& Bartel, 2001; Lewis, Burge, \& Bartel, 2005; Sohel, 2016).

The miRNAs are transcribed from individual genes containing their own promoter as a primary-miRNAs (pri-miRNAs) in nucleus by RNA polymerase II (pol II) or polymerase III (pol III). The biogenesis of pre-miRNA (with a $22-\mathrm{bp}$ stem) in the nucleus from pri-miRNA involves cleavage of the hairpin structure by the microprocessor protein complex consisting of Ribonuclease III enzyme DROSHA and DGCR8 (Czech \& Hannon, 2011; Gregory \& Shiekhattar, 2005; Sullivan, Leong, \& Fehniger, 2013). The pre-miRNA is exported to the cytoplasm by Exportin-5 for further cleavage where another RNase III enzyme, DICER, cleaves pre-miRNA to remove the loop and yields an 18-25-nucleotides miRNA duplex. Following unwinding, one strand is degraded and other strand of miRNA acts as the functional guide strand for binding to the target mRNA (Fig. 22) (Bartel, 2004; Y. Huang et al., 2010).


Fig. 22. Pathways of miRNA editing. Adopted from (Cai, Yu, Hu, \& Yu, 2009).
The segment of the primary transcript (pri-miRNA) contains the mature miRNA sequence (blue) that resides in one of the arms in the stem-loop precursor structure. Editing (red dot) starts at the pri-miRNA stage, and the edited pri-miRNAs may not be processed into precursor miRNA (pre-miRNA). The canonical biogenesis pathway of miRNAs (black arrows; the excised RNA fragments during miRNA biogenesis are indicated with dashed arrows) and the possible miRNA editing events (orange arrows) both happen in the cytosol where pre-miRNA may be subject to further editing events, resulting in the identification of different mRNA target (mRNA').

### 5.1. Mechanism of Action

The miRNA-mRNA targeting occurs predominantly at the 3 'UTR of the target mRNA where target sequence of miRNA is located. Pairing of miRNA and mRNA (mostly in plant cells) results in miRISC-mediated endonucleolytic cleavage leading to mRNA degradation. In animal cell, complete complementarity base pairing is lacking but still adequate enough to repress mRNA translation (Bartel, 2004).

Two posttranscriptional mechanisms involved in gene regulation are demonstrated (a) mRNA cleavage and degradation (b) Translational repression (Y. Huang et al., 2010). The seed region is 2-8 nucleotide segment of miRNA matching to its complementary sequence within the 3 'UTR of mRNA. The stabilization of the duplex depends on the presence of complementarity between half of the miRNA and 3'UTR of the target mRNA (W. Sun, Julie Li, Huang, Shyy, \& Chien, 2010). Several lines of evidence indicate that miRNAs can also bind to other regions in the target mRNA (Lytle, Yario, \& Steitz, 2007).

The translational repression occurs during initiation of translation (miRISCs compete with elF4E for binding to the mRNA 5' cap structure), formation of ribosome assembly (preventing 60S ribosomal subunit to join the 40S subunit), post initiation steps (miRISCs prevent the mRNA from circularizing) and termination of translation process. Depending on the degree of homology to the 3 'UTR target sequence, miRNAs can induce the translational repression or degradation of mRNAs (Bertoli et al., 2015; W. Sun et al., 2010; Wahid, Shehzad, Khan, \& Kim, 2010).

Several computational as well as biochemical approaches are used to identify the transcriptionally active promoter of miRNA. Epigenetic changes like trimethylation of Lys 4 of histone 3 ( H 3 K 4 me 3 ) is used to identify the transcription start site of active promoters in human \& mouse genome (Marson et al., 2008). The transcription of miRNA is stimulated by numerous soluble factors e.g Transforming Growth Factor- $\beta$ (TGF- $\beta$ ) and Platelet-Derived Growth Factor (PDGF). Epigenetic changes like DNA methylation and histone modification, acetylation and deacetylation controls miRNA promoter, e.g the use of HDAC inhibitor suppresses miR-27a leading to up-regulation of its target genes (Scott, Mattie, Berger, Benz, \& Benz, 2006). The study of transcriptional factors (TF) to regulate miRNA promoters is very limited in normal cells and most studies were done in tumor cells to understand TF regulation of miRNA promoter (W. Sun et al., 2010). The transcription factor induces transcription of miRNA cluster results in modulation of the function of various targeted proteins. One such example is MEF2, which regulates transcription of miR-23a cluster promoting antioxidant response via targeting KEAP1 gene (Davis-Dusenbery \& Hata, 2010; Khan et al., 2016).

### 5.2. Role of miRNAs in health and disease

The miRNAs have significant roles in physical health and pathophysiological progressions in diseases. In a biological system, coordinating networks of genes regulate organ development, immune response, metabolism, homeostasis and pathological conditions. The miRNAs govern these genetic networks either by positive or negative feedback loop. Various functions of identified miRNAs by computational and bioinformatics studies are yet to be investigated (Bartel, 2004; Bertoli et al., 2015; Khan et al., 2016; Slack, 2011).

The miRNAs are attaining growing focus in cell proliferation, differentiation and development of tissues and organs since first discovery in C. elegans. The essential role in embryogenesis appeared as first evidence (Bernstein et al., 2003). The embryonic stem cells
(ESC) isolated from mice deficient in Dicer showed poor differentiation with G1 cell cycle arrest rendering miRNA involvement in pleuripotency (Kanellopoulou \& Monticelli, 2008). Latter on it was observed that the master regulators of stem cell pleuripotency like OCT-4, NANOG and TCF3 are post-transcriptionally regulated by miRNA (C. Liu \& Tang, 2011). Several studies have shown the regulatory effects of miRNA in maturation \& differentiation of muscles and neurons (W. Sun et al., 2010). A cluster of miRNAs is upregulated in brain like; miR-107 is reported to target $\beta$-site Amyloid Precursor Protein Cleaving Enzyme 1(BACE1) initiating Alzheimer disease. Similarly, in heart failure, more than 12 miRNAs are found to be deregulated (Almeida, Reis, \& Calin, 2011). Upregulation of miR-181 expression in T-cells raises sensitivity to antigen showing role of miRNAs in immunity (Kanellopoulou \& Monticelli, 2008). The deletion of miR-1 is associated with cellular hyperplasia (sign of heart enlargement) and knocking down of miR-133 causes dilated cardiomyopathy in mice, whereas miR-21 protects cardiomyocytes from oxidative stress. The similar protective function is described for miR-221 and miR-222 (Cheng et al., 2009; W. Sun et al., 2010).

The miRNA profile can be helpful in disease diagnosis as cancer diagnosis and prognosis has been demonstrated using miRNAs as biomarker (Almeida et al., 2011; Bertoli et al., 2015).

### 5.3. Role of miRNA in Cancer

Dysregulation of microRNAs is involved in the initiation and progression of several human cancers (Bertoli et al., 2015). The transformation of normal cells into tumor cells resulting in uncontrolled proliferation and abnormal gene expression is a complex process and yet to be fully explored. Gene expression can be dysregulated by miRNAs leading to tumor development since each miRNA can modify the control of its target genes. If one of the target genes is an oncogene, the cancer does not develop (oncosuppressor-miRs) while if the target gene is a tumor suppressor, the cancer most likely can develop (oncomiRs). Similarly due to deletion, each miRNA can reduce the control over its target gene. If the target gene is an oncogene, the cancer develops (oncomiRs); if the target gene is a tumor suppressor, the cancer does not develop (oncosuppressor-miRs) (Bertoli et al., 2015). Modification (like deletion, mutation or epigenetic changes such as DNA methylation, histone modification) in cancer related transcription factors can results in cancer progression hence; miRNAs can indirectly contribute in tumor onset. For example, in prostate cancer, miR-101 is found to regulate histone methyltransferase EZH2 (Lujambio \& Lowe, 2012).

Significant evidence have established that miRNAs can act as oncogenes or tumor suppressor genes and defects in miRNA biogenesis pathways could alter miRNA expression levels making cells suitable for oncogenic changes (Fig-23) (Bertoli et al., 2015). The first evidence for the involvement of miRNA in cancer appeared with the discovery of miRNA. The loss of function of lin-4 (first identified miRNA) results in abnormal development and differentiation of cell lineage indicative of tumor like characteristics (R. C. Lee et al., 1993). In B-cell chronic lymphocytic leukemia (BCLL) deletion in chromosomal region was observed where presence of tumor suppressor genes was assumed. Latter on presence of two tumor suppressing miRNA, miR-15 and miR-16, was observed in this region, which are either deleted or down regulated in CLL patients (Calin et al., 2002).


Fig. 23. Altered steps in miRNA biogenesis lead to cancer. Adopted from (Bertoli et al., 2015).

A schematic representation of altered steps of the miRNA biogenesis pathway commonly deregulated in cancer: 1. miRNA genes contain upstream regulator elements (enhancers/repressors) and promoter regions, indicating that miRNAs are subjected to CpG methylation (CpG promoter met); 2. The alteration in the copy number of miRNA (due to genomic amplification or deletion, activating or repressing mutation, loss of epigenetic silencing and transcriptional activation) could increase the oncogenic miRNAs or decrease the tumor suppressor miRNAs; 3. Alteration in the miRNA processing machinery, i.e. down regulation of DROSHA, could decrease the cropping of pri-miR to pre-miR; 4. XPO5 mutation could prevent pre-miR export to the cytoplasm; 5. Mutation of TARBP2 or downregulation of DICER1 decreases mature miRNA levels, causing finally a loss of tumor
suppressor miRNAs; 6 and 7. Accumulation of oncogenic miRNAs or loss of tumor suppressor miRNAs could finally lead to cancer development.

The expression of miRNA is related to tumor origin thus accurate miRNA profiling could be a powerful tool for classification of poorly differentiated tumors by flow cytometry (Hwang \& Mendell, 2006). Tumor cells like stem cells undergo continuous division without enduring senescence hence share a similar miRNA profiling in order to maintain excessive growth. Several reports highlight the role of cancer stem cell (CSC) in genesis of certain types of cancers and miRNAs involvement in regulating CSC. First reported CSCs in solid tumors were breast cancer cells and let-7 which is important for regulating stem cells self-renewal and differentiation was observed down regulated in breast CSC (C. Liu \& Tang, 2011). Another miRNA down regulated in breast cancer SCs is miR-30 and its up regulation reduces self-renewal and increases apoptosis (F. Yu et al., 2007; J. Yu \& Auwerx, 2010).

Patients with reduced DROSHA and DICER have poor clinic prognosis suggests that biogenesis of miRNAs and oncogenesis are critical and closely related (Merritt et al., 2008). Any alteration in DICER during miRNA biogenesis results in disorganized heterochromatin formation and pericentromeric silencing impairing its functionality leading to tumor onset (Kanellopoulou \& Monticelli, 2008). Cancer cells bearing (sh) RNA of DICER and DROSHA show decrease miRNA levels and hasten proliferation and cellular transformation reflecting the association of miRNA-processing machinery and tumor development. In lung cancer single mutation in DICER1 allele fosters KRAS-driven carcinoma while complete extirpation proves more lethal (Kumar, Lu, Mercer, Golub, \& Jacks, 2007).

Under homeostatic physiological conditions miRNAs act as a buffer and regulates various processes either positively or negatively thus depending on the cellular context and microenvironment they can play a role as tumor suppressors or in oncogenesis (Table 9). For example, miR-29 acts as oncogene in breast cancer and tumor suppressor in lung cancers (Lujambio \& Lowe, 2012). The overexpression of miR-17 accompanied with c-MYC in human B cell lymphomas was the first evidence of miRNAs as oncogenes (He et al., 2005). The $c-M Y C$ gene was also associated to repress certain miRNA possessing properties like proapoptotic and antiproliferative resulting in tumorogenisis (Lujambio \& Lowe, 2012). Another example is miR-21 retaining anti-apoptotic properties and its overexpression results in B cell lymphoma in mice while its inhibition represses tumor growth (Medina, Nolde, \& Slack, 2010). Certain miRNAs also play a critical role in tumor metastasis like overexpression of
miR-10 results in tumor invasion and migration in vivo by repressing the HOXD10 (Ma, Teruya-Feldstein, \& Weinberg, 2007). Similarly miR-373 and miR-520c stimulate invasion and migration of breast tumor cells (Q. Huang et al., 2008).

Table 9: miRNA as oncogene or tumor suppressor. Adapted from (Lujambio \& Lowe, 2012).

| MiRNA | Function | Target gene | Mechanism | Expression in Patients |
| :---: | :---: | :---: | :---: | :---: |
| miR-15 and miR 16 | Tumor suppressor | BCL-2, MCL1 | Genomic loss, Mutation | CLL, prostate cancer |
| let $7 \mathrm{la}-2$ | Tumor suppressor | Myc, KRAS, CDK6 | Negetive regulation of Myc | Down in lung, colon; breast cancers |
| miR 34 family | Tumor suppressor | CDK4, CDK6, Mtc, MET, CREB | Epigenetic silencing, deletion, positive regulation by Myc | Down in hang, colon; kidney cancers, melonoma cell lines, neuroblastome |
| miR-26a | Tumor suppressor | CCND2, CCNE2 | Negetive regulation by Myc | Down in liver cancers |
| miR-29 family | Tumor suppressor | CDK6, MCL1, DNMT1, DNMT3 | Genomic loss, Negetive regulation by Myc | Down in CLL, AML, lymphoma, hepatocarcinoma, lung and breast cancers |
| miR-29 family | Oncogene | 2FP36 | Transcriptional activation | Breast cancer and indolent CLL |
| miR 155 | Oncogene | SHIP1, CEBPB | Positive regulation by NF-kB | Up in high risk AML, CLL, lymphomas |
| miR-17-92 | Oncogene | BIM, PTEN, CDKN1A | Positive regulation by Myc and E2F | Up in hang, colon, prostate and stomach cancer, CLL, AML |
| miR-21 | Oncogene | PDCD4, TPM1, PTEN | Positive regulation by IL6 and GFla. | Up in CLL, AML, myeloma and gliobastoma |
| miR 372 and miR 373 | Oncogene | LATS2 | Unknown | Up in testicular germ cell tumors and breast cancer |

Besides oncogenic properties, some miRNAs possess anti-proliferative and pro-apoptotic characteristics. They target genes involved in oncogenesis, proliferation and differentiation. let-7 targets an oncogene $R A S$, is down regulated in lung cancer patients, while, its overexpression in lung adenocarcinoma cell line overwhelms tumor growth (Johnson et al., 2005; Takamizawa et al., 2004). On the other hand, in breast cancer miR-206 and miR-355
decrease cell motility by modifying cellular morphology while miR-126 expression reduces tumor growth (Almeida et al., 2011).

## 5.4. miRNA regulation of metabolism

Cellular metabolism is a dynamic process that involves complex pathways and networks. Cells need energy for synthesis of DNA, RNA and protein and cell metabolism provides energy in form of different metabolites for these proposes. The role of miRNAs as a posttranscriptional regulator in regulating metabolic networks is attaining attention (Lynn, 2009; Rottiers \& Näär, 2012) in several metabolic pathways like insulin release, cholesterol biosynthesis, lipid metabolism and amino acid catabolism (Table 10). First evidence arose with the observation of the role of miR-14 in fat metabolism (P. Xu, Vernooy, Guo, \& Hay, 2003). Recently miR27b was reported to inhibit LDLR and ABC1 expression playing role in lipid metabolism (Goedeke et al., 2015). Certain miRNAs (miR-9, miR-196, miR-124a) are also involved in regulation of insulin vesicle docking (Kolfschoten, Roggli, Nesca, \& Regazzi, 2009). Similarly, blocking of let-7 suppresses diabetes development in high fat diet (Zhu et al., 2011).

Table 10: miRNAs involved in metabolism and their target genes. Adapted from (Rottiers \& Näär, 2012).

| miRNA | Target gene | Targettissue |
| :---: | :---: | :---: |
| let 7 | IGF1R, IRS2, HMGA2 | Muscle, Adipose |
| miR-29 | INSIG1, CAV2, SLC16A1 | Muscle, Adipose, Liver |
| miR-223 | SLC2A4 (GLUT4) | Muscle |
| miR-143 | ERK5 | Adipose, Liver, Pancreas |
| miR 335 | STXBP1 | Adipose, Liver, Pancreas |
| miR-103 and miR-107 | CAV1, DICER | Adipose, Liver |
| miR27a | PPARG | Adipose |
| mir 130 | PPARG | Adipose |
| miR-378 and miR 378 * | GABPA, ESRRG | Adipose |
| miR-9 | Sirt1 | Pancreas |
| miR 375 | USP1, MTPN, JAK2, PDPK1 | Pancreas |
| miR-124a | FOXA2, RAB27A | Pancreas |
| miR-34a | Sirt1, VAMP2, ACSL1 | Liver, Pancreas |
| miR-122 | SLC7A1, ADAM17 | Liver |
| miR-33a and miR 33 b | ABCA1, NPC1, CPT1A, CROT, IRS2 | Liver |

### 5.5. Cluster of miR-23a~miR-27a~miR-24-2

Most of miRNAs are found in clusters and cluster of miR23a is located on chromosome 9q22. All members have individual as well as combined effect. Despite same origin, the expression pattern is diverse under different conditions e.g overexpression of this cluster in HEK293T cells showed increase in miR-27a and miR-24-2 while miR-23a expression was unaffected (R. Chhabra, Adlakha, Hariharan, Scaria, \& Saini, 2009). The paralog miR-23b-27b-24-1 is located on chromosome 19 p13 signifying diverse transcriptional regulation. Surprisingly miR-24-1 and miR-24-2 are similar while only a single nucleotide is different for miR-23a
and miR-27a from its paralog (Fig. 24) (Ravindresh Chhabra, Dubey, \& Saini, 2010). Related homologous clusters display strikingly similar evolutionary expression and functional relationships between them (Liang, Yu, Liu, \& Guo, 2014).

Various physiological as well as pathological conditions are associated with miR23a cluster. This cluster regulates multiple aspects of T cell biology and low expression of this family confers proper effector T cell function at both physiological and pathological conditions (L.F. Lu et al., 2016). Overexpression of this cluster was observed in hepatic carcinomas and lymphocytic leukemia and its non activity can leads to cancer progression (Ravindresh Chhabra et al., 2010). Overexpression of this cluster results in growth promotion in hepatocellular carcinoma cells by avoiding TGF- $\beta$ induced tumor suppression and repressing glucose-6-phosphate (G6P) along with peroxisome proliferators activated receptor gamma coavtivator 1 alpha (PGC1 $\alpha$ ) (B. Wang, Hsu, Frankel, Ghoshal, \& Jacob, 2012). Knock-down of miR-23 cluster in fetal and new born liver promotes bile duct differentiation and block TGF- $\beta$ induced liver fibrosis (Rogler, Matarlo, Kosmyna, Fulop, \& Rogler, 2016). This cluster also regulates physiological functions of osteogenesis by targeting SATB2, an osteogenesis activator. Contrarily, RUNX2 activates osteogenesis by suppressing this cluster while, by a feedback mechanism miR23 cluster targets RUNX2 at the terminal stage to maintain specific phenotype (Hassan et al., 2010).


```
Human UGGCUCAGUUCAGCAGGNACAG-22
Mouse UGGCUCAGUUCAGCAGGAACAG- 22
RAT UGGCUCAGUUCAGCAGGAACAG- }2
Horse UGGCUCAGUUCAGCAGGAACAG- 22
Dog UGGCUCAGUUCAGCAGGAACAGG 23
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Fig.24. miR-23 cluster. Adopted from (Ravindresh Chhabra et al., 2010).
Mature sequence of members of cluster in five different species. The structure of pre-miR transcript is visible in detail. The TSS is from 0 to 124 bp and followed by upstream promoter region ( -603 to +36 bp ), which contains a GC rich region. The end of transcript has a poly A tail (1752-1757 bp).

### 5.5.1. miR-23a

Oncogenes like $c$-MYC and PML-RARA suppress miR-23a for tumor progression. PML-RARA repress whole cluster and is associated with acute promyelocytic leukemia (APL) (Saumet et al., 2009), whereas, c-MYC represses miR-23a and miR-23b resulting in up regulation of their target i.e. glutaminase (GLS) to increasing glutamine catabolism (Gao et al., 2009). Overexpression of miR-23a in leukemic cells impaired glutamine usage and induces mitochondrial dysfunction leading to cell death (Moeez G Rathore et al., 2012). Increased miR-23 level was also associated with the severity of coronary artery disease in patients ( Di , Zhang, $\mathrm{Hu}, \& \mathrm{Li}$, 2015) and upregulation of miR-23a was observed in cardiac hypertrophy due to repression of muscle specific ring finger protein 1 (MuRF1) (Z. Lin et al., 2009). Interestingly, MAFbx/atrogin-1 atrophy related protein is repressed by miR-23a in skeletal muscle cells showing the diverse role of miR23 (Ravindresh Chhabra et al., 2010). In endothelial cells (ECs) overexpressed miR-23a regulates caspases-7 and STK-4-caspase-3 pathways activating tumor necrosis factor- $\alpha$ (TNF- $\alpha$ ) induced ECs apoptosis (Ruan, $\mathrm{Xu}, \mathrm{Li}$, Yuan, \& Dai, 2011), while it targets SPROUTY2 and SEMA6A and promote angiogenesis
(Q. Zhou et al., 2011). Metastasis suppressor 1 (MTTS1) is also a target of miR-23a rendering metastasis of colorectal cancer (Jahid et al., 2012). Expression of miR-23a was shown to be positively regulated by MEF2A and MEF2C in human vascular smooth muscle cells undergoing oxidative stress-induced senescence (W. Zhao, Zheng, Peng, \& Zhao, 2015) or in hematopietic cells (Moeez G Rathore et al., 2012). Recently we have demonstrated that miR23a targets KEAP1 thus triggering NRF2 antioxidant activity in human leukemic cells (Khan et al., 2016). MiR-23 also controls cathepsinc C expression aprotein essential for cytotoxic lymphocytes (Sanchez-Martínez et al., 2014).

### 5.5.2. miR-27a

This member is also involved in the development of cancers like breast, renal and gastric carcinoma (Ravindresh Chhabra et al., 2010; S. Zhou et al., 2016). Excessive miR-27 expression in T cells severely impairs regulatory T (Treg) cell development and functional effects (Cruz et al., 2016). In breast cancer development this miRNA coordinates with miR196 and miR-182. All three miRNAs are upregulated in MCF-7 breast cancer preventing apoptotic response due to down regulation of FOXO-1 protein (Guttilla \& White, 2009). This miRNA along with its paralog also play a critical role in lipid metabolism and targets PPAR $\gamma$ and $\mathrm{C} / \mathrm{EBP} \alpha$ hence effeting adipogensis (Sacco \& Adeli, 2012). Critical role in cell cycle regulation was also observed by miR-27a thus contributing tumor progression (Ravindresh Chhabra et al., 2010). Sensitivity of breast cancer cells to cisplatin treatment was also associated by miR-27a expression while this miRNA is involved in resistance to treatment in mutant lung cancer (Montes, Reyes, Sharma, \& Huang, 2016; S. Zhou et al., 2016).

### 5.5.3. miR-24-2

Both miR-24-2 and its paralog miR-24-1 share many biological functions. Upregulation of miR-24 in squamous carcinomas targets tumor suppressor genes thus favoring tumor growth (S.-C. Lin et al., 2010) and its overexpression was reported in early breast cancer along with other oncomiRs while circulation level of this is considered as a biomarker in non-small cell in lung cancer (Franchina et al., 2014; Sochor et al., 2014). This miRNA targets CHEK1 gene (involve in G2-M checkpoint), VHL (tumor suppressor gene), cyclin D inhibitor (CDKN1B), c-MYC and E2F therefor supporting tumor growth (Ravindresh Chhabra et al., 2010; Lal et al., 2009). Hyperglycemia-induced repression of miR-24 increases the risk of thrombotic
cardiovascular events (Xiang et al., 2015). Tumor evasion and metastasis is also associated with miRNA 24 (Du et al., 2013).

## Les microARNs (miARNs)

Les microARNs (miARNs) sont de petits ARN non codants qui possèdent entre 18 et 22 nucléotides. Les miARNs sont des régulateurs important du développent physiologique de divers espèces par leur liaison à l'ARN messager (ARNm) du gène cible qui conduit à la répression traductionnelle ou à la dégradation de cet ARNm. La plupart des miRNAs sont situés dans la cellule. Certains type de miRNAs sont trouvés dans l'espace extracellulaire (liquides biologiques et milieux de culture) appelés miARNs circulants. Environ $30 \%$ du génome humain est contrôlé par les miARNs et jusqu'à présent plus de 28000 miARNs ont été identifiés chez les humains, les mammifères et les plantes.

Les gènes de miARN sont transcrits sous la forme de longs précurseurs nommés «primiARN». Ces précurseurs sont clivés dans le noyau pour former un pré-miARN. Le prémiARN est transporté vers le noyau puis clivé pour produire un miARN mature et fonctionnel. L'hybridation des miARNs à leur ARNm cible concerne principalement une région plus restreinte du miARN s'étendant sur 2 à 8 nucléotides. Le site d'hybridation se trouve dans la région 3'UTR de l'ARNm.

Les miRNAs jouent un rôle important dans la santé physique et pour établir de nouveaux mécanismes physiopathologiques et de nouvelles cibles thérapeutiques. Ils jouent aussi un rôle critique dans divers fonctions biologiques allons du métabolisme au développement des cancers. Leur profil peut être utile dans le pronostique et le diagnostique des cancers via l'utilisation des miARNs comme biomarqueurs. Le profilage des miARNs dans le plasma des patients est utile pour étudier la progression de la maladie et la réponse au traitement.

Certains miARN sont impliqués dans la progression du cancer (oncomiRs), tandis que d'autres jouent un rôle clé dans la suppression tumorale (oncosuppressor-miRs). Des défauts dans la biosynthèse du miARN peuvent conduire à des effets délétères sur divers processus biologiques.

Il a été démontré que plusieurs fonctions biologiques ont été régulés par les miARNs (miR$23 \mathrm{a} \sim \mathrm{miR}-27 \mathrm{a} \sim \operatorname{miR}-24-2$ ) d'une manière groupé ou individuel. Dans la condition physiologique homéostatique, les miRNAs agissent comme un tampon et régulent divers processus soit positivement ou négativement. En fonction du contexte cellulaire et du microenvironnement, les miARNs peuvent jouer un rôle de suppresseurs de tumeurs ou dans l'oncogenèse. L'expression de miARN est également liée à l'origine de la tumeur, donc le
profilage précis des miARN pourrait être un outil puissant pour la classification des tumeurs peu différenciées. Diverses fonctions des miRNA identifiés par les études informatiques et bioinformatiques est en cours d'invistigation.

## 6. Low Density Lipoprotein Receptors

Cholesterol being an essential component of the animal plasma membrane functions as barrier between cells and environment and serves as precursor for manufacturing of all steroid hormones and finally also protects axons by providing a cushion. Cholesterol transportation occurs through lipoprotein ligands such as: chylomicron, low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL) or very low-density lipoprotein (VLDL) mediated by family of membrane receptors. Low-density lipoprotein (LDL) also called as bad cholesterol and high-density lipoprotein (HDL) termed as good lipids. Majority of heart attacks are due to elevated concentrations of LDL, while elevated HDL are protective and in familial hypercholesterolaemia (FH) patients excess circulating cholesterol is LDL, not HDL (G.-w. Go \& A. Mani, 2012; Goldstein \& Brown, 2009).

The Low-Density Lipoprotein Receptor (LDL-R) is a cell surface receptor ubiquitously expressed in mammals belonging to the low density lipoprotein receptor gene family (Goldstein \& Brown, 1974; Nykjaer \& Willnow, 2002). LDLR is involved in lipoproteins trafficking and mainly responsible for the endocytosis of cholesterol-rich Low-Density Lipoprotein (LDL) thus plays a critical role in LDL level in plasma. Other members of family are structurally and functionally connected to LDLR, which is the patriarch of the entire family. All members of the family share structurally common motifs in proteins as shown in Fig. 25 (G.-w. Go \& A. Mani, 2012; T. P. Leren, 2014). LDL receptors are translated by ribosomes on the endoplasmic reticulum and then before transferring to cell surface are further modified by the Golgi apparatus. These receptors are involved in endocytic process of lipoproteins that includes clustering of lipoprotein receptors in coated pits, transport to early and late endosomes followed by hydrolysis in lysosomes, release of the lipid to cytoplasm and finally recycling of receptors back to cell surface (G.-w. Go \& A. Mani, 2012). The gene of LDLR was identified by Michael S. Brown and Joseph L. Goldstein (Michael S Brown \& Goldstein, 1984) and were awarded Nobel Prize in Physiology or Medicine in 1985. The LDLR gene is present on chromosome 19 and produces 6 isoforms and its mutation results in inherited disease know as familial hypercholesterolaemia.

Familial Hypercholesterolemia (FH) is an autosomal dominant genetic disorder and approximately, $\sim 1$ in 500 individuals suffer from this worldwide (Liyanage, Burnett, Hooper, \& van Bockxmeer, 2011). Patients with heterozygotes FH have higher concentrations of plasma LDL cholesterol and they are at higher risk of atherosclerosis and coronary heart
disease. They have several fold elevated level of cholesterol in blood compared to normal concentration resulting in heart attacks early in life (Felson, 2001; Goldstein \& Brown, 2009). This build up concentrations in the inner walls of the arteries is oxidized and leads to foam cells formation, consequently blocking the vessels and initiating atherosclerosis (Gu \& Zhang, 2015).


Fig. 25. Low-density lipoprotein receptor family. Adopted from (G.-w. Go \& A. Mani, 2012).

LDLR is the patriarch of the LDLR family. Members of the LDLR family share common structural motifs: LDLR type A repeats (responsible for binding of ligands), epidermal growth factor (EGF)-like domain (involved in pH -dependent release of ligands in endosome), transmembrane anchor, and cytoplasmic domain (binding of NPxY and ARH mediates clustering of the receptors into clathrin coated pit). LDLR, VLDLR, and LRP8 (ApoER2) additionally contain o-link sugar domain outside the plasma membrane and NPxY motif in the cytoplasmic domain. LRP1 and LRP2 have relatively large extracellular domains. LRP5/6 has PPPSP motif in cytoplasmic domain.

Based on $L D L R$ mutations it can be classified into five various groups depending on the functional characteristics of the encoded proteins: 1) null alleles resulting in receptor synthesis-defect; 2) transport-defective alleles leading to defect in shipping receptor to cell surface; 3) binding-defective alleles responsible for encoding proteins which fail to bind ligands; 4) internalization-defective alleles that encode proteins that fail to interact with clathrin coated pit; and 5) recycling-defective alleles, which fail to dissociate in the acidic pH of lysosomes ruining recycling process (Hobbs, Russell, Brown, \& Goldstein, 1990). These
defect consequently impair LDLR function or expression and results in elevated LDL cholesterol leading towards development of major atherosclerotic diseases (G.-w. Go \& A. Mani, 2012).

### 6.1. Mechanism of Action

These receptors (LDLR) are mainly involved in transporting cholesterol-containing lipoprotein particles from the circulation into cells. Although this process prevail in all nucleated cells, the liver clears approximately $\sim 70 \%$ of LDL from the circulation (Goldstein \& Brown, 2009). These receptors attach to apo-protein B100 in the outer phospholipid layer of LDL particles and also have ability to recognize the apoE protein (Lindgren, Luskey, Russell, \& Francke, 1985). Other than these very low density lipoprotein (VLDL), Intermediate density lipoprotein (IDL), high-density lipoprotein (HDL) and chylomicron remnant are also recognizable by LDLR at neutral pH (Innerarity, Mahley, Weisgraber, \& Bersot, 1978; Innerarity \& Mahley, 1978). Recognition of apoB-100 of LDL particles occurs with a stoichiometry of a single copy of apoB-100 per one LDL particle per receptor monomer (Wiklund, Dyer, Tsao, \& Curtiss, 1985).

Following binding this complex undergo endocytosis. These receptors have clathrin-coated pits on their cytoplasmic end which pinch off from the surface on LDL binding to receptors in order to form coated endocytic vesicles carrying LDL and inside the cell this vesicle is fused to endosomes. This process also involves low density lipoprotein receptor related protein 6 (LRP6) and autosomal recessive hypercholesterolemia protein (ARH, also known as LDLR adaptor protein) (Goldstein \& Brown, 2009). Inside endosomes LDL is dissociated once exposed to low pH and released ligand particles further transported to the lysosome for enzymatic degradation. After detachment, the receptor folds back on it self and gained a closed conformation before recycling to the cell surface for further function (Fig. 26).

LDLR comprises of seven LDLR type A repeats in its binding domain, immediately followed by EGF-like modules, transmembrane anchor and NPxY-repeats, which contain cytoplasmic domain. Once a LDL particle is internalized it activates three processes: 1) gene expression of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) is reduced in order to suppress cholesterol biosynthesis; 2) activity of acyl-CoA cholesteryl acyl transferase (ACAT) to reduce toxic free cholesterol is enhanced; and finally 3) LDLR synthesis is repressed at transcriptional level via SREBPs to reduce LDL uptake (Beglova \& Blacklow, 2005; Michael

S Brown \& Goldstein, 1976; G.-w. Go \& A. Mani, 2012; Rudenko et al., 2002; Shimomura et al., 1997; Tamura \& Shimomura, 2005).


Fig. 26. LDLR pathway. Adopted from (Beglova \& Blacklow, 2005).

### 6.2. LDLR Regulation

The expression of LDLR is regulated at the transcriptional, post-transcriptional and posttranslational levels (Yuma Tanaka, Shimada, \& Nagaoka, 2014). Primarily, the expression of LDLR is regulated at the transcriptional level through a negative feedback mechanism with respect to the intracellular cholesterol pool, subsequently when cellular cholesterol increases, the production of LDL receptors is reduced (Michael S Brown \& Goldstein, 1975; Weijia Kong et al., 2004). Sterol regulatory element-binding proteins (SREBPs) are transcription factors involved indirectly in lipid homeostasis through regulating the LDLR expression at transcriptional level by directly binding at promoter of LDLR (Briggs, Yokoyama, Wang, Brown, \& Goldstein, 1993; Gu \& Zhang, 2015; Parraga, Bellsolell, Ferre-D'Amare, \& Burley, 1998). SREBs are essential in regulation of LDL receptors for the action of statin drugs in lowering plasma LDL-cholesterol levels. When the cholesterol level within cells is at normal range SREBP are present in endoplasmic reticulum. In the inactive state, SREBP associates with another transmembrane protein, SREBP-cleavage activating protein (SCAP) that
contains a cholesterol-sensing domain and provides conditional chaperone activity to the SREBP (Michael S Brown \& Goldstein, 1999; Nohturfft, DeBose-Boyd, Scheek, Goldstein, \& Brown, 1999; Nohturfft, Yabe, Goldstein, Brown, \& Espenshade, 2000). Cellular depletion of sterol results in activation of the SCAP-SREBP transportation to Golgi apparatus, where the N-terminal transcription activation domain of the SREBP is released from the precursor protein through specific cleavages leading to SREBP activation (Michael S Brown \& Goldstein, 1999; Goldstein, Rawson, \& Brown, 2002; T. Yang, Goldstein, \& Brown, 2000). The active form of the SREBP translocates to the nucleus where it binds to its cognate SRE-1 site and hence stimulates transcription of the $L D L R$ gene. While under cholesterol-replete conditions, the SCAP-SREBP complex remains in an inactive form in the ER through active repression by sterols and $L D L R$ gene transcription is maintained at a minimal constitutive level (Weijia Kong et al., 2004). The post-transcriptional regulation of LDLR mRNA is still not clear but one observed mechanism is the modulation of LDLR mRNA stability by a group of AU-rich element (ARE) binding proteins (J. Liu et al., 2007).

Certain amino acids are also involved in LDLR regulation, as glutamine is known to upregulate hepatic LDLR expression via the stimulation of SREBP processing (H. Li et al., 2009). Sulphur containing amino acids like L-cysteine were also observed to up-regulates the LDLR mRNA expression by activating the promoter via the activation of ERK and p38 MAPK signaling pathways by releasing the transforming growth factor alpha (TGF- $\alpha$ ) in hepatic cells (Yuma Tanaka et al., 2014).

Statins are lipid-lowering drugs and inhibit the enzyme HMG-CoA reductase in liver that plays a central role in the cholesterol synthesis. Inhibition of this enzyme lower the cholesterol level in the cell and consequently activates SREBP resulting in up-regulation of LDLR on cell surface to enhance cholesterol uptake from plasma. Hence, cholesterol in the liver is sustained at normal level by inhibiting its synthesis on one side while clearing blood cholesterol through increased LDLR (Michael S Brown \& Goldstein, 2004; Collaboration, 2007; Costet et al., 2006; P. C. J. L. Santos \& Pereira, 2015). Several statins are available in the market: atorvastatin, fluvastatin, lovastatin, rosuvastatin, pitavastatin, pravastatin and simvastatin (Sweetman, 2009). Atorvastatin is the best-selling statin.

LDLR turnover is also regulated by a proprotein convertase subtilisin/kexin type 9 (PCSK9), a serine protease (Attie \& Seidah, 2005), which targets these receptors for lysosomal degradation inhibiting their recycling to the cell surface (Fig. 27) (Zaid et al., 2008). PCSK9
is mainly synthesized in liver and quickly secreted into plasma after its maturation in the endoplasmic reticulum and regulates LDLR at posttranslational level (Gu \& Zhang, 2015; Seidah et al., 2003). Interestingly, PCSK9 gene expression is also positively regulated by SREBP through an SRE motif of the proximal promoter in reaction to low level of intracellular sterols (Dong, Li, Singh, Cao, \& Liu, 2015). PCSK9 dependent LDLR degradation is primarily through interaction with the receptor on the cell surface and does not involve the proteolytic activity (McNutt et al., 2009; McNutt, Lagace, \& Horton, 2007). The exact mechanism by which binding of PCSK9 to the LDLR directs the receptor to lysosomes for degradation is still not elucidated and believed to be complex (Gu \& Zhang, 2015). Plasma levels of this protein directly influence the level of circulating LDL-cholesterol (Lambert et al., 2008). LDLR degradation through PCSK9 is dose and incubation dependent both intracellularly and extracellularly which is also cell type specific (Poirier et al., 2009), since it did not disturb LDLR expression in Chinese hamster ovarian cells (CHO-K1), monkey kidney cells (COS-7) and rat liver cells while in human hepatoma cells (HepG2 and HuH7) noticeable reduction of LDLR level was observed (Lagace et al., 2006; Maxwell \& Breslow, 2005; Park, Moon, \& Horton, 2004). PCSK9 mediated LDLR degradation is inhibited by annexin A2 that interact the C-terminal cysteine- and histidine-rich domain of PCSK9 subsequently disturbing its function. Higher expression of annexin 2 in COS-7 cells may explain the impair efficiency of PCSK9 function (Mayer, Poirier, \& Seidah, 2008; Nguyen, Kosenko, \& Lagace, 2014).

It is demonstrated that gain-of-function mutations of PCSK9 leads to elevated plasma LDL-C consequently in accelerated atherosclerosis resulting in premature coronary heart disease. While, loss-of-function mutations lead to low concentrations of LDL-C consequently protecting from coronary heart disease. Thus it is very much clear in several genetic studies that PCSK9 plays a chief monitoring part in cholesterol homeostasis (Abifadel et al., 2009; Abifadel et al., 2003; Cohen, Boerwinkle, Mosley Jr, \& Hobbs, 2006; T. Leren, 2004; Seidah, Khatib, \& Prat, 2006). Inhibition of PCSK9 dramatically reduces LDL-Cholesterol levels in plasma in a phase II trial (Cannon et al., 2015; Robinson et al., 2015). Interestingly, PCSK9 clearance is mediated by LDLR and $L d l r$ knock out mice express markedly increased plasma level of PCSK9 showing a feed back mechanism (Melroy X Miranda et al., 2015; Tavori et al., 2013).


Fig. 27. PCSK9 mediated LDLR degradation pathway. Adopted from (Gu \& Zhang, 2015). (1) The catalytic domain and prodomain of PCSK9 bind to EGF-A and YWTD repeats of the LDLR, respectively. (2) PCSK9-LDLR complex enters into cells via clathrin-dependent endocytosis and is delivered to the endosome. (3) PCSK9 strongly binds to the LDLR at the acidic endosomal environment, which blocks recycling of the LDLR to the cell surface. (4) PCSK9-LDLR complex is transported to the lysosome for degradation.

Berberine (BBR) is an alkaloid extracted from the Chinese herb Coptis chinensis, widely used for the treatment of gastrointestinal infections was also observed to have cholesterol-lowering effect (Weijia Kong et al., 2004). Initially, it was observed that BBR regulates cholesterol through up-regulating expression of hepatic LDLR by stabilizing mRNA level involving an extracellular regulated kinase (ERK), a mechanism distinct from statins. The up-regulation of LDLR by BBR was independent of intracellular cholesterol levels suggesting that SREBPs were not involved in the actions of BBR. But sooner it was revealed that BBR also inhibit cholesterol and triglycerides (TG) synthesis through a mechanism involving activation of AMPK (P. Abidi, Y. Zhou, J.-D. Jiang, \& J. Liu, 2005; J.-M. Brusq et al., 2006; Weijia Kong et al., 2004). Recently it has been demonstrated that BBR regulates LDLR expression through
another mechanism since it reduces PCSK9 transcription via down-regulation of hepatic HNF1 alpha protein expression inducing its proteasomal degradation and consequently inhibiting LDLR degradation (Dong et al., 2015). Thus three distict mechanism are involved in BBR mediated cholesterol suppression.

Sirtuin1 (SIRT1) has also been associated with LDLR regulation because clinical studies with administration of SIRT1 activators outcome in decline of total cholesterol and LDLcholesterol plasma levels (Libri et al., 2012; Venkatasubramanian et al., 2013). In mice pharmacological activation of SIRT1 results in stimulation of LDLR through reduction of PCSK9 developing in atheroprotection. When mice were fed SRT3025 (Sirtuin1 activator) hepatic release of PCSK9 was attenuated preventing LDLR degradation and hence enhancing LDL-cholesterol clearance (Melroy X Miranda et al., 2015).

### 6.3. Clinical Significance

Heterozygous FH is the most common monogenic disorder of lipoprotein metabolism disease affecting one in every 500 individual worldwide and is due to genetic mutation in $L D L R$ gene (Liyanage et al., 2011). Till now more than 1600 mutations in the LDLR gene have been identified (Schaefer, Kurt, Sattler, Klaus, \& Soufi, 2012). The importance of the disease can be identified that in the USA alone it is estimated that more than 640,000 people are suffering from heterozygous FH and are at higher risk of heart attack despite of early diagnosis (P. C. J. L. Santos \& Pereira, 2015). It is observed that about 50 percent of heterozygote FH patients acquire various forms of cardiovascular disease during the fourth or fifth decades of their life span (Goldstein \& Brown, 1973).

Atherosclerosis and other cardiovascular diseases are the foremost causes of mortality and morbidity throughout the world. LDL-Receptors have a vital role in blood lipid profile playing a critical role in the regulation of plasma LDL-cholesterol thus a key determinant of many cardiovascular disorders like atherogenesis since plasma levels of cholesterol especially LDL-Cholesterol are directly correlated with the risk of atherosclerosis and almost 65-70\% of plasma cholesterol is transported by LDL in humans (Goldstein \& Brown, 2009; Gu \& Zhang, 2015; Melroy X Miranda et al., 2015; Osono, Woollett, Herz, \& Dietschy, 1995; F. J. Raal et al., 1999; Shuldiner \& Pollin, 2010). Augmented LDLR expression can improve the clearance of plasma LDL cholesterol through receptor-mediated endocytosis, which is strongly related to reduce risk of developing cardiovascular disease (Ansell, Watson, \& Fogelman, 1999;

Grundy, 1998). Blockage of arteries feeding the heart causes coronary artery disease while in the arteries feeding the brain can results in stroke (Michael S. Brown \& Goldstein, 2006; Goldstein \& Brown, 2009). LDLR genes mutation at loci functionally related to APOA5, i-e lipoprotein lipase and apolipoprotein C-III has also been associated with myocardial infarction risk in population leading to higher plasma LDL cholesterol (Jørgensen, FrikkeSchmidt, Nordestgaard, \& Tybjærg-Hansen, 2014; Rose-Hellekant et al., 2007; The, of the Exome, \& Project, 2014). Mutation of $L D L R$ gene is associated with augmented risk for both incident and recurrent coronary artery disease events (Mega et al., 2015). Majority of cardiovascular diseases including atherosclerosis are caused by accumulation of LDL cholesterol in blood vessels. Hyperthyroidism stimulates LDLR and hence is associated with hypocholesterolaemia. LDL receptors have been associated with the metabolomic syndrome, pathophysiology of atherosclerosis and steatohepatitis (Consortium, 2015; Hsieh et al., 2016).

Hepatic overexpression of LDLR in mice results in reduced plasma LDL-cholesterol levels thus protecting from plaque formation, while genetic deficiency up-regulates plasma LDLand VLDL-cholesterol making them vulnerable to atherosclerosis with high-cholesterol diet (Gorenne et al., 2013; Ishibashi et al., 1993).

Statins are widely used as cholesterol lowering drugs in cardiovascular disorders with uncertain results (Mihaylova et al., 2012) since they also have several side effects. The most important adverse side effects are muscle problems, an increased risk of diabetes and increased liver enzymes in the blood due to liver damage (Naci, Brugts, \& Ades, 2013). The efficacy of statins also depends upon type of mutation as described by table 11 .

Table 11 : Statin drug lowering efficacy in relation LDLR mutation type. Adopted from (P. C. J. L. Santos \& Pereira, 2015).

Data from studies that evaluated the association of the type of LDLR mutation with response to lipid-lowering treatment or with cardiovascular disease in familial hypercholesterolemia patients.

| Country | No. of FH <br> Patients | Finding | Ref |
| :---: | :---: | :---: | :---: |
| Brazil | 156 | In 1 year of follow-up with lipid-lowering <br> therapy, LDLR nullmutation patient group had: <br> higher baseline TC and LDL-C and lower <br> proportion of patients attaining an LDL-C $<130$ <br> mg/dl | (P. C. J. L. <br>  <br> Pereira, 2015) |
| Spain | 387 | CVD was significantly higher in patients carrying <br> a null-mutation compared with those carrying a <br> defective mutation | (Mata et al., <br> 2011) |
| Spain | 811 | Patients carrying null-mutation had significantly | (Alonso et al., <br> 2008) |


| higher frequency of premature CVD and recurrence of CV events |  |  |  |
| :---: | :---: | :---: | :---: |
| Greece | 49 | Patients carrying class V mutations had higher percentage decrement in LDL-C and apoB levels compared with patients carrying class II mutation, after atorvastatin $20 \mathrm{mg} /$ day during 12 weeks of therapy | (Miltiadous et al., 2005) |
| Canada | $\begin{gathered} 63 \\ \text { adolescents } \end{gathered}$ | After 6 weeks of treatment with simvastatin 20 $\mathrm{mg} /$ day, the mean reduction in LDL-C was higher in patients with null-mutation than in patients with defective mutation | $\begin{aligned} & \text { (Vohl et al., } \\ & 2002 \text { ) } \end{aligned}$ |
| Spain | 55 | Patients with null-mutation had lower reduction of LDL-C treating with simvastatin $20 \mathrm{mg} /$ day, for 6 weeks, compared with patients with defective mutation | $\begin{aligned} & \text { (Chaves et al., } \\ & \text { 2001) } \end{aligned}$ |
| UK | 109 | Patients with a 'severe' LDLR mutation achieved a lower proportion of LDL-C levels below 160 $\mathrm{mg} / \mathrm{dl}$ compared with patients with a 'mild' mutation, after simvastatin therapy | (Heath, Gudnason, Humphries, \& Seed, 1999) |
| UK | 42 | Both baseline LDL-C and after treatment LDL-C were higher in patients with 'severe' mutations than in patients with 'mild' mutations, during treatment with simvastatin and bile acid sequestrant | $\begin{aligned} & \text { (X.-M. Sun et } \\ & \text { al., 1998) } \end{aligned}$ |
| Number of FH patients was considered index case patients (some studies selected relatives too). <br> CVD: Cardiovascular disease; FH: Familial hypercholesterolemia; LDL-C: Low-density lipoprotein cholesterol; TC: <br> Total cholesterol |  |  |  |

Another undesirable effect of statins are they also up-regulate the transcription of PCSK9 along with LDLR thus depressing cholesterol lowering efficacy (F. Raal, Panz, Immelman, \& Pilcher, 2013; Welder et al., 2010) Lowering circulating PCSK9 levels and consequently upregulating hepatic LDLR is beneficial for reducing the risk of cardiovascular disease in humans and is demonstrated to be a promising approach in recent years, proved through neutralizing anti-PCSK9 antibodies that lowered serum LDL-C levels in dyslipidemic and hypercholesterolemic patients (Ling, Burns, \& Hilleman, 2014). Therefore now combinations of statins along with PCSK9 inhibitors are gaining attentions (Cannon et al., 2015; Robinson et al., 2015).

## Le Récepteur Au LDL

Le cholestérol ne sert pas seulement de source d'énergie pour différents processus cellulaires mais est aussi un constituant structural fondamental. Cependant toutes les sortes de cholestérol ne sont pas aussi bénéfiques les unes que les autres et le mauvais cholestérol en excès peut générer des maladies cardiovasculaires. La métabolisation du cholestérol s'effectue majoritairement dans le foie grâce à l'activation des récepteurs aux LDL (LDLR).

Michael S. Brown et Joseph L. Goldstein ont identifié ces récepteurs et ont reçu le prix Nobel de Physiologie ou de Médecine en 1985 pour cette découverte. Des mutations ou des délétions de ces récepteurs engendrent une maladie héréditaire appelée hypercholestérolémie familiale (HF).

Les LDLR sont des récepteurs à la surface cellulaire et participent à l'endocytose des lipoprotéines de faible densité (Low-Density Lipoproteins; LDL) depuis le plasma. Après attachement aux LDLR les LDL sont internalisés dans la cellule et transportés dans des endosomes où la présence d'un faible pH permet leur détachement des récepteurs. Ensuite les récepteurs sont modifiés et recyclés à la surface cellulaire et en parallèle le LDL riches en cholestérol est dégradé et utilisé pour la production d'énergie nécessaire à diverses fonctions cellulaires.

L'expression des LDLR est contrôlée au niveau traductionnel et post-traductionnel. Quand le niveau de cholestérol intracellulaire est haut la synthèse endogène de cholestérol est inhibée ainsi que l'expression des LDLR, qui en parallèle sont dégradés grâce à la protéine PCSK9. À l'inverse quand le taux de cholestérol intracellulaire chute, l'expression des LDLR est augmentée et leur dégradation est inhibée via un rétrocontrôle négatif. La dérégulation de ce processus chez des patients ayant des mutations conduit à des taux plasmatiques en cholestérol élevés et donc augmente le risque de maladies cardiovasculaires et d'attaques cardiaques.

L'athérosclérose et les maladies cardiovasculaires sont les causes principales de mortalité et de morbidité à travers le monde. Les LDLR ont un rôle critique dans le profil lipidique sanguin ayant un impact direct sur la régulation des LDL plasmatiques. Les niveaux plasmatiques de cholestérol et plus particulièrement de LDL-cholestérol sont directement corrélés avec le risque d'athérosclérose et près de 65 à $70 \%$ du cholestérol plasmatique est
transporté par les LDL chez l'homme. Les statines sont des médicaments hypocholestérolémiants utilisés pour contrôler les taux de cholestérols dont les résultats sont incertains et peuvent avoir des effets secondaires. De plus ils augmentent la transcription de PCSK9 ce qui impacte l'expression des LDLR, diminuant ainsi leur efficacité pour baisser les taux de cholestérol. Baisser les taux circulants de PSCK9 permettrait d'augmenter l'épuration du cholestérol plasmatique en inhibant la dégradation des LDLR et cette approche suscite actuellement beaucoup d'attention.

## 7. Materials and Methods

This chapter describes in detail certain techniques and protocols use for experimentation.

### 7.1. In vivo mouse experiments

NSG mice, 6 to 8 week old, were injected with 1 million AML cells. At day 80, when human cells reached $1 \%$ in blood, mice were separated in 2 groups of 4 mice control and DCA. Mice were treated with DCA ( $50 \mathrm{mg} / \mathrm{kg}$, 1 dose/day by gavage, starting at day 1 for 16 consecutive days). Human tumor AML cells gather in mouse spleen and bone marrow, hence we isolated mRNA from these organs. We used human-specific primers to visualize expression of human mRNA.
B) B6 wt mice were treated with a single dose of DCA ( $50 \mathrm{mg} / \mathrm{kg} /$ day $)$ intraperitoneally and mouse LDLR mRNA was analyzed in spleen and liver after different times. Experimental procedures were conducted according to the European guidelines for animal welfare (2010/63/EU). Protocols were approved by the Animal Care and Use Committee "LanguedocRoussillon" (approval number: CEEA-LR-12163).

### 7.2. Human liver samples and preparation of PHHs cultures

Liver samples were obtained from liver resections performed in adult patients for medical reasons or from donors when the liver was considered unsuitable for organ transplantation. The use of human specimens for scientific purposes was approved by the French National Ethics Committee. Written or oral informed consent was obtained from each patient or family prior to surgery. After liver perfusion, hepatocytes were counted and cell viability was assessed by trypan blue exclusion test. A suspension of $1 \times 10^{6}$ cells $/ \mathrm{mL}$ per well was added in 12-well plates pre-coated with type I collagen (Beckton Dickinson) and cells were allowed to attach for 12 h . Then, the supernatant containing dead cells and debris was carefully removed and replaced with 1 mL of serum-free long-term culture medium (Lanford medium, LNF). The number of confluent attached cells was estimated at $\sim 1.5 \times 10^{5}$ cells $/ \mathrm{cm}^{2}$.

### 7.3. Cell lines and culture conditions

The human leukemic Jurkat T cells, AML (OCI-AML3, MOLM13 and NB4) were grown in RPMI 1640-Glutamax (GIBCO) supplemented with 5\% (Jurkat) or 10\% (AML) FBS. All samples from cancer patients were collected at diagnosis. Primary cells from a lymphoma B
cell patient (BCL-P2) were grown in the same medium. Data and samples from patients with different hematological cancers were collected at the Oncology and Clinical Hematology Department of the CHU Montpellier, France, after patient's informed consent. Patients were enrolled in two independent clinical programs approved by the "Comités de Protection des Personnes Sud Méditerranée I (ref 1324)" and ID-RCB: 2011-A00924-37. In certain experiments cells were grown in RPMI 1640 without glucose (GIBCO 11879) with the addition of 2 mM glutamine and 10 mM galactose (OXPHOS medium) (Khan et al., 2016; Moeez G Rathore et al., 2012). The cell lines were incubated at $37^{\circ} \mathrm{C}$ in a humid environment supplied with $5 \% \mathrm{CO}_{2}$. The Jurkat TAg cells carry the SV40 large T Ag to facilitate cell transfection. HepG2C and HuH7 cells were grown MEM and DMEM respectively supplemented with FBS, sodium pyruvate, glutamine, penicillin and streptomycin. The HCT116 human colon cancer cells were cultured in low glucose ( 5 mM ) DMEM medium supplemented with 10 \% FBS. Cellular confluency was between 80-85\%.

### 7.4. Reagents and antibodies

RIPA buffer to prepare protein extracts was from Euromedex. The complete protease inhibitors cocktail (Complete EDTA-free) and the phosphatases inhibitor cocktail (PhosSTOP) were from Roche. $\mathrm{H}_{2} \mathrm{O}_{2}$ was from SIGMA and DCA was purchased by Santa Cruz. Galactose and glutamine were from GIBCO. The antibody against KEAP1 and MEF2 (E-17) were from Santa Cruz Biotechnology. ERK5 antibody was from Cell Signaling Technology. The antibody against $\beta$-Actin and HRP-labeled secondary antibodies were from Sigma. Human anti-LDLR-PE and IgG were from BD Biosciences and 7AAD from Beckman. The MEK5 inhibitor BIX02189 and ERK5 inhibitor XMD8-92 were from Selleck.

### 7.5. Cell proliferation, viability and apoptosis

The cell viability and the number of cells were determined by the Muse ${ }^{\circledR}$ Cell Analyzer (Millipore) or trypan blue exclusion method as indicated. Analysis of apoptotic cells (Annexin V assay) was performed using the Muse ${ }^{\circledR}$ Cell Analyzer.

### 7.6. Transient transfection

Jurkat cells in logarithmic growth phase were used for transfection. Jurkat cells ( $5 \times 10^{6}$ / condition) were centrifuged and washed with cold PBS. A second washing was performed with RPMI 1640 Glutamax without FBS. Cells were resuspended in RPMI 1640 Glutamax without FBS. Sterile electroporation cuvettes were prepared and in each experiment, cells
were transfected with the same total amount of DNA by supplementing with empty vector. Cells were incubated for 10 min at RT with the DNA mix and electroporated using the Gene Pulser Xcell ${ }^{\mathrm{TM}}$ Electroporation system (Bio-Rad) at $260 \mathrm{mV}, 960 \mathrm{mF}$ in $400 \mu \mathrm{l}$ of RPMI 1640. Expression of the different proteins was confirmed by western blot or GFP was detected by excitation with 488 Blue Laser and emission FL1 530/30 by FACS. In all experiments related to luciferase measurement, cells were transfected with a $\beta$-Galactosidase reporter plasmid as previously described (Johan Garaude et al., 2008). The relative luciferase units (RLU) were calculated by dividing the luciferase values between the $\beta$-Galactosidase values to avoid differences in transfection. The transfection efficiency in Jurkat TAg cells is between 60 and $80 \%$. In adherent cells, transfection of $30-50 \mathrm{nM}$ siRNAs was carried out using Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM (Invitrogen), according to the manufacturer's instructions. Primary hepatocytes were transfected twice at day first and third. Cells were harvested 48 to 96 h post-transfection.

All siRNA duplexes used for Control, ERK5, MEF2A, MEFC and AMPK $\alpha 1$ knockdown were ON-TARGETplus modified (Dharmacon).

### 7.7. Plasmids

The 3'UTR of KEAP1 was a generous gift of Dr. Qun Zhou, University of Maryland School of Medicine (Eades et al., 2011). The sequence corresponding to the miR-23a/24-2 promoter (2046 bp upstream miR-23a precursor) was cloned into the BgIII/HindIII and NheI/XhoI restriction sites of pGL3-basic vector (Saumet et al., 2009). The wild type miR-23a/24-2 locus was cloned into the EcoRI/BamHI sites of the MIE retroviral vector (containing the IRESGFP cassette) using the following primers: sense, ggaattcgccatgcaagttgctgtage and antisense, cgggatccggctgctaggaaggtgcg. The locus expressing only miR-24 and miR-27a (miRD23) was similarly cloned using the primers ggaattcctgagctctgccaccgagga (sense) and cgggatccggctgctaggaaggtgcg (antisense). The locus expressing only miR-23a (miRD24-27) was cloned using the primers ggattcgccatgcaagttgctgtagc (sense) and cgggatccgccaggcacaggcttcgg (antisense) (Moeez G Rathore et al., 2012). The MEF2C constructs were a generous gift from Dr. T. Gulick and has been previously used (Nuria Lopez-Royuela et al., 2014). The expression vectors for ERK5, the pSUPER expression vector for GFP alone or GFP plus shERK5 and the pSiren-retroQ-puro (BD Biosciences) retroviral vectors for shERK5 and control have been previously described (Johan Garaude et al., 2006). The expression vectors for the catalytically inactive ERK5 mutant (ERK5KM:

K84 mutated to M) in pLZR (Johan Garaude et al., 2006) was a generous gift of Dr. Atanasio Pandiella (Centro de Investigación del Cáncer, IBMCC/CSIC-Universidad de Salamanca, Spain).

### 7.8. ROS measurement

Cells lines were plated at 300,000 cells $/ \mathrm{ml}$ and treated with different $\mathrm{H}_{2} \mathrm{O}_{2}$ or DCA concentrations for the indicated times, harvested and counted to perform further analysis. To evaluate ROS levels, we labeled cells with CellROX® Deep Red Reagent or with CHH2DCFDA (Life Technologies) for 30 minutes and analyzed them by FACs following manufacturer's instructions.

### 7.9. Nuclear Fractionation

For preparation of nuclear extracts, Jurkat cells were grown in RPMI 1640 Glutamax supplemented with $6 \%$ FBS. Six million cells were taken and washed twice in cold PBS. Cells were resuspended in $400 \mu \mathrm{l}$ wash buffer containing 10 mM HEPES ( pH 7.4 ), 10 mM $\mathrm{KCl}, 2 \mathrm{mM} \mathrm{MgCl} 2,0.1 \mathrm{mM}$ EDTA, and protease inhibitors (Roche). Cells were incubated in wash buffer containing NP-40 ( $0.2 \%$ ) for 30 min at $4^{\circ} \mathrm{C}$. This incubation was followed by centrifugation at $6,000 \mathrm{rpm}$ for 5 min . Supernatant were taken at this step for cytoplasmic fraction and the pellets were washed twice with wash buffer. Nuclear extracts were prepared by resuspending the pellets in $50 \mu \mathrm{l}$ of extraction buffer containing 50 mM HEPES ( pH 7.8 ), $50 \mathrm{mM} \mathrm{KCl}, 300 \mathrm{mM} \mathrm{NaCl}, 0.1 \mathrm{mM}$ EDTA, $10 \%$ glycerol, and protease inhibitors (Roche). Proteins were extracted by agitation for 20 min at $4^{\circ} \mathrm{C}$, the insoluble fractions were removed by centrifugation at $13,000 \mathrm{rpm}$ for 10 min , and the soluble proteins were analyzed by immunoblotting.

### 7.10. ImmunoBlotting

Briefly, cells were washed with PBS and lysed in SDS Laemmle sample buffer (2\% [w/v] SDS, $2 \%[\mathrm{v} / \mathrm{v}]$ glycerol, $25 \mathrm{mMTris-HCl}[\mathrm{pH} 6.8]$, and $1 \%[\mathrm{v} / \mathrm{v}] 2-\mathrm{ME}$. Cell fractions were resuspended in SDS Laemmle buffer to a final 1x concentration. Extracts were boiled for 5 min , and proteins were separated by SDS-PAGE before electrotransfer on poly- vinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked at room temperature for 1 h in TBST ( $140 \mathrm{mMNaCl}, 10 \mathrm{mM}$ Tris- $\mathrm{HCl}[\mathrm{pH} 7.5], 0.05 \%[\mathrm{v} / \mathrm{v}]$ Tween 20), and $5 \%[\mathrm{w} / \mathrm{v}]$ low fat milk powder) or TPBS and incubated overnight at $4^{\circ} \mathrm{C}$ with the
indicated Abs (diluted in blocking buffer or 5\% Bovine serum albumin (BSA) (w/v)-TBST). After several washes in TPBS, the membranes were incubated for 1 h at room temperature with peroxidase- conjugated secondary Abs diluted in blocking buffer. After being washed with TPBS, protein-Ab complexes were detected by chemiluminescence, using Millipore ECL Western blotting kit.

### 7.11. Reporter assay

In all experiments, jurkat cells were transfected with $\beta$-galactosidase reporter plasmid. The transfected cells were harvested after 2 days and centrifuged at 1000 g for 5 min . The cell pellet was suspended in 1 ml cold PBS and transferred to 1.5 ml Eppendorf tube for washing. Cells were lysed with $100 \mu \mathrm{l}$ luciferase lysis buffer (Promega) and incubated at room temperature for 10 min . The lysates were centrifuged and luciferase assays ( $40 \mu \mathrm{l}$ ) performed according to the manufacturer's instructions (Promega, Charbonnières, France) using a Berthold luminometer. For $\beta$-Galactosidase assays, $40 \mu 1$ of lysates were added to $200 \mu 1$ of $\beta$ Galactosidase assay buffer ( 50 mM phosphate buffer pH 7.4; ONPG $200 \mu \mathrm{~g} ; 1 \mathrm{mM} \mathrm{MgCl2}$; $50 \mathrm{mM} \beta$-Mercaptoethanol) and the absorbance measured at 405 nm . The results were expressed as luciferase units normalized to the corresponding $\beta$-galactosidase activity. The expression level of the transfected proteins was routinely control by immunoblot analysis

### 7.12. RT-PCR

Cells ( $1 \times 10^{6}$ ) were washed with cold PBS and total RNA was extracted using NucleoSpin RNA isolation columns (Macherey-Nagel). RNA was quantified by Nanodrop and one microgram of each sample was reverse transcribed into cDNA using iScript ${ }^{T M}$ cDNA Synthesis Kit (Biorad). Quantitative PCR was performed with KAPA SYBR Green qPCR SuperMix (Cliniscience) and a CFX Connect ${ }^{\text {TM }}$ Real-Time qPCR machine (Biorad) according to the manufacturer specifications. Reactions were carried out ( 5 min at $95^{\circ} \mathrm{C}$ followed by 40 cycles of 30 sec at $95^{\circ} \mathrm{C}, 30 \mathrm{sec}$ at $64^{\circ} \mathrm{C}$ and 30 sec at $72^{\circ} \mathrm{C}$ ). All samples were normalized to $\beta$-actin mRNA levels. Primers were designed and selected using the Primer3 (v. 0.4.0) program.

### 7.13. Immunofluorescence

Control or treated cells were washed with cold buffer and fixed with paraformaldehyde (3.2\% in PBS) for 20 minutes. Cells were washed 3 times with PBS and stored at $4^{\circ} \mathrm{C}$ until
labelling. Cells were permeabilized with Triton ( $0.1 \%$ in TBS) for 5 minutes and washed with TBS (TBS + Tween $0.05 \%$ ). Cells were labelled with primary antibody (for one hour at room tempreture (dilution in TBS $+2 \% \mathrm{SVF}$ ) and washed with TBS (TBS + Tween $0.05 \%$ ). Cells were labelled with secondery antibody + Hoechst or DAPI $1 / 1000$ (dilution in TBS $+2 \%$ SVF) for 30 minutes. Cells were washed with TBS (TBS + Tween $0.05 \%$ ) and finally washed with $\mathrm{H}_{2} \mathrm{O}$ before montage.

### 7.14. LDL Intake

Cells after treatment were incubated with BODIPY FL LDL (Invitrogen) in PBS with 2\% FBS and incubated at $37^{\circ} \mathrm{C}$ for 30 min . Cells were then washed and suspended in $200-250 \mu \mathrm{l}$ PBS 2\% FBS and staining was analyzed using a Gallios flow cytometer (Beckman) and the Kaluza software.

### 7.15. Flow Cytometry

Briefly, $1 \times 10^{6}$ cells were stained with antibody in PBS with $2 \%$ FBS and incubated at $37^{\circ} \mathrm{C}$ for 30 min . Cells were then washed and suspended in 200-250 $\mu \mathrm{l}$ PBS $2 \%$ FBS and staining was analyzed using a Gallios flow cytometer (Beckman) and the Kaluza software.

### 7.16. Chromatin Immunoprecipitation Assay

The detailed process used for ChIP assay is given below.

## Nuclei preparation ad Digestion of Chromatin

Approximately $4 \times 10^{7}$ Jurkat cells were used for single chromatin preparation and it generated 10 separate immunoprecipitations. To crosslink proteins to DNA, $37 \%$ fresh formaldehyde ( $540 \mu \mathrm{l}$ for 15 ml medium) was added to culture flask and incubated at RT for 10 min. This gave final formaldehyde concentration of $1 \%$. This reaction was stopped by adding 2 ml of 10x glycine. Cells were recovered in 50 ml conical falcon and centrifuged at 1500 rpm for 5 min . The cell pellet was washed twice with cold PBS and suspended in 10 ml ice cold lysis buffer A (containing $5 \mu \mathrm{l}$ of 1 M DTT $+50 \mu \mathrm{l}$ of 200x Protease Inhibitor Cocktail (PIC) $+100 \mu \mathrm{l}$ of 0.1 M PMSF$)$. The suspended cells in lysis buffer were incubated on ice for 10 min and mixed every 3 min by inverting tube. The nuclei were pelleted by centrifugation at $3,000 \mathrm{rpm}$ for 5 minutes at $4^{\circ} \mathrm{C}$. Remove supernatant and resuspend pellet in 10 ml ice-cold Buffer B + DTT $(5 \mu \mathrm{l})$. The nuclei were pelleted again by centrifugation at $3,000 \mathrm{rpm}$ for 5
minutes at $4^{\circ} \mathrm{C}$ and resuspended in 1 ml ice-cold Buffer B. The enzymatic digestion of chromatin was performed by adding $2.5 \mu \mathrm{l}$ of Micrococcal Nuclease, mix by inverting tube several times and incubated for 20 minutes at $37^{\circ} \mathrm{C}$ with frequent mixing to digest DNA to length of approximately $150-900 \mathrm{bp}$. The reaction was stopped by adding $100 \mu \mathrm{l}$ of 0.5 M EDTA and nuclei were pelleted by centrifugation at $13,000 \mathrm{rpm}$ for 1 minute at $4^{\circ} \mathrm{C}$. The nuclear pellet was resuspended in 1 ml of 1 x ChIP buffer (containing $5 \mu \mathrm{l}$ of PIC $+10 \mu \mathrm{l}$ PMSF) and incubated on ice for 10 minutes in a microcentrifuge. Following incubation, the digestion of chromatin was completed by sonication of lysate in TPX tubes in a sonicator (Diagenode) at high speed for $4 \mathrm{~min}(30 \mathrm{sec}$ pause and 30 sec pulse). The lysate was cleared by centrifugation at $10,000 \mathrm{rpm}$ in a microcentrifuge for 10 minutes at $4^{\circ} \mathrm{C}$. The DNA concentration was measured by a spectrophotometer (eppendorf). DNA concentration should ideally be between 50 and $200 \mu \mathrm{~g} / \mathrm{ml}$ and 5 to $10 \mu \mathrm{~g}$ of chromatin DNA was used for each immunoprecipitation.

## Immunoprecipitation (IP)

According to desired number of immunoprecipitations, 1x ChIP Buffer was prepared. Each precipitation contains $400 \mu \mathrm{l}$ of 1X ChIP Buffer plus $2 \mu \mathrm{I}$ PIC and the cross-linked chromatin preparation ( $100 \mu \mathrm{l}$ chromatin DNA per IP) was added. A small volume ( $10 \mu \mathrm{l}$ ) of diluted chromatin was transferred to a microfuge tube. This was $2 \%$ Input Sample, which can be stored at $-20^{\circ} \mathrm{C}$ until further use. For each immunoprecipitation, diluted chromatin ( $500 \mu \mathrm{l}$ ) was transferred to a new microcentrifuge tube and the immunoprecipitating antibody was added. The amount of antibody required for IP varies for each antibody. The IP samples were incubated overnight at $4^{\circ} \mathrm{C}$ with rotation. ChIP Grade Protein G Magnetic Beads were added and incubated for 2 hours at $4^{\circ} \mathrm{C}$ with rotation. Positive and negative control antibody also used in preparation of IP.

## Washing of the Immunoprecipitated Chromatin

The tubes were placed in magnetic Separation Rack to pellet the Protein G magnetic beads and supernatants were removed carefully. The low salt wash buffer (1x ChIP Buffer) was added and incubated at $4^{\circ} \mathrm{C}$ for 5 min with rotation. This washing was repeated twice. The high salt wash buffer ( 1 x ChIP Buffer plus $70 \mu \mathrm{l}$ of 5 M NaCl ) was added and incubated at $4^{\circ} \mathrm{C}$ for 5 min with rotation. The tubes were placed in magnetic Separation Rack to pellet the Protein G magnetic beads and supernatants were removed carefully.

## Elution of Chromatin from Antibody/Protein G Beads

The ChIP Elution Buffer $(150 \mu \mathrm{l})$ was added to each IP sample and the samples were incubated at $65^{\circ} \mathrm{C}$ for 30 minutes with gentle vortexing ( $1,200 \mathrm{rpm}$ ). The tubes were placed in magnetic Separation Rack to pellet the Protein G magnetic beads and eluted chromatin supernatants were transferred to a new tube. To all tubes, including the $2 \%$ input, 5 M NaCl ( 6 $\mu \mathrm{l})$ and PIC $(2 \mu \mathrm{l})$ were added and incubated at $65^{\circ} \mathrm{C}$ for 2 hours.

## DNA Purification Using Spin Columns

For each DNA sample, DNA Binding reagent ( $600 \mu \mathrm{l}$ ) was added and transferred to the DNA purification spin column in collection tubes. The purification columns were centrifuged at $14,000 \mathrm{rpm}$ for 30 sec . DNA wash Reagent B was added to the spin column and a second centrifugation was done at $14,000 \mathrm{rpm}$ for 30 sec . The spin columns were replaced in a new collection tube and finally, $50 \mu 1$ of DNA Elute Reagent was added to each spin column and centrifuged at $14,000 \mathrm{rpm}$ for 30 sec to elute DNA. Eluates were purified DNA and can be stored at $-20^{\circ} \mathrm{C}$.

## Quantification of DNA by PCR

DNAs were amplified using the SyberGreen PCR Master Mix from Invitrogen. Amplification products were detected by Real Time PCR using the light cycler 480 (Roche) according to the manufacturer specifications. Reactions were carried out ( $3^{\prime}$ at $95^{\circ} \mathrm{C}$ followed by 40 cycles of $15^{\prime \prime}$ at $95^{\circ} \mathrm{C}, 15^{\prime \prime}$ at $64^{\circ} \mathrm{C}$ and $30^{\prime \prime}$ at $72^{\circ} \mathrm{C}$ ). RT-PCR data for mRNAs were calculated with the Light cycler 480 Softwear release 1.5 (Roche) according to the manufacturer specifications.

### 7.17. Statistical analysis

The statistical analysis of the difference between means of paired samples was performed using the paired t test. The results are given as the confidence interval ( ${ }^{*}$ : $\mathrm{p}<0.05,{ }^{* *}$ : $\mathrm{p}<0.01$, $* * *: \mathrm{p}<0.005$ ). All the experiments described in the figures with a quantitative analysis have been performed at least three times in duplicate. Other experiments were performed three times with similar results. We used actin as a loading control and the histograms represent the ratio (value of protein of interest)/(value of actin).

## Primers Used

## LDLR (H):

Forward: AGGGACCCAACAAGTTCAAG
Reverse: AAGAAGAGGTAGGCGATGGA
NRF2 (H):
Forward: AAA CCA CCC TGA AAG CAC AG
Reverse: AGT GTT CTG GTG ATG CCA CA

## HO-1 (H):

Forward: ACA AGG AGA GCC CAG TCT TC
Reverse: AGA CAG GTC ACC CAG GTA GC
NQO-1 (H):
Forward: CCT CTA TGC CAT GAA CTT
Reverse: TAT AAG CCA GAA CAG ACTC

## KEAP1 (H):

Forward: GAGCGCCTGGACGTAGAACCG
Reverse: GCTGCGAGTCCGAGGTCTTCC
ERK5 (H):
Forward: CGCTACTTCCTGTACCAACTGC Reverse: AGCCATACCAAAGTCACCAATC

MEF2A (1) (H):
Forward: CCAGCTGCTCCGGAGATACG
Reverse: GGAGTGAGTGCGGGAGACAA
MEF2A (H):
Forward: TGAGAGCCCTGATGCTGACG
Reverse: ACATCCCACCTGCATTGCCA
MEF2C (1) (H):
Forward: CATCCCAGTGTCCAGCCACA
Reverse: CCAGGTGAGACCAGCAGACC

## MEF2C (H):

Forward: GCGTGCTGTGTGACTGTGAG
Reverse: TGTGGCTGGACACTGGGATG

## ACTIN (H):

Forward: GAGGGAAATCGTGCGTGACA
Reverse: AATAGTGATGACCTGGCCGT
GAPDH (H):
Forward: CTGCACCACCAACTGCTTAG
Reverse: AGGTCCACCACTGACACGTT

## LDLR (M):

Forward: GAATCTACTGGTCCGACCTGTC
Reverse: CTGTCCAGTAGATGTTGCGGTG

## NRF2 (M):

Forward: CCATTTACGGAGACCCACCGCCTG
Reverse: CTCGTGTGAGATGAGCCTCTAAGCGG

## NQO-1 (M):

Forward: GGCATCCTGCGTTTCTGTG
Reverse: GGTTTCCAGACGTTTCTTCCAT

## ACTIN (M):

Forward: GCGGACTGTTAGTGAGCTGCG
Reverse: TGTTTGCTCCAACCAACTGCTGTC

## GAPDH (M):

Forward: CTACAGCAACAGGGTGGTGGAC
Reverse: GGGTGCAGCGAACTTTATTGATGG

## 8. Results

## Results

This thesis embraces following publication

### 8.1. Human Leukemic Cells performing Oxidative Phosphorylation

 (OXPHOS) Generate an Antioxidant Response Independently of Reactive Oxygen species (ROS) Production
## Graphical Abstract



Research Paper

# Human Leukemic Cells performing Oxidative Phosphorylation (OXPHOS) Generate an Antioxidant Response Independently of Reactive Oxygen species (ROS) Production 

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#### Abstract

Tumor cell metabolism is altered during leukemogenesis. Cells performing oxidative phosphorylation (OXPHOS) generate reactive oxygen species (ROS) through mitochondrial activity. To limit the deleterious effects of excess ROS, certain gene promoters contain antioxidant response elements (ARE), e.g. the genes NQO-1 and $\mathrm{HO}-1$. ROS induces conformational changes in KEAP1 and releases NRF2, which activates AREs. We show in vitro and in vivo that OXPHOS induces, both in primary leukemic cells and cell lines, de novo expression of NQO-1 and $\mathrm{HO}-1$ and also the MAPK ERK5 and decreases KEAP1 mRNA. ERK5 activates the transcription factor MEF2, which binds to the promoter of the miR-23a-27a-24-2 cluster. Newly generated miR-23a destabilizes KEAP1 mRNA by binding to its $3^{\prime}$ UTR. Lower KEAP1 levels increase the basal expression of the NRF2-dependent genes NQO-1 and $\mathrm{HO}-1$. Hence, leukemic cells performing OXPHOS, independently of de novo ROS production, generate an antioxidant response to protect themselves from ROS.


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## 1. Introduction

Eukaryotic cells perform oxidative phosphorylation (OXPHOS), which uses the energy released by the mitochondrial oxidation of certain metabolites, i.e. glucose, to produce adenosine triphosphate (ATP). OXPHOS is an efficient way of releasing energy, however it produces reactive oxygen species (ROS) through mitochondrial activity. In fact, ROS and mitochondria are functionally linked in several ways (Willems et al., 2015). Most cancer cells change their metabolism from respiration/OXPHOS to anaerobic glycolysis and, hence, do not completely oxidize glucose. This is called the Warburg effect. This metabolic change is not absolute and tumor cells continue, at least partially, to perform OXPHOS (Jose and Rossignol, 2013; Obre and Rossignol, 2015; Villalba et al., 2014). Tumor cell metabolism depends on the tumor origin, patient and period, with several waves of gene regulation that modify it (Smolkova et al., 2011). In addition, tumor cell

[^1]metabolism is a dynamic process with a wide remodeling of the metabolic pathways that likely occurs during tumorigenesis (Jezek et al., 2010; Bellance et al., 2009; Jose and Rossignol, 2013; Villalba et al., 2013). During these waves cells can increase OXPHOS and need to protect themselves from ROS production. In fact, low ROS levels could facilitate tumorigenesis while excessive levels are deleterious (Devasagayam et al., 2004). Therefore, they should be tightly regulated before high levels are produced.

Why most tumor cells continue performing a certain degree of OXPHOS, in spite of the dangers of high ROS levels, is still an enigma. However, it is plausible that cells possess an anti-ROS mechanism when performing OXPHOS. The MAPK extracellular signal-regulated kinase-5 (ERK5) is essential for mitochondrial function and for generating efficient antioxidant responses in leukemic cells (Chami et al., 2010; Lopez-Royuela et al., 2014). In fact, several types of oxidative stress activate ERK5 (Zhao et al., 2011), which can be considered a redox MAPK. Nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or NRF2) binds to anti-oxidant response elements (ARE) in gene promoters and, consequently, regulates oxidative stress (Kensler and Wakabayashi, 2010). In endothelial cells, steady laminar blood flow (s-flow) activates

ERK5 that induces up-regulation of NRF2-dependent gene expression, although the mechanism is not fully elucidated (Kim et al., 2012; Nigro et al., 2011). Therefore, ERK5 could link OXPHOS and the antioxidant response.

MicroRNAs (miRNAs), a class of short, non-coding RNA molecules, regulate multiple physiological processes through regulating posttranscriptional gene expression by annealing to the $3^{\prime}$ untranslated regions of target mRNAs to generally promote mRNA degradation or translational repression (Chhabra et al., 2010). The microRNA-200a (miR200a) activates Nrf2 transcriptional activity by degrading Kelch-like ECH-associated protein 1 (Keap1) mRNA (Eades et al., 2011). The decrease in Keap1 allows Nrf2 to escape ubiquitination and subsequent proteolysis, thus inducing its nuclear translocation. Another miR that has been linked to regulation of metabolism and the production of ROS is miR-23 (Rathore et al., 2012; Gao et al., 2009; Kulshreshtha et al., 2007). The unconventional promoter region of the miR-23a-27a-24-2 cluster lacks common promoter elements (Chhabra et al., 2010), but it contains several putative MEF2 binding sites (Rathore et al., 2012). This transcription factor mediates several ERK5 functions, including metabolic regulation (Lopez-Royuela et al., 2014) and activates a reporter plasmid driven by the 2.4 kb upstream of the miR-23a-27a-24-2 cluster promoter (Rathore et al., 2012).

We performed transcriptome analysis of Jurkat cells expressing a small hairpin RNA for ERK5 (shERK5) and compared the genes down regulated in shERK5 expressing cells with the predicted targets of miR-23a. We identified KEAP1 as a possible candidate to mediate the antioxidant response in cells performing OXPHOS. We next tested this hypothesis; the results elucidated the molecular mechanism. ERK5mediated miR-23 upregulation controls the antioxidant response during OXPHOS by decreasing KEAP1 mRNA independently of ROS.

## 2. Materials \& Methods

### 2.1. Reagents and Antibodies

RIPA buffer to prepare protein extracts was from Euromedex. The complete protease inhibitor cocktail (Complete EDTA-free) and the phosphatase inhibitor cocktail (PhosSTOP) were from Roche. $\mathrm{H}_{2} \mathrm{O}_{2}$ was from SIGMA and DCA from Santa Cruz Technologies. Galactose and glutamine were from GIBCO. The antibody against KEAP1 and MEF2 (E-17) were from Santa Cruz Biotechnology. ERK5 antibody was from Cell Signaling Technology. The antibody against $\beta$-Actin and HRP-labeled secondary antibodies were from Sigma.

### 2.2. Cell lines and Culture Conditions

The leukemic T Jurkat TAg and the OCI-AML3, NB4 and MOLM-13 AML human cell lines were grown in RPMI 1640-GlutaMAX (GIBCO) supplemented with 5\% (Jurkat) or 10\% (AML) FBS. In certain experiments cells were grown in RPMI 1640 without glucose (GIBCO 11879) with the addition of 2 mM glutamine and 10 mM galactose (OXPHOS medium). The Jurkat TAg cells carry the SV40 large T Ag to facilitate cell transfection.

### 2.3. Primary Leukemic Cells

Data and samples from patients with different hematological cancers were collected at the Oncology and Clinical Hematology Department of the CHU Montpellier, France, after patient's informed consent (Allende-Vega et al., 2015; Krzywinska et al., 2015). Patients were enrolled in two independent clinical programs approved by the "Comités de Protection des Personnes Sud Méditerranée I (ref 1324)" and ID-RCB: 2011-A00924-37. All samples from cancer patients were collected at diagnosis.

### 2.4. ChIP Analysis

Jurkat cells growing exponentially were cross-linked in situ and subjected to nuclear isolation, DNA shearing, preclearing, and immunoprecipitation. Procedures used were modified from the ChIP-IT kit (Active Motif) using enzymatic DNA shearing as described previously in detail (Ramachandran, 2008; Rathore et al., 2012). The Pol II (8WG16 monoclonal) ChIP antibody was from Covance. The MEF2 antibody was from Santa Cruz Technologies and the histone H3 (D2B12) XP® Rabbit mAb (ChIP Formulated) from Cell Signaling Technology.

### 2.5. Transient Transfection

Jurkat cells in logarithmic growth phase were transfected with the indicated amounts of plasmid by electroporation (Garaude et al., 2006; Garaude et al., 2008). In each experiment, cells were transfected with the same total amount of DNA by supplementing with empty vector. Cells were incubated for 10 min at RT with the DNA mix and electroporated at $260 \mathrm{mV}, 960 \mathrm{mF}$ in $400 \mu \mathrm{l}$ of RPMI 1640. Expression of the different proteins was confirmed by Western blot. In all experiments related to luciferase measurement, cells were transfected with a $\beta$-Galactosidase reporter plasmid as previously described (Garaude et al., 2008). The relative luciferase units (RLU) were calculated by dividing the luciferase values between the $\beta$-Galactosidase values to avoid differences in transfection. The transfection efficiency in Jurkat TAg cells is between 60 and $80 \%$.

### 2.6. Plasmids

The 3'-UTR of KEAP1 was a generous gift of Dr. Qun Zhou, University of Maryland School of Medicine (Eades et al., 2011). The sequence corresponding to the miR-23a/24-2 promoter (2046 bp upstream miR-23a precursor) was cloned into the BgIII/HindIII and NheI/XhoI restriction sites of pGL3-basic vector (Saumet et al., 2009). The wild type miR-23a/24-2 locus was cloned into the EcoRI/BamHI sites of the MIE retroviral vector (containing the IRES-GFP cassette) using the following primers: sense, ggaattcgccatgcaagttgctgtagc and antisense, cgggatccggctgctaggaaggtgcg. The locus expressing only miR-24 and miR-27a (miRD23) was similarly cloned using the primers ggaattcctgagctctgccaccgagga (sense) and cgggatccggctgctaggaaggtgcg (antisense). The locus expressing only miR-23a (miRD24-27) was cloned using the primers ggaattcgccatgcaagttgctgtage (sense) and cgggatccgccaggcacaggcttcgg (antisense) (Rathore et al., 2012).

The MEF2C constructs were a generous gift from Dr. T. Gulick and has been previously used (Lopez-Royuela et al., 2014). The expression vectors for ERK5, a constitutively active MEK5 mutant (S313D/ T317D, termed MEK5D), $\beta$-galactosidase, the pSUPER expression vector for GFP alone or GFP plus shERK5 and the pSiren-retroQpuro (BD Biosciences) retroviral vectors for shERK5 and control have been previously described (Garaude et al., 2006). The expression vectors for the catalytically inactive ERK5 mutant (ERK5KM: K84 mutated to M) in pLZR (Garaude et al., 2006) was a generous gift of Dr. Atanasio Pandiella (Centro de Investigación del Cáncer, IBMCC/CSICUniversidad de Salamanca, Spain).

### 2.7. Counting and Determination of Cell Viability

Cell number, viability and cell death were analyzed with the Muse Cell Analyzer (Millipore) by incubating cells with Muse Count \& Viability and Annexin V and Dead Cell kits respectively, following the manufacturer's instructions.

### 2.8. Induction of Oxidative Stress and ROS Measurement

Cell lines were plated at 300,000 cells $/ \mathrm{ml}$ and treated with different $\mathrm{H}_{2} \mathrm{O}_{2}$ concentrations for the indicated times, harvested and counted to


Fig. 1. Different ROS production by cells performing OXPHOS. Different cell lines growing in glucose (control cells) were treated with $\mathrm{H}_{2} \mathrm{O}_{2}(100 \mu \mathrm{M})$ for 1 h or $\mathrm{DCA}(20 \mathrm{mM})$ for 12 h or were kept in OXPHOS medium for at least 1 month. Cells were labeled with CellROX@ Deep Red Reagent and analyzed by FACs.
perform further analysis. To evaluate ROS levels, we labeled cells with CellROX® Deep Red Reagent or with CH-H2DCFDA (Life Technologies) for 30 min and analyzed them by FACs following manufacturer's instructions.

### 2.9. Immunoblotting

Protein analysis by immunoblotting was performed essentially as previously described (Garaude et al., 2006; Garaude et al., 2008). Briefly,


Fig. 2. Cells performing OXPHOS activate an antioxidant response. A) Different cell lines were grown in OXPHOS medium for at least 1 month before mRNA extraction. mRNA expression was quantified by qPCR and represented as the \% of mRNA compared to control cells. B) Cells were treated with 20 mM DCA for 24 and 48 h and $\mathrm{KEAP1}$ and NQ01 mRNA levels were quantified by qPCR. C) The expression of different proteins was analyzed in cells growing in OXPHOS medium or treated with DCA as described above. The data represent means $\pm$ $S D ;{ }^{*} p<0.05, * p<0.01, * * p<0.001$ Student's $t$-test compared to control cells or as depicted in the graphic.
samples were collected, washed out with PBS and lysed with RIPA buffer. Protein concentration was determined by BCA assay (Pierce) before electrophoresis in $4-15 \%$ TGX gels (BioRad) and equal amount of protein was loaded in each well. Protein transfer was performed in TransTurbo system (BioRad) in PVDF membranes. After blocking for 1 h with $5 \%$ non-fat milk, membranes were incubated overnight at $4^{\circ} \mathrm{C}$ in agitation with primary antibodies, washed three times with TBS-Tween $0,1 \%$ and incubated with the appropriate HRP-labeled secondary antibody for 1 h . Membranes were washed out three times with TBS-Tween $0,1 \%$ and developed with Substrat HRP Immobilon Western (Millipore). Band quantification was performed using the "ImageLab" software from BioRad and represented as the ratio between the protein of interest and a control protein i.e. actin. The value of 1 is arbitrarily given to control cells. One blot representative of several experiments is shown.

### 2.10. In Vivo Mouse Experiments

In vivo experiments were carried out using 6 to 8 week/old NSG mice. Mice were bred and housed in pathogen-free conditions in the animal facility of the European Institute of Oncology-Italian Foundation for Cancer Research (FIRC), Institute of Molecular Oncology (Milan, Italy). All animal experiments were carried out in accordance with national and international laws and policies. For induction of acute leukemia in mice, 1 million AML cells were injected intravenously (i.v.)
through the lateral tail vein in non-irradiated mice. At day 80 , when human cells reached $1 \%$ in blood, mice were separated into two groups of four mice: control and DCA ( $50 \mathrm{mg} / \mathrm{kg}$, 1 dose/day by gavage, starting at day 1 for 16 consecutive days). At day 140 post-graft, bone marrow and spleen were collected and mRNA isolated for analysis. We used the following human primers that did not recognize mouse mRNAs:

ERK5: Forward: (5'-CGCTACTTCCTGTACCAACTGC-3') Reverse: (5'-AGCCATACCAAAGTCACCAATC- $3^{\prime}$ ), KEAP1: Forward: ( $5^{\prime}$-GAGCGCCTGG ACGTAGAACCG-3') Reverse: (5'-GCTGCGAGTCCGAGGTCTTCC-3'), NQ0-1: Forward: ( $5^{\prime}-C C T$ CTA TGC CAT GAA CTT-3') Reverse: (5'-TAT AAG CCA GAA CAG ACTC-3'), H0-1: Forward: ( $5^{\prime}$-ACA AGG AGA GCC CAG TCT TC-3') Reverse: ( $\left.5^{\prime}-A G A ~ C A G ~ G T C ~ A C C ~ C A G ~ G T A ~ G C-3 '\right), ~$ Actin: Forward: (5'-GAGGGAAATCGTGCGTGACA-3') Reverse: (5'-AATAGTGATGACCTGGCCGT-3').

### 2.11. Statistical Analysis

The statistical analysis of the difference between means of paired samples was performed using the paired $t$ test. The results are given as the confidence interval ( ${ }^{*} \mathrm{p}<0.05,{ }^{* *} \mathrm{p}<0.01,{ }^{* * *} \mathrm{p}<0.005$ ). All the experiments described in the figures with a quantitative analysis have been performed at least three times in duplicate. Other experiments were performed three times with similar results. We used actin as a loading control and the histograms represent the ratio (value of protein of interest)/(value of actin).


Fig. 3. Cells performing OXPHOS activate an antioxidant response in vitro and in vivo in primary leukemic cells. A) Tumor cells from 4 hematological cancer patients ( $2 \mathrm{MM}, 1 \mathrm{~B}$-CLL and 1 T cell lymphoma) were treated with various concentrations of DCA for 24 h and mRNA was analyzed by $q$ PCR. B) NSG mice were engrafted with primary human AML cells. At day 80 postgraft, they were treated with DCA ( $n=4$ ) or left untreated ( $n=4$ ). At day 140 mRNA from AML tumor cell from bone marrow or spleen was isolated and the expression of different proteins was quantified by $q$ PCR. The data represent means $\pm S D ;{ }^{*} p<0.05,{ }^{* *} p<0.01$, **p $<0.001$ Student's $t$-test compared to non treated cells or mice.

## 3. Results

### 3.1. OXPHOS-Induced Increase in ROS Levels is Cell Type Dependent

Leukemic cells performing OXPHOS increase ERK5 levels (Charni et al., 2010). This enzyme is essential for the antioxidant response that keeps ROS under control (Lopez-Royuela et al., 2014). To investigate if leukemic cells stably performing OXPHOS showed higher ROS levels, we incubated cells in a glucose-free culture medium with a final glutamine concentration of 4 mM and 10 mM galactose. Glutamine was used to drive mitochondria to utilize OXPHOS and galactose allowed cells to synthesize nucleic acids through the pentose phosphate pathway (Reitzer et al., 1979; Rossignol et al., 2004; Charni et al., 2010; Lopez-Royuela et al., 2014). We called it 'OXPHOS medium', because it forced leukemic cells to use OXPHOS as primary ATP source (Charni et al., 2010; Rathore et al., 2012; Allende-Vega et al., 2015). Three out
of four leukemic cell lines cultured in OXPHOS medium for several weeks showed increased ROS levels (Fig. 1 and Supplemental Fig. 1A and B). The exception was the acute promyelocytic leukemia (APL) NB4 cell line. We also treated cells with dichloroacetate (DCA), a PDK1 inhibitor that induces OXPHOS through PDH activation in all of these leukemic cell lines (Charni et al., 2010; Allende-Vega et al., 2015). OCI and MOLM-13 AML cell lines responded by increasing ROS levels whereas NB4 did not. Jurkat, a T cell leukemia cell line, showed a minor increase. $\mathrm{H}_{2} \mathrm{O}_{2}$, which served as a positive control, increased ROS in all cell lines. Hence, not all leukemic cell lines increased ROS levels when performing OXPHOS.

### 3.2. OXPHOS Induces an Antioxidant Response

All cell lines performing OXPHOS increased ERK5 mRNA levels (Fig. 2A). This was associated with decreased KEAP1 mRNA and


Fig. 4. Increase in ROS levels is not essential for KEAP1 downregulation. A) Jurkat cells were treated with increasing concentrations of $\mathrm{H}_{2} \mathrm{O}_{2}$ for 1 h and mRNA expression was analyzed. B) OCI-AML3 cells (left) or primary tumor cells from a BCL patient (right) were treated with 1.5 mM NAC 1 h before adding DCA ( 20 mM ) for 24 h . Cells were labeled with CH-H2DCFDA and analyzed by FACs for ROS production. Keap1 mRNA and protein were analyzed as described in Fig. 2. C) Primary tumor cells from 2 BCL patients were treated as in (B) before analyzing KEAP1 mRNA expression, results represent the means $\pm$ SD of these two patients in triplicate. The data represent means $\pm S D ;{ }^{*} p<0.05,{ }^{* *} p<0.01,{ }^{* * *} p<0.001$ Student's $t$-test compared to non-transfected cells.
increased mRNA levels for the antioxidant genes $\mathrm{NQO}-1$ and $\mathrm{HO}-1$. Induction of OXPHOS with DCA also caused a decrease in KEAP1 and increase in NQO-1 mRNA (Fig. 2B). This was concentration and timedependent (Supplemental Fig. 2). Interestingly, NB4 and Jurkat cells, which did not increase ROS after DCA treatment, still produced this antioxidant response. Protein expression correlated with mRNA levels in cells performing OXPHOS (Fig. 2C).

### 3.3. OXPHOS Induces an Antioxidant Response in Primary Leukemic Cells In Vitro and In Vivo

We validated these results in primary leukemic cells derived from 4 patients with hematological neoplasias (Fig. 3A). These cells also increased ERK5 and NQO-1 and decreased KEAP1 mRNAs, on average, following DCA treatment.

To test this in vivo, we engrafted AML primary cells in non-obese diabetic/severe combined immunodeficient (NOD/SCID)-interleukin-2 receptor $\gamma$ null (NSG) mice, as previously described (Allende-Vega et al., 2015). Mice with established tumors (day 80 post-graft) were treated with DCA (Fig. 3B). The treatment was not toxic and did not show any notable effect on cell survival (Allende-Vega et al, 2015). Human tumor AML cells gather in mouse spleen and bone marrow, hence we isolated mRNA from these organs. We used human-specific primers to analyze the expression of the selected mRNAs and found an increase in ERK5 and NQO-1 and a decrease in KEAP1 mRNAs (Fig. 3B).

### 3.4. OXPHOS-Induced Antioxidant Response was ROS Independent

NB4, and partially Jurkat, cells did not increase ROS when performing OXPHOS, although they mounted an antioxidant response similar to other cell lines (Figs. 1 and 2). To investigate further if ROS were essential for the antioxid ant response, we induced oxidative stress with $\mathrm{H}_{2} \mathrm{O}_{2}$ in Jurkat cells and observed similar effects to those produced by OXPHOS: increase in ERK5 and NQO-1 and decrease in KEAP1 mRNAs (Fig. 4A and Supplemental Fig. 1). Hence, the increase in ROS levels could also mediate this antioxidant response. To explore this possibility, we blocked DCA-induced ROS production with the antioxidant N -acetyl-cysteine (NAC). We focused in OCI-AML3 (Fig. 4B left panels), in which DCA significantly increased ROS levels (Fig. 1). To firmly establish that DCA had a significant effect, we used a different dye to monitor ROS from that in Fig. 1. While NAC efficiently blocked the DCA-induced increase in ROS (Fig. 4B, upper left panel and Supplemental Fig. 1B), it failed to affect DCA effects on KEAP1 mRNA or protein (Fig. 4B, bottom left panels). As described above, DCA ineffectively induced ROS in Jurkat cells but decreased KEAP1 expression (Figs. 1 and 2). NAC blocked the former but not the latter effect, that is the decrease in KEAP1 expression (Supplemental Fig. 3). Next we used tumor cells from a BCL patient (BCL-P2) that could be maintained in vitro for several weeks. NAC effectively blocked the DCA-induced ROS increase (Fig. 4B right top panel and Supplemental Fig. 1B). However, in contrast to cell lines, NAC decreased KEAP1 mRNA levels without affecting protein expression (Fig. 4B right bottom panels). In any case, NAC did not affect DCA-

B)

C)


Fig. 5. ERK5 controls Keap1 mRNA expression.A) $10^{7}$ Jurkat-TAg cells were transfected with $5 \mu \mathrm{~g}$ of the empty pcDNA vector, ERK5 or a pSUPER Neo vector containing a small hairpin RNA for ERK5 (shERK5). Forty-eight hours later mRNA expression was analyzed by qPCR and presented as the \% of mRNA compared to cells transfected with the control vector. B) Protein expression of cells transfected in (A).C) $10^{7}$ Jurkat-TAg cells were transfected with $5 \mu$ of the empty pSUPER Neo vector or with the vector encoding for the shERK5. Protein expression was analyzed by WB at different times after transfection. The data represent means $\pm$ SD; ${ }^{*} \mathrm{p}<0.05,{ }^{* *} \mathrm{p}<0.01,{ }^{* * *} \mathrm{p}<0.001$ Student's t-test compared to empty vector transfected cells (control).
induced decrease in either KEAP1 mRNA or protein. This was confirmed in freshly primary leukemic cells of another two BCL patients (Fig. 4C).

### 3.5. ERK5 Controls Keap1 Expression

We investigated the molecular mechanisms responsible for the decrease in KEAP1 expression. Overexpression of ERK5 in Jurkat cells decreased KEAP1 mRNA and this correlated with an increase in NQO-1 (Fig. 5A and B). Conversely, expression of a small hairpin RNA for ERK5 (shERK5), which decreased ERK5 protein levels (Fig. 5B), increased KEAP1 and decreased NQO-1 mRNA levels (Fig. 5A). KEAP1 protein levels corresponded to those of its mRNA (Fig. 5B). The shERK5mediated increase in KEAP1 protein occurred after the decrease in

ERK5 (Fig. 5C), showing that ERK5 was necessary and sufficient to activate this antioxidant response.

We next investigated the effect of shERK5 on cells performing OXPHOS by growing shERK5-transfected Jurkat cells in OXPHOS media or by treating them with DCA. We could not transfect cells growing in OXPHOS media because the survival rate was very low. The OXPHOS-induced increase in ERK5 levels was totally abrogated in shERK5-expressing cells, which actually showed a time-dependent decrease (Fig. 6A and B). In contrast to cells transfected with empty vector, KEAP1 mRNA levels did not decrease in shERK5-expressing cells. The level of NQO-1 showed a much lower increase in these cells. shERK5expressing cells failed to down regulate KEAP1 and up regulate NQO-1 after $\mathrm{H}_{2} \mathrm{O}_{2}$ treatment (Fig. 6C). This showed that ERK5 could mediate several antioxidant responses.


Fig. 6. ERK5 enables NRF2-mediated antioxidant response in cells performing 0XPHOS. $10^{7}$ Jurkat-TAg cells were transfected with $5 \mu \mathrm{~g}$ of the empty pcDNA vector or shERK5 as described in Fig. 4. A) Transfected cells were placed on glucose or OXPHOS media after 24 h and left for the indicated times before mRNA expression was analyzed by qPCR. B) Cells were prepared as in (A), kept in glucose and treated with 20 mM DCA before mRNA analysis. C) Cells prepared as in (B) but they were treated with different concentrations of $\mathrm{H}_{2} \mathrm{O}_{2}$ for 1 h before analyzing mRNA. The data represent means $\pm \mathrm{SD}$; ${ }^{*} \mathrm{p}<0.05,{ }^{* *} \mathrm{p}<0.01, * * \mathrm{p}<0.001$ Student's t -test compared to non-treated cells or to empty vector transfected cells when depicted in the graphic.

### 3.6. ERK5 Mediated OXPHOS-Induced miR-23a Up Regulation

As previously described, the decrease in KEAP1 mRNA could be due to miR-23-mediated inhibition. In fact, cells growing in OXPHOS up regulate miR-23 by an unknown mechanism (Rathore et al., 2012). The promoter of the miR-23a-27a-24-2 locus contains consensus sites for the transcription factor MEF2, which increases expression of a reporter plasmid driven by the miR-23a-27a-24-2 locus proximal promoter (Rathore et al., 2012). Chromatin immunoprecipitation (ChIP) assays showed that MEF2 bound to the miR-23a-27a-24-2 locus promoter in Jurkat cells (Fig. 7A). Anti-Histone-3 and an irrelevant IgG antibody were used as positive and negative controls. CHIP assays also showed that RNA Pol II bound to this promoter (Fig. 7A). In summary, we concluded that MEF2 also activated its transcription.

To further study the role of the ERK5/MEF2 pathway in miR-23 expression, we overexpressed either ERK5 WT, the inactive ERK5 mutant ERK5 KM, or shERK5 and investigated the effect on the activity of a reporter plasmid driven by a 2.4 kb fragment of the miR-23a promoter (Fig. 7B). ERK5 increased expression of the promoter. Both ERK5 KM, which functions as a dominant negative construct in Jurkat cells (Charni et al., 2009), and shERK5 decreased the expression of the reporter (Fig. 7B). As previously described (Rathore et al., 2012), cells performing OXPHOS increased expression of this reporter (Fig. 7B). Inhibition of the ERK5 pathway inhibited OXPHOS-induced reporter activation and ERK5 overexpression increased it. Strong activation of the ERK5 pathway by co-overexpression of a constitutively active mutant of MEK5 (MEK5D), the upstream kinase of ERK5, and ERK5 induced a greater response (Fig. 7C). This was enhanced by cotransfection with MEF2C, which alone also significantly increased the expression of the reporter. In contrast, expression of a dominant negative form of MEF2C (MEF2C-DN) decreased the effect of ERK5 and MEK5D on cells in OXPHOS medium (Fig. 7C). Thus, ERK5 controls miR-23a expression through MEF2. We then investigated if miR-23a regulates KEAP1 mRNA levels.

## 3.7. miR23a Targets KEAP1 mRNA

We used transcriptomic data of Jurkat cells expressing shERK5 and compared the genes downregulated by shERK5 with the predicted targets of the miR-23a-27a-24-2 cluster (compiling miRBase/microcosm and TargetScan predictions). This identified KEAP1 as a potential target of miR-23a (according to miRBase/microcosm). To investigate this possibility, we overexpressed the miR-23a locus in leukemic Jurkat T cells. This construct also expressed GFP as a control for transfection (Rathore et al., 2012). To evaluate the role of individual miRNAs in this locus, we used mutants of the miR-23a-27a-24-2 cluster: one mutant deleted of the pre-miR-24 and pre-miR-27 (miR-23 $\Delta 24-27$ ) and one mutant deleted of the pre-miR-23a (miR-23 $\Delta 23$ ). All these constructs significantly overexpressed the encoded miRNAs (Rathore et al., 2012).

Full miR-23a-27a-4-2 locus and mutant miR-23 $\Delta 24-27$, but not miR- $23 \Delta 23$ mutant, reduced expression of KEAP1 mRNA and protein (Fig. 8A and B), suggesting that this mRNA was a genuine target of miR-23a. To prove it, we used a reporter plasmid containing the $3^{\prime} U T R$ of KEAP1 mRNA fused downstream of the luciferase gene (Eades et al., 2011). We transfected it into Jurkat cells together with the different constructs of the miR-23a-27a-24-2 locus. miR-23a overexpression led to a statistically significant decrease in luciferase expression, showing that KEAP1 mRNA was a direct miR-23a target (Fig. 8C). This regulation was physiologically relevant, because cells that overexpressed miR23a increased expression of endogenous NQO-1 mRNA (Fig. 8D).

## 4. Discussion

A wide remodeling of the metabolic pathways is likely to occur during tumorigenesis (Jezek et al., 2010; Bellance et al., 2009; Jose and

Rossignol, 2013; Villalba et al., 2013; Smolkova et al., 2011). During this process, cells adapt in different ways to obtain energy, including OXPHOS. In this case, they must protect themselves from excessive ROS production, preferably before this occurs. While we have only investigated this using leukemic cells, it is possible that all cells, transformed and non-transformed, have this pathway. In the future, it will be important to investigate this idea, although this requires knowing the "initial" metabolic status of the selected cells in order to be able to alter it.


Fig. 7. The ERK5/MEF2 pathway regulates expression of the miR-23a-27a-24-2 locus. A) $40 \times 10^{7}$ Jurkat cells growing in glucose were used to perform ChIP analysis using antibodies against proteins depicted on the $x$-axis. Two different miR23a promoter oligonucleotides were used to study MEF2 binding to the promoter. IL-2 and Rpl30 promoters were used as a control. B-C) $10^{7}$ Jurkat-TAg cells were co-transfected with $5 \mu \mathrm{~g}$ of the following vectors shERK5, ERK5 wild type (wt) and ERK5 K/M (KM) together with $2 \mu \mathrm{~g}$ of a luciferase reporter plasmid driven by the promoter of the miR-23a-27a-24-2 locus along with $1 \mu$ g of $\beta$-galactosidase expression vector. Cells were incubated in glucose (gray bars) or OXPHOS (black bars) media 16 h after transfection and analyzed 2 days later for luciferase and $\beta$-galactosidase activities. The graphic represents the relative luciferase units (RLU). C) Cells were transfected and treated as in (B) but we additionally used $5 \mu \mathrm{~g}$ of a constitutively active MEK5 mutant (MEK5D), MEF2C and MEF2 with dominant negative function MEF2-DN. Experiments in panels (B) and (C) were done in parallel but the scale is different to visualize differences in (B). The data represent means $\pm$ SD; *p<0.05, "p $<0.01,{ }^{* * *} p<0.001$ Student's $t$-test compared to empty vector transfected cells or as depicted in the graphic.

We describe here a mechanism that regulates the antioxidant response in cells performing OXPHOS in the absence of ROS increase. Mitochondria are the first source of ROS, although it is also the main organelle that assures their removal. ERK5 is essential for OXPHOS in leukemic cells (Charni et al., 2010), but also for protecting cells from excessive ROS (Lopez-Royuela et al., 2014). The last function involves NRF2 activation (Kim et al., 2012; Nigro et al., 2011), which can be mediated through SIRT1 expression (Lopez-Royuela et al., 2014) or by decreasing KEAP1 levels, as we describe here. How OXPHOS activates ERK5 is uncertain, but ERK5 translocates to mitochondria in cells performing OXPHOS (Charni et al., 2010), from where it could regulate activation of nuclear respiratory factors NRF1 and NRF2. NRF1 can regulate MEF2 expression, leading to expression of mitochondrial genes (Ramachandran, 2008). But ERK5 can also regulate mitochondrial activity by directly regulating MEF2, which mediates several mitochondrial functions (el Azzouzi et al., 2010; Ramachandran, 2008).

The mechanism described here involved miR-23a. Its expression must be finely regulated because high levels induce cell death through impairment of glutamine metabolism and mitochondrial activity and hence also affect ROS control (Gao et al., 2009; Rathore et al., 2012; Chhabra et al., 2011; Safdar et al., 2009; Kulshreshtha et al., 2007). In fact, transient overexpression of the whole locus is toxic in lymphocytes several days post-transfection (Rathore et al., 2012). In contrast, too low a level could also originate an impaired antioxidant response, as we illustrated here. In addition, miRNAs have multiple targets and their
effects depend on the relative expression of their targeted mRNAs in each specific tissue. miR- 23 expression is cell-dependent: miR-23a is downregulated during lymphoid development and upregulated during myeloid development (Jin et al., 2008); and it promotes myeloid development and blocks lymphoid development (Kong et al., 2010). This suggests that miR-23a differentially affects different tissues, although it can promote the same pathways in different cell types.

In this work we have developed a leukemic cell model for investigating the antioxidant response in cells performing OXPHOS. Understanding the mechanism(s) should facilitate the development of novel therapeutic approaches for leukemia (Villalba et al., 2014). In fact, metabolism is now seen as a good candidate for specifically targeting tumor cells (Obre and Rossignol, 2015), although expression or mutation of proteins regulating metabolism, e.g. p53, could greatly affect the efficacy of anti-metabolic tumor treatments (Allende-Vega et al., 2015). Leukemic cells show high ROS levels that can contribute to disease development and progression. However, high levels can be deleterious for cells and hence, leukemic cells also express increased levels of antioxidant proteins, which detoxify ROS (Rushworth and MacEwan, 2011; Rushworth et al., 2012). Although initially NRF2 can protect nonmalignant cells from malignant transformation, after that NRF2 can protect the tumor cells from oxidative stress and chemotherapy-induced cytotoxicity (Rushworth and MacEwan, 2011; Hayes and McMahon, 2009). In AML, high NRF2 expression is driven by NF-кB (Abdul-Aziz et al., 2015). Interestingly, ERK5 activates NF-KB (Garaude et al., 2006)


Fig. 8. miR-23a targets KEAP1 mRNA. A) Jurkat cells were transfected with the whole miR-23a-27a-24-2 locus or with the constructs miR-23 $\Delta 24-27$ and miR-23 $\Delta 23$. The expression of KEAP1 mRNA was analyzed by qPCR and represented as the \% of mRNA compared to cells transfected with the control vector. B) Expression of KEAP1 protein and the quantification. C) Jurkat cells were transfected with the different constructs together with a reporter plasmid containing the $3^{\prime}$ UTR of KEAP1 mRNA downstream of the luciferase mRNA. Data are represented as the \% of luciferase expression in cells transfected with the empty vector. D) The expression of NQO-1 mRNA was analyzed by qPCR in cells transfected as in (A). The data represent means $\pm S D ;{ }^{*} p<0.05, * p<0.01, * " p<0.001$ Student's $t$-test compared to empty vector transfected cells or as depicted in the graphic.
and also controls miR-23 expression (Rathore et al., 2012). Moreover, ERK5 protects AML cells from oxidative stress (Lopez-Royuela et al., 2014). In summary, the ERK5/MEF2/miR-23/KEAP1 pathway represents a potential target for therapeutic intervention. Moreover, our results help explain how NRF2 supports leukemogenesis: it would reinforce leukemic cell survival under different metabolic conditions.

DCA can induce an antioxidant response through NRF2 activation, which can drive cytoprotective gene expression. However, DCA has other effects on cell biology: it changes metabolism and induces p53 activation (Allende-Vega et al., 2015; Charni et al., 2010). Moreover, DCA synergizes with genotoxic drugs, such as doxorubicin or vincristine, to activate p53. Hence, we cannot attribute the effect of DCA on tumor cells uniquely to its role in NRF2 activation. DCA has been used to treat several types of cancer with some positive results, including leukemia. Therefore, we believe that its anti-tumorigenic activity is higher than its pro-tumorigenic action.

During tumorigenesis, the immune system exerts additional pressure that likely contributes to the selection of certain tumor clones. Hence, the immune system may be a factor in selecting tumor cells with a defined metabolic state (Villalba et al., 2013; Catalán et al., 2015). Notably, ERK5 is an important factor in both tumor immune evasion and tumor cell metabolism (Charni et al., 2009; Charni et al., 2010). The fact that ERK5 is involved in several phenomena described here, namely tumor cell evasion, metabolism and antioxidant response, shows that several phenomena converge to generate a specific phenotype in a clinical tumor.

## Potential Conflict of Interest

The authors declare no competing financial or other interests.

## Author Contributions

A. K., M. G. R., N. A-V, D-N. V., S. B., S. O., G. T., performed experiments; F. B., C-H. L. and M. V. conceived experiments; G. C. provide reagent; M. V. secured funding.

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## Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx. doi.org/10.1016/j.ebiom.2015.11.045.

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Supplemental Figure 1A. Mean Fluorescence Intensity of ROS production was calculated for different cell lines under various treatments


Supplemental Figure 1B. Mean Fluorescence Intensity of ROS production was calculated for different cell lines under various treatments


Supplemental Figure 2. Jurkat cells were treated with different concentrations of DCA for different times and mRNA expression was analyzed as described in Fig. 2.


Supplemental Figure 3. Jurkat cells were treated with 1.5 mM NAC 1 h before adding DCA $(20 \mathrm{mM})$ for 24 h . Cells were labeled with CHH2DCFDA and analyzed by FACs for ROS production. Keap1 mRNA and protein were analyzed as described in Fig. 4.

## Les Cellules Leucémiques Humaines Qui Réalisent Une Phosphorylation Oxydante (OXPHOS) Génèrent Une Réponse Antioxydante Indépendamment De La Production Des Espèces Réactives De L'oxygène (ROS)

Les cellules eucaryotes obtiennent de l'énergie grâce à la phosphorylation oxydative (OXPHOS) qui se déroule dans les mitochondries. Cette OXPHOS permet la production des espèces réactives de l'oxygène (ROS) lors de l'activité mitochondriale. Les cellules tumorales utilisent l'OXPHOS, mais elles ont une préférence pour la glycolyse anaérobie (Effet Warburg). Pourquoi la plupart des cellules tumorales continuent à effectuer l'OXPHOS en dépit des dangers de niveaux élevés de ROS. Il est possible que les cellules possèdent un mécanisme anti-ROS lors de l'exécution de l'OXPHOS. La MAP-kinase ERK5 (extracellular signal-regulated kinase-5) est essentielle pour la fonction mitochondriale et pour générer des réponses antioxydantes efficaces dans les cellules leucémiques par l'activation de Nrf2, facteur de transcription intervenant dans la réponse antioxydante.

Nous avons observé que lorsque les cellules sont forcées à effectuer l'OXPHOS, elles augmentent le niveau de ROS qui dépend du type cellulaire. Cependant, les cellules activent la réponse antioxydante pendant l'exécution de l'OXPHOS dans les cellules leucémiques primaires in vitro et in vivo. Cette réponse antioxydante médiée par OXPHOS était indépendante de ROS. Nous avons étudié plus en détaille le mécanisme et nous montrons que les cellules sous OXPHOS sur-expriment ERK5. Nos résultats révèlent que l'OXPHOS induit une surexpression du miR-23a via l'activation de la voie ERK5-MEF2. Ce miR23a cible la protéine Keap1 impliquée dans la dégradation cytoplasmique de Nrf2. Cette dégradation a pour conséquence la translocation nucléaire de Nrf2 et une activation supplémentaire de la réponse antioxydante via la liaison à des éléments de réponse antioxydants (ARE) présents dans les promoteurs des gènes-cibles.

Nous décrivons ici le mécanisme qui régule la réponse antioxydante dans les cellules effectuant l'OXPHOS sans augmentation du ROS. ERK5 est essentiel pour OXPHOS dans les cellules leucémiques vu son implication dans la régulation de l'activité mitochondriale. L'activation de Nrf2 peut protéger les cellules saines de la transformation maligne, comme elle peut aussi protéger les cellules tumorales contre le stress oxydatif et la cytotoxicité induite par la chimiothérapie. En résumé, la voie ERK5 / MEF2 / miR-23 / KEAP1 représente une cible thérapeutique potentielle. En outre, nos résultats aident à expliquer comment NRF2
soutient la leucémogenèse en favorisant la survie des cellules leucémiques dans différentes conditions métaboliques.

### 8.2. Mitochondrial Activity Signals Antioxidant Response Through ERK5

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## Running title: mitochondrial complex I induces NRF2 expression

Keywords: oxidative phosphorylation; mitochondria; ERK5, MEF2; anti-oxidant response elements (ARE), mitochondrial complexes

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#### Abstract

Eukaryotes obtain most of their energy by oxidative phosphorylation (OXPHOS) of different substrates through the mitochondrial electron transport chain (ETC). This is a very efficient way to produce energy but it generates reactive oxygen species (ROS). To limit the deleterious effects of excess ROS, a large amount of ROS-detoxifying enzymes contain in their gene promoters antioxidant response elements (ARE), e.g. the genes NQO-1 and HO-1. The transcription factor NRF2 binds to AREs and induce an anti-oxidant response. We show here in vitro and in vivo that mitochondrial activity induces NRF2 expression, its translocation to the nucleus and its transcriptional activity in the absence of de novo ROS production. The NRF2 promoter contains MEF2 binding sites and the MAPK ERK5 induces MEF2-dependent NRF2 expression. Cells lacking functional mitochondrial complex I activity do not induce ERK5 expression and fail to generate the anti-oxidant response. Hence, we describe that complex I induces an antioxidant response to block the ROS that it is going to generate.


## Introduction

Energy efficiency should be finely regulated to spare resources. The vast majority of eukaryotic cells reach this by performing oxidative phosphorylation (OXPHOS), which uses the energy produced by mitochondrial oxidation to produce adenosine triphosphate (ATP). This is a very efficient way to obtain energy, but it produces reactive oxygen species (ROS), which are chemically reactive chemicals containing oxygen. They are involved in normal cell signaling and homeostasis; but under stress, when levels are relatively high, they originate oxidative stress, which damage cell structures. Hence, cells using mitochondria as first energy source must regulate ROS levels. Logically, ROS and mitochondria are functionally linked in several ways and ROS in the short-term regulate mitochondrial morphology and function via non-transcriptional pathways (P. H. Willems, R. Rossignol, C. E. Dieteren, M. P. Murphy, \& W. J. Koopman, 2015). On the other hand, tumor cells forced to perform OXPHOS generate an anti-ROS response in the absence of ROS increase (Khan et al., 2016). The strongest antioxidant cellular response is mediated by nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or NRF2), which binds to anti-oxidant response elements (ARE) in gene promoters and, consequently, regulates oxidative stress (Kensler \& Wakabayashi, 2010). Nrf2 regulates ROS production by mitochondria (Kovac et al., 2015) and mitochondrial function (DinkovaKostova \& Abramov, 2015; Strom, Xu, Tian, \& Chen, 2016). Interestingly the opposite is also found and mitochondrial activity induced by acute exercise promotes Ref1/Nrf2 signaling and increases mitochondrial antioxidant activity in myocardial and skeletal muscle and this activation was correlated with increased antioxidant capacity (Muthusamy et al., 2012; P. Wang, Li, Qi, Cui, \& Ding, 2016). However, how mitochondria transcriptionally signal the genetic program to block the ROS it produces or is going to produce is unknown. The MAPK extracellular signal-regulated kinase-5 (ERK5) regulates the choice of metabolic substrates in hematopoietic cells (Catalán, Charni, Aguiló, et al., 2015; S. Charni et al., 2010;

Khan et al., 2016; N. Lopez-Royuela et al., 2014; M. Villalba et al., 2014; M. Villalba et al., 2013). But at the same time, it is essential for mitochondrial function and for generating efficient antioxidant responses in leukemic cells (S. Charni et al., 2010; Khan et al., 2016; N. Lopez-Royuela et al., 2014). In fact, several types of oxidative stress activate ERK5 (J. Zhao et al., 2011), which can be considered a redox MAPK. In endothelial cells, steady laminar blood flow (s-flow) activates ERK5 that induces up-regulation of NRF2-dependent gene expression, although the mechanism is not fully elucidated (M. Kim et al., 2012; Nigro, Abe, \& Berk, 2011). We have shown that in hematopoietic cells ERK5, through the transcription factor MEF2, induces expression of miR-23 that inhibits KEAP-1 mRNA leading to NRF2 activation (Khan et al., 2016). Therefore, ERK5 link OXPHOS and the antioxidant response and, moreover, ERK5 translocates to mitochondria when cells are forced to perform OXPHOS (S. Charni et al., 2010). But the effect of KEAP1 is partial and we realized that NRF2 promoter contains MEF2 binding sites that have been validated by ChIP (http://genome.ucsc.edu/). Moreover, predicted networks of transcription factor interactions in skeletal muscle unveil direct regulation of NRF2 by MEF2A (Mysickova \& Vingron, 2012). Hence, we speculated that ERK5 could transcriptionally induce NRF2 expression.

Here we show that mitochondrial complex I activity induces the transcriptional expression of ERK5. ERK5 protein through MEF2 induces NRF2 de novo expression. Therefore, mitochondrial activity is directly linked to the most important antioxidant response in the absence of de novo increase in ROS levels. This means that eukaryotic cells have managed a genetic program to prevent oxidative stress directly linked to OXPHOS and not requiring ROS.

## Results

## OXPHOS-induced de novo expression of NRF2

Leukemic cells performing OXPHOS generate an anti-oxidant response independently of ROS increase, which is partially mediated by ERK5-induced increase in miR-23 that impairs expression of KEAP-1 mRNA (Khan et al., 2016). However, during this work we realized that NRF2 mRNA was also increased in cells, 3 hematopoietic cell lines and primary cells obtained from a BCL patient, growing in OXPHOS medium (Fig. 1A). This glucose-free culture medium has a final glutamine concentration of 4 mM and 10 mM galactose. Glutamine is used to drive mitochondria to utilize OXPHOS and galactose allows cells to synthesize nucleic acids through the pentose phosphate pathway (S. Charni et al., 2010; N. Lopez-Royuela et al., 2014; L. J. Reitzer, B. M. Wice, \& D. Kennell, 1979; R. Rossignol et al., 2004). We called it 'OXPHOS medium', because it forced leukemic cells to use OXPHOS as primary ATP source (N. Allende-Vega et al., 2015; S. Charni et al., 2010; M. G. Rathore et al., 2012). The PDK1 inhibitor dichloroacetate (DCA), which stimulates OXPHOS in all tested leukemic cells (N. Allende-Vega et al., 2015; Catalán, Charni, Jaime, et al., 2015; S. Charni et al., 2010; Khan et al., 2016; N. Lopez-Royuela et al., 2014; M. Villalba et al., 2014), also induced NRF2 mRNA expression (Fig. 1A). Both ways to stimulate OXPHOS also induced NRF2 protein measured by western blot (Fig. 1B, upper panels) or intracellular staining and FACs analysis (Fig. 1B, lower panel). DCA also increased NRF2 mRNA and protein in two hepatic cell lines (supplemental Fig. 1A).

DCA also increased NRF2 mRNA in a group of 4 different primary leukemic cells (Supplemental Fig. 1B). Of relevance, we observed that in primary human hepatocytes DCA also increased ERK5 and NRF2 mRNAs leading to HO-1 and NQO-1 expression (Fig. 1C). In summary OXPHOS induced expression of NRF2 mRNA in multiple cell contexts.

## OXPHOS induced NRF2 translocation to the nucleus

NRF2 must translocate to the nucleus to transcribe its target genes and generate the antioxidant response. We observed that the adherent HuH 7 hepatic cells treated with DCA showed NRF2 accumulation in the nucleus (Fig. 2A). These results were confirmed in Jurkat cells that grow in suspension by western blotting (Fig. 2B) and in the hepatic cell line HepG2 (supplemental Fig. 2).

### 3.3 OXPHOS induced de novo expression of NRF2 in vivo

To test this in vivo, we engrafted AML primary cells in non-obese diabetic/severe combined immunodeficient (NOD/SCID)-interleukin-2 receptor $\gamma$ null (NSG) mice, as previously described (N. Allende-Vega et al., 2015). Mice with established tumors (day 80 post-graft) were treated with DCA (Fig. 3A). The treatment was not toxic and did not show any notable effect on mice survival (N. Allende-Vega et al., 2015). Human tumor AML cells gather in mouse spleen and bone marrow, hence we isolated mRNA from these organs. We used human-specific primers to analyze the expression of the selected mRNAs and found an increase in NRF2 mRNA (Fig. 3A) and also in ERK5 and NQO-1 (Khan et al., 2016). DCA also induced mouse Erk5, Nrf2 and Nqo1 mRNA in liver and spleen in a separate experiment in which wt mice were treated for 1 and up to 3 days with DCA (Fig. 3B). The effect was first observed in hematopoietic cells gathering in spleen and later on in liver tissue. Nrf2 was probably active because we observed an increase on its target gene Nqo-1. Hence DCA induced NRF2 expression in multiple cell populations in vitro and in vivo.

### 3.4. Increase in ROS levels is not essential for NRF2 expression.

The cellular oxidative state can regulate NRF2 expression (Kensler \& Wakabayashi, 2010). DCA induces ROS production in some hematopoietic cell lines, e.g. OCI-AML3, but not all,
e.g. Jurkat (N. Allende-Vega et al., 2015; Khan et al., 2016). In contrast both cell lines increases NRF2 expression suggesting that ROS production was not essential for this (Fig. 4 and supplemental Fig. 3). Next we incubated both cell lines with the antioxidant N -acetylcysteine (NAC), which failed to affect DCA-induced NRF2 mRNA or its target gene NQO-1 (Fig. 4 and supplemental Fig. 3). This was equally observed in hepatic cell lines in which HepG2 did not increase ROS but Huh7 did (Khan et al, unpublished observations). However, DCA increased NRF2 mRNA in both and NAC did not block its expression (Fig. 4 and supplemental Fig. 3). We confirmed this in primary leukemic cells from a B cell lymphoma patient (Fig. 4). These results excluded a major role for ROS in NRF2 expression after DCA treatment. Although this could be consider partially unexpected; in AML cells, there was no relationship between high ROS levels and high nuclear NRF2 (S. A. Rushworth et al., 2012). Furthermore, use of the ROS quencher N-acetyl cysteine (NAC), which successfully sequesters endogenous ROS in AML, had no effect on nuclear NRF2 levels (S. A. Rushworth et al., 2012). Taken together, this excludes oxidative stress as the mechanistic cause of high nuclear NRF2 in resting human AML cells (S. A. Rushworth et al., 2012).

## ERK5/MEF2 controls NRF2 expression

We further investigated the underlined mechanism responsible of mitochondrial-induced NRF2 expression. Reducing expression of ERK5 with a small hairpin RNA (shERK5) reduced NRF2 mRNA expression in hematopoietic cells under resting conditions (Fig. 5A). We could not treat shERK5-expressing cells with DCA because they die due to lack of appropriate mitochondrial functions and antioxidant response (Catalán, Charni, Aguiló, et al., 2015; S. Charni et al., 2010; Khan et al., 2016; N. Lopez-Royuela et al., 2014; M. Villalba et al., 2014; M. Villalba et al., 2013). Conversely, overexpression of ERK5 increased NRF2 mRNA (Fig. 5A). NRF2 protein was also reduced after shERK5 transfection (Fig. 5B).

Reducing expression of ERK5 in primary human hepatocytes (Fig. 5C) and HuH7 and HepG2 (supplemental Fig. 4A-B) hepatic cell lines with small interference RNA for ERK5 (siERK5) also impaired expression of NRF2 and its target genes NQO-1 and HO-1.

To further study the role of the ERK5/MEF2 pathway in NRF2 expression, we overexpressed several proteins of this pathway. Strong activation of the ERK5 pathway by cooverexpression of a constitutively active mutant of MEK5 (MEK5D), the upstream kinase of ERK5, and ERK5 induced a greater increase in NRF2 mRNA (supplemental Fig. 4C). This was enhanced by cotransfection with the ERK 5 target MEF2C. These results show be taken carefully because only a percentage ( $30-60 \%$ ) of cells are effectively transfected.

To partially solve this problem, we use a luciferase reporter plasmid driven by a DNA fragment of 1.5 kb of the human NRF2 promoter (S. A. Rushworth et al., 2012). In this context, cells expressing the reporter plasmid also contain the overexpressed proteins. ERK5 significantly activated the reporter and MEK5D increased this effect (Fig. 5D). Expression of a dominant negative form of MEF2C (MEF2C-DN) decreased the effect of ERK5 and MEK5D. This DN construct also diminished basal or DCA-stimulated reporter expression. In contrast, MEF2C overexpression increased both basal and DCA-induced activity (Fig. 5D). DCA, which induced strong activation, did not show a synergistic, but rather an additive, effect with the activating proteins on the reporter. These results, and the fact that MEF2 binds to NRF2 promoter in several cell lines (see above), suggested that ERK5 controls NRF2 expression through MEF2. To test it, we transfected siMEF2 in the hepatic cell line HepG2 and the AML cell line ACI-AML3 (Fig. 5E). The siMEF2 efficiently decreased MEF2 mRNA (data not shown) and also decreased both basal and DCA-induced NRF2 mRNA.

## Mitochondrial complex I activity signals ERK5 expression

The previous experiments had shown that mitochondrial activity generated a signal that induced ERK5 expression, which is responsible of inducing NRF2-mediated antioxidant response. Next, we investigated the mechanism.

Firstly, we inhibited the mitochondrial complex I with metformin and observed a strong inhibition on the antioxidant response, including ERK5 and NRF2 mRNA and protein (Fig. 6A). Both metformin and DCA induce AMPK activation (Allende-Vega et al.); however, they blocked or induced ERK5 expression, respectively. This suggested that AMPK and the metabolic changes it induced were not involved on ERK5 expression. We confirmed it by reducing expression of the catalytic subunit of AMPK, AMPK $\alpha$, with 2 different siRNA that effectively blocked several AMPK-mediated metabolic changes (Allende-Vega et al.). This did not affect expression of ERK5 or NRF2 mRNA (Fig. 6B). In summary, AMPK activation was not responsible of generating the antioxidant response in cells performing OXPHOS.

Fig. 6A showed that complex I inhibition decreased ERK5 mRNA. The electron transport chain complex III removes electrons from ubiquinol $\left(\mathrm{QH}_{2}\right)$ and sequentially transferred to cytochrome c . The reduction of ubiquinone $(\mathrm{Q})$ to $\mathrm{QH}_{2}$ is made by mitochondrial complex I , which removed electrons from NADH or by mitochondrial complex II, which removed them from succinate and transferred through FAD. Then, we investigated the effect of the complex II inhibitor thenoyltrifluoroacetone (TTFA). Fig. 6C showed that this drug highly induced ERK5. DCA did not increase TTFA effects suggesting that both shared the same target.

TTFA was slightly toxic (supplemental Fig. 5A) and, like metformin, could have off-target effects. Therefore, we used an array of cell lines with impaired activity of the different mitochondrial complexes (supplemental table 1). Cells lacking a functional ETC, rho0 cells, did not induce ERK5 expression after DCA treatment, in agreement with our previous results showing that mitochondrial activity induced ERK5 expression (S. Charni et al., 2010). We next used 3 different cell lines in 2 different mitochondrial backgrounds with defects in
mitochondrial complex I and observed that DCA treatment did not induce ERK5 expression and in 2 out of 3 cell lines we observed a decrease in ERK5 expression (Fig. 6D) similar to that observed with metformin (Fig. 6A). In agreement with Fig. 6C, mutation in mitochondrial complex II did not inhibit DCA-induced ERK5 expression. Cells with mutations in complex III and V, but not in complex IV, increased ERK5 expression after DCA treatment. Mutation in the mitochondrial tRNA Ile in the L929 cell line (mB77), which produces more ROS (Moreno-Loshuertos et al., 2011), did not show increase in ERK5 mRNA. This supported our results in Fig. 4 showing that de novo ROS production was not involved in ERK5 expression.

During mitochondrial activity, different complexes can form supercomplexes and depending on the substrate availability complex I or complex II associate to complexes III or IV for electron transport. This means that the activity of complex I inhibits complex II activity. DCA, by inhibiting PDK1, activates PDH and the formation of acetyl-CoA that enters cell cycle and produce 3 NADH and $1 \mathrm{FADH}_{2}$ molecules. Therefore, whereas both complexes could have the initial source of electrons, complex I seems favored. Complex II, or succinate dehydrogenase (SDH), is also part of the Krebs cycle and catalyzes the conversion of succinate on fumarate. Hence, complex II inhibition by complex I activity induces succinate accumulation and a change in $\mathrm{NADH} / \mathrm{FADH}_{2}$ ratio. Both phenomena are well-known for transducing intracellular signaling. Fumarate and succinate are unstable compounds in solution. Hence, we used monomethylsuccinate (MMS) and dymethylfumarate (DMF), which are stable, to investigate their role on ERK5 expression. In 2 different cell lines MMS failed to increase ERK5 levels, whereas DMF did it (Fig. 7A). When used together they increased expression. In OCI-AML3 cells, MMS decreased ERK5 levels. Next we used metformin to inhibit complex I, forcing the use of complex II and MMS. When used together we did not
find any additional effect Fig 7 B. In summary, we did not find any role of succinate on ERK5 expression although fumarate could play a role.

Complex I activity mainly uses NADH, in contrast to complex II that employs FADH. Therefore the ratio $\mathrm{NADH} / \mathrm{FADH}_{2}$ decreases with the use of complex I versus complex II through NADH oxidation. The main producer of $\mathrm{FADH}_{2}$ is FAO in which for each decarboxylation of 2 carbons from the acyl-CoA molecule, 1 NADH and 1 FADH2 molecule are produced. Etomoxir inhibits FFAA transport into the mitochondria and blocks FAO and then should increase the ratio $\mathrm{NADH} / \mathrm{FADH}_{2}$ by decreasing FAD reduction. Interestingly, Etomoxir decreased ERK5 mRNA and blocked DCA-induced increase (Fig. 7C). Therefore, there is not a correlationship between ERK5 expression and the expected changes in $\mathrm{NADH} / \mathrm{FADH}_{2}$ ratio. Etomoxir and DCA were not toxic to OCI-AML3 cells, although they decreased cell proliferation (supplemental Fig. 5B). However when combined they induced cell death suggesting that DCA treatment requires FAO for cell survival.

## Discussion

The activity of the electron transport chain generates ROS. Complex I is considered one of the main sites at which premature electron leakage to oxygen occurs and give rise to superoxide anion (Lenaz et al., 2006). We show here that complex I activity originates an antioxidant response mediated by ERK5-induced NRF2 expression. It is interesting to note that the main generator of ROS is also the responsible of creating the mechanism to eliminate them. Of relevance, ROS de novo production is not require, but the cell "anticipates" ROS formation and generates the pathway to avoid them. Once produced ROS quickly originate biochemical reactions that generate damage to cell structures. Hence, it is on cell benefit to create the antioxidant response when ROS production is going to occur.

NRF2 mediates the main antioxidant response and ROS induce NRF2 activation (Kensler \& Wakabayashi, 2010). However, new data have challenged that ROS are the unique way to activate NRF2. For example, NRF2 expression in AML depends on NF-кB and is independent of ROS (S. A. Rushworth et al., 2012). Interestigngly, ERK5 activates NF-кB in leukemic cells (J. Garaude et al., 2006). Hence, ERK5 could handle the NRF2-mediated antioxidant response by at least 3 mechanisms independently of de novo ROS generation: i) direct transcription through MEF2 (the results presented here); ii) direct transcription through NF-кB (J. Garaude et al., 2006; S. A. Rushworth et al., 2012); iii) upregulation of miR-23 and downregulation of KEAP1 mRNA (Khan et al., 2016). This explains the central role of ERK5 in the antioxidant response (S. Charni et al., 2010; Khan et al., 2016; N. Lopez-Royuela et al., 2014).

## Material and Methods.

## Ethical statement

Experimental procedures were conducted according to the European guidelines for animal welfare (2010/63/EU). Protocols were approved by the Animal Care and Use Committee "Languedoc-Roussillon" (approval number: CEEA-LR-12163).

## In vivo mouse experiments

In vivo experiments were carried out using 6 to 8 weeks/old male NSG mice. Mice were bred and housed in pathogen-free conditions in the animal facility of the European Institute of Oncology-Italian Foundation for Cancer Research (FIRC), Institute of Molecular Oncology (Milan, Italy). For engraftment of human cells, 1 million AML cells were injected intravenously (i.v.) through the lateral tail vein in non-irradiated mice. NSG mice with established human AML tumors (day 80 post-graft) were treated with DCA ( $50 \mathrm{mg} / \mathrm{kg}, 1$ dose/day by gavage, starting at day 1 for 16 consecutive days). Human tumor AML cells gather in mouse spleen and bone marrow, hence we isolated mRNA from these organs. We used human-specific primers to visualize expression of human mRNA. In a different experiment B6 wt mice were treated with a daily single dose of DCA ( $50 \mathrm{mg} / \mathrm{kg} / \mathrm{day}$ ) intraperitoneally and mouse LDLR mRNA was analyzed in spleen and liver after different times.

## Cell lines and culture conditions

The leukemic human cell lines T Jurkat Tag, NB4 and OCI-AML3 were grown in RPMI 1640-Glutamax (GIBCO) supplemented with $5 \%$ (Jurkat) or $10 \%$ (OCI and NB4) FBS. Primary cells from a lymphoma B cell patient (BCL-P2) were grown in the same medium with $10 \%$ FBS. In certain experiments cells were grown in RPMI 1640 without glucose (GIBCO 11879) with the addition of 2 mM glutamine and 10 mM galactose (OXPHOS medium). The Jurkat TAg cells carry the SV40 large T Ag to facilitate cell transfection.

HepG2-C3A and HuH7 cells were grown in MEM and DMEM respectively supplemented with $10 \%$ FBS, sodium pyruvate, glutamine, penicillin and streptomycin. The HCT116 human colon cancer cells were cultured in low glucose ( 5 mM ) DMEM medium supplemented with $10 \%$ FBS. Cellular confluence during experiments was between $80-85 \%$.

## Primary Leukemic Cells

Data and samples from patients with different hematological cancers were collected at the Oncology and Clinical Hematology Department of the CHU Montpellier, France, after patient's informed consent. Patients were enrolled in two independent clinical programs approved by the "Comités de Protection des Personnes Sud Méditerranée I (ref 1324)" and ID-RCB: 2011-A00924-37. All samples from cancer patients were collected at diagnosis.

## Human liver samples and preparation of PHHs cultures

Liver samples were obtained from liver resections performed in adult patients for medical reasons. The use of human specimens for scientific purposes was approved by the French National Ethics Committee. Written informed consent was obtained from each patient prior to surgery. Human hepatocytes isolation and culture were performed as described previously (Ref). Briefly, after liver perfusion, hepatocytes were counted and cell viability was assessed by trypan blue exclusion test. A suspension of $1 \times 10^{6}$ cells $/ \mathrm{mL}$ per well was added in 12 -well plates pre-coated with type I collagen (Beckton Dickinson) and cells were allowed to attach for 12 h . Then, the supernatant containing dead cells and debris was carefully removed and replaced with 1 mL of serum-free long-term culture medium (Lanford medium, LNF). The number of confluent attached cells was estimated at $\sim 1.5 \times 10^{5}$ cells $/ \mathrm{cm}^{2}$.

## Reagents and antibodies

DCA was from Santa Cruz Technologies. Galactose and glutamine were from GIBCO. The MEK5 inhibitor BIX02189 and the ERK5 inhibitor XMD8-92 were from Selleck. RIPA buffer to prepare protein extracts was from Euromedex. The complete protease inhibitor
cocktail (Complete EDTA-free) and the phosphatase inhibitor cocktail (PhosSTOP) were from Roche. $\mathrm{H}_{2} \mathrm{O}_{2}$, DMF and MMS were from Sigma. ERK5 and Nrf2 antibodies were from Cell Signaling Technology and Santa Cruz respectively. The antibody against $\beta$-Actin and HRP-labeled secondary antibodies were from Sigma.

## Plasmids

The luciferase reported plasmid driven by a DNA fragment of 1.5 kb of the human Nrf2 promoter was a kind gift from Stuart Rushworth. (Ref). The expression vectors for ERK5, the pSUPER expression vector for GFP alone or GFP plus shERK5 and the pSiren-retroQ-puro (BD Biosciences) retroviral vectors for shERK5 and control have been previously described. Control, MEF2A and C and ERK5 siRNA were ON-TARGETplus SMARTpools (mixture of 4 siRNA) from Dharmacon.

## Transient transfection

Jurkat cells in logarithmic growth phase were transfected with the indicated amounts of plasmid by electroporation. In each experiment, cells were transfected with the same total amount of DNA by supplementing with empty vector. Cells were incubated for 10 min at RT with the DNA mix and electroporated using the Gene Pulser Xcell ${ }^{\mathrm{TM}}$ Electroporation system (Bio-Rad) at $260 \mathrm{mV}, 960 \mathrm{mF}$ in $400 \mu \mathrm{l}$ of RPMI 1640. Expression of the different proteins was confirmed by western blot. The transfection efficiency in Jurkat TAg cells is between 60 and $80 \%$. OC-AML-3 cells were transfected using Amaxa ${ }^{\text {TM }}$ D-Nucleofector ${ }^{\text {TM }}$ Lonza Kit according to manufactured protocol. In $\mathrm{HuH7}$ and HCT 116 cells, transfection of $30-50 \mathrm{nM}$ siRNAs was carried out using Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM (Invitrogen), according to the manufacturer's instructions. Primary hepatocytes were transfected twice at day first and third post-seeding. Cells were harvested 48 to 96 h posttransfection.

## Reporter assay

In all experiments, jurkat cells were transfected with $\beta$-galactosidase reporter plasmid. The transfected cells were harvested after 2 days and centrifuged at 1000 g for 5 min . The cell pellet was suspended in 1 ml cold PBS and transferred to 1.5 ml Eppendorf tube for washing. Cells were lysed with $100 \mu \mathrm{l}$ luciferase lysis buffer (Promega) and incubated at room temperature for 10 min . The lysates were centrifuged and luciferase assays ( $40 \mu \mathrm{l}$ ) performed according to the manufacturer's instructions (Promega, Charbonnières, France) using a Berthold luminometer. For $\beta$-Galactosidase assays, $40 \mu \mathrm{l}$ of lysates were added to $200 \mu \mathrm{l}$ of $\beta$ Galactosidase assay buffer ( 50 mM phosphate buffer pH 7.4 ; ONPG $200 \mu \mathrm{~g} ; 1 \mathrm{mM} \mathrm{MgCl2}$; $50 \mathrm{mM} \beta$-Mercaptoethanol) and the absorbance measured at 405 nm . The results were expressed as luciferase units normalized to the corresponding $\beta$-galactosidase activity. The expression level of the transfected proteins was routinely control by immunoblot analysis.

## Nuclear fraction

For preparation of nuclear extracts, Jurkat cells were grown in indicated medium. Ten million cells were taken and washed twice in cold PBS. Nuclear and cytoplasmic proteins were extracted using according to manufactured instruction of Bio Basic Inc ${ }^{\circledR}$. Extracted soluble proteins were analyzed by immunoblotting.

## Counting and determination of cell viability

Cell number, viability and cell death was analyzed with the Muse Cell Analyzer (Millipore) by incubating cells with Muse Count \& Viability and Annexin V and Dead Cell kits respectively, following manufacturer's instructions.

## Immunofluorescent assay

Control or treated cells were washed with cold buffer and fixed with paraformaldehyde (3.2\% in PBS) for 20 minutes. Cells were washed 3 times with PBS and stored at $4^{\circ} \mathrm{C}$ until labelling. Cells were permeabilized with Triton ( $0.1 \%$ in TBS) for 5 minutes and washed with TBS (TBS + Tween $0.05 \%$ ). Cells were labelled with primary antibody (for one hour at room
tempreture (dilution in TBS $+2 \%$ SVF) and washed with TBS (TBS + Tween 0.05\%). Cells were labelled with secondery antibody + Hoechst or DAPI $1 / 1000$ (dilution in TBS $+2 \%$ SVF) for 30 minutes. Cells were washed with TBS (TBS + Tween $0.05 \%$ ) and finally washed with $\mathrm{H}_{2} \mathrm{O}$ before montage.

## RT-PCR

Total RNA was extracted using NucleoSpin RNA isolation columns (Macherey-Nagel), reverse transcription was carried out using iScript ${ }^{\mathrm{TM}}$ cDNA Synthesis Kit (Biorad). Quantitative PCR was performed with KAPA SYBR Green qPCR SuperMix (Cliniscience) and a CFX Connect ${ }^{\text {TM }}$ Real-Time qPCR machine (Biorad) with ERK5, NRF2, NQO1, HO-1 and actin primers (supplemented Fig. x). All samples were normalized to $\beta$-actin mRNA levels. Results are expressed relative to control values arbitrarily set at 100 . Supplemental Figure 6 shows all primers used in this study.

## Immunoblotting

Protein analysis by immunoblotting was performed essentially as previously described (Ref). Briefly, samples were collected, washed out with PBS and lysed with RIPA buffer. Protein concentration was determined by BCA assay (Pierce) before electrophoresis in 4-15\% TGX gels (BioRad) and equal amount of protein was loaded in each well. Protein transfer was performed in TransTurbo system (BioRad) in PVDF membranes. After blocking for 1 h with $5 \%$ non-fat milk, membranes were incubated overnight at $4^{\circ} \mathrm{C}$ in agitation with primary antibodies, washed three times with PBS-Tween $0,1 \%$ and incubated with the appropriate HRP-labeled secondary antibody for 1 h . Membranes were washed out three times with PBSTween $0,1 \%$ and developed with Substrat HRP Immobilon Western (Millipore). Band quantification was performed using the "ImageLab" software from BioRad and represented as the ratio between the protein of interest and a control protein i.e. actin. The value of 1 is arbitrarily given to control cells. One blot representative of several experiments is shown.

## Statistical analysis

The statistical analysis of the difference between means of paired samples was performed using the paired t test. The results are given as the confidence interval ( ${ }^{*}$ : $\mathrm{p}<0.05,{ }^{* *}$ : $\mathrm{p}<0.01$, ***: $\mathrm{p}<0.005$ ). All the experiments described in the figures with a quantitative analysis have been performed at least three times in duplicate. Other experiments were performed three times with similar results. We used actin as a loading control and the histograms represent the ratio (value of protein of interest)/(value of actin).

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## Figure Legends

Figure 1. Cells performing OXPHOS upregulated NRF2 expression. A) Different hematopoietic cell lines were incubated in OXPHOS medium or treated with $5 \mu \mathrm{M}$ DCA for for 2 weeks. NRF2 mRNA expression was quantified by qPCR and represented as the $\%$ of mRNA compared to control cells. B) NRF2 protein expression was analyzed in these cell lines by western blotting (upper panel) or by FACs. C) Hepatocytes from 4 donors were treated with indicated concentration of DCA for 24H and ERK5, NRF2, NQO-1 and HO-1 mRNA were analyzed. Bars show average $\pm$ SD of the four donors performed in duplicate.

Figure 2. OXPHOS induced NRF2 translocation into the nucleus. A) Huh7 cells were treated with 10 mM DCA for 48 h and nuclear translocation was revealed by immunofluorescence. B) Jurkat cells were treated with 10 mM DCA for 48 h and NRF2 nuclear translocation was revealed by subcellular fractionation and WB.

Figure 3. Cells performing OXPHOS induce NRF2 expression in vivo. A) NSG mice were engrafted with primary human AML cells. At day 80 post-graft, they were treated with DCA $(\mathrm{n}=4)$ or leave untreated $(\mathrm{n}=4)$. At day 140 mRNA from AML tumor cell from bone marrow or spleen was isolated and the expression of different proteins was quantified by qPCR. The data represent means $\pm \mathrm{SD} ;{ }^{*} \mathrm{p}<0.05,{ }^{* *} \mathrm{p}<0.01,{ }^{* * *} \mathrm{p}<0.001$ student t -test compare to non treated cells or mice. B) B6 wt mice ( $\mathrm{n}=4 / 5$ per group) were treated with a dose of DCA (50 $\mathrm{mg} / \mathrm{kg}$ ) everyday intraperitoneally and mouse NRF2 mRNA was analyzed in spleen and liver after different times after initial treatment. The data represent means $\pm$ SD; * $\mathrm{p}<0.05$, ** $\mathrm{p}<0.01,{ }^{* * *} \mathrm{p}<0.001$ student t -test compare to non treated cells or mice.

Figure 4. Increase in ROS levels is not required for NRF2 expression. A) OCI-AML and $\mathrm{HuH7}$ cell lines and primary leukemic cells from a BCL patient were treated with 2 mM NAC

1 h before adding DCA ( 10 mM ) for 24 h . mRNA was analyzed as described in Fig. 1. Results represent the means $\pm$ SD of 3 independent experiments performed in triplicate. The data represent means $\pm \mathrm{SD} ; * \mathrm{p}<0.05,{ }^{* *} \mathrm{p}<0.01,{ }^{* * *} \mathrm{p}<0.005$ student t -test compare to nontransfected cells.

Figure 5. ERK5 controls NRF2 expression. A) $10^{7}$ Jurkat-TAg cells were transfected with 5 $\mu \mathrm{g}$ of the empty pSUPER Neo vector or with this vector containing a small hairpin RNA for ERK5 (shERK5) or with a pcDNA vector expressing ERK5. Forty-eight hours later mRNA expression was analyzed by qPCR and represented as the \% of mRNA compared to cells transfected with the control vector. B) Cell transfected with control or shERK5 were analyzed for protein expression by western blotting. Graphic bars show the NRF2/actin ratio of the depicted experiment. C) Primary human hepatocytes were double transfected with control siRNA or with siRNA against ERK5 (siERK5). 96 h later mRNA was collected and mRNA expression was analyzed by qPCR. D) $10^{7}$ Jurkat-TAg cells were co-transfected with $5 \mu \mathrm{~g}$ of the following vectors ERK5 wild type (wt), a constitutively active MEK5 mutant (MEK5D), MEF2C and MEF2C with dominant negative function MEF2-DN together with $2 \mu \mathrm{~g}$ of a luciferase reporter plasmid driven by the NRF2 promoter along with $1 \mu \mathrm{~g}$ of $\beta$-galactosidase expression vector. Cells were incubated in glucose alone (gray bars) or 10 mM DCA (black bars) 24 h after transfection and analyzed 2 days later for luciferase and $\beta$-galactosidase activities. The graphic represents the relative luciferase units (RLU). Experiments were done in triplicate and data represent means $\pm \mathrm{SD} ; * \mathrm{p}<0.05,{ }^{* *} \mathrm{p}<0.01,{ }^{* * *} \mathrm{p}<0.005$ student t -test compare to empty vector transfected cells (control). E) Both cell lines were transfected with a siRNA for MEF2 (A and C) and 24h later treated with 10 mM DCA for 36 h . NRF2 mRNA was analyzed as fig. 1.

Figure 6. Inhibition of mitochondrial complex II signals ERK5 expression. A) Different hematopoietic cell lines were incubated for 24 h with 5 mM metformin. mRNA expression was quantified by qPCR and represented as the $\%$ of mRNA compared to control cells. NRF2 protein expression was analyzed in these cell lines by western blotting (lower panel). B) HCT116 cells were transfected with 2 small interference RNA (siRNA) for AMPK $\alpha$ or with control siRNA and treated with 20 mM DCA for 6 h before mRNA analysis. C) Jurkat and OCI-AML3 cells were treated with 10 mM DCA and $300 \mu \mathrm{M}$ TTFA for 24 h . NRF2 mRNA expression was quantified by qPCR and represented as the $\%$ of mRNA compared to control cells. D) Different cell lines described in supplemental Table 1 were treated with 20 mM DCA during 24 hours. NRF2 mRNA expression was quantified by qPCR and represented as the \% of mRNA compared to non-mutant control cells. Experiments were done in triplicate and data represent means $\pm \mathrm{SD} ; * \mathrm{p}<0.05,{ }^{* *} \mathrm{p}<0.01,{ }^{* * *} \mathrm{p}<0.005$ student t -test compare to empty vector transfected cells (control).

Figure 7. A) Jurkat and OCI-AML3 cells were treated with 10 mM DCA, 5 mM MMS and $100300 \mu \mathrm{M}$ DMF for 24 h B ) OCI-AML3 cells were treatew with 5 mM metformin and 10 mM MMS. C) OCI-AML3 cells were treated with 5 mM DCA and $100 \mu \mathrm{M}$ Etomoxir for 48h. The expression of ERK5 mRNA was analyzed by qPCR. The data represent means $\pm$ SD; * $\mathrm{p}<0.05,{ }^{* *} \mathrm{p}<0.01,{ }^{* * *} \mathrm{p}<0.005$ student t -test compare to empty vector transfected cells or as depicted in the graphic.


Figure 1


## B



Figure 2


Figure 3
A)

OCI-AML
Huh7
BCL-P2


Figure 4


Figure 5


Figure 6


Figure 7



| $\substack{\text { HEPG2C } \\ \text { Cont } \\ 1.0}$ | DCA |
| :---: | :---: |
|  | 1.51 |
| 1.0 | 1.27 |
|  |  |
|  |  |
|  |  |

Supplemental Figure 1. A) Hepatic Cells were were treated with 10 mM DCA for 24 h and NRF2 mRNA (upper) and protein was measure (lower). The ratio ERK5/actin and NRF2/actin is depicted in the figure.
B)Tumor cells from patients ( $2=\mathrm{MM}, 1=\mathrm{B}-\mathrm{CLL}$ and $\mathrm{l}=$ Lymphoma T ) were treated with various concentration of DCA for 24 H and Nrf2 mRNA was analyzed with qPCR.

Sup. Fig. 1


B


Supplemental Figure 2. OXPHOS induced NRF2 translocation into the nucleus. A) HepG2 cells were treated with 10 mM DCA for 48 h and nuclear translocation was revealed by immunofluorescence.
B) Jurkat cells were treated with 10 mM DCA for 48 h or kept in OXPHOS for 1 week and NRF2 nuclear translocation was revealed by subcellular fractionation and WB.

Sup. Fig. 2


Sup. Fig. 3

A) Huh7 cells were transfected with control or siERK5 plasmid and NRF2, NQO-1 and HO-1 mRNA was analyzed.
B) HepG2 cells were transfected with control or siERK5 plasmid and ERK5 and NRF2 protein was analyzed
C) Jurkat cells were transfected with indicated plasmid and NRF2 and NQO-1 mRNA was analyzed.

Sup. Fig 4


Supplemental Figure 5: A) Jurkat and OCI-AML3 cells were treated with 10 mM DCA and $300 \mu \mathrm{M}$ TTFA for 24 h and viability was counted by MUSE.
B) OCI-AML3 cells were treated with 5 mM DCA and $100 \mu$ M Etomoxir for indicated time and cell number (upper) and viability (lower) were calculated by MUSE.

Sup. Fig 5

| Name | Nuclear Background | mtDNA <br> Background | Mutation | Oxphos Complex |
| :---: | :---: | :---: | :---: | :---: |
| L929 | $\begin{aligned} & \mathrm{L} 929 \\ & (\mathrm{C} 3 \mathrm{H} / \mathrm{An}) \end{aligned}$ | $\begin{aligned} & \mathrm{L} 929 \\ & (\mathrm{C} 3 \mathrm{H} / \mathrm{An}) \end{aligned}$ | Wt | N/A |
| L929-Rho0 | $\begin{aligned} & \mathrm{L} 929 \\ & (\mathrm{C} 3 \mathrm{H} / \mathrm{An}) \end{aligned}$ | no mtDNA | no mtDNA | No mETC |
| E9 | $\begin{aligned} & \mathrm{L} 929 \\ & (\mathrm{C} 3 \mathrm{H} / \mathrm{An}) \end{aligned}$ | $\begin{aligned} & \text { L929 } \\ & (\mathrm{C} 3 \mathrm{H} / \mathrm{An}) \end{aligned}$ | $\begin{aligned} & \text { mt-COXI } \\ & (\mathrm{T} 6589 \mathrm{C}) \end{aligned}$ | Complex IV |
| A22 | $\begin{aligned} & \mathrm{L} 929 \\ & (\mathrm{C} 3 \mathrm{H} / \mathrm{An}) \end{aligned}$ | $\begin{aligned} & \mathrm{L} 929 \\ & (\mathrm{C} 3 \mathrm{H} / \mathrm{An}) \end{aligned}$ | $\begin{aligned} & \text { mt-Cytb } \\ & (\mathrm{G} 15263 \mathrm{~A}) \end{aligned}$ | Complex III |
| FL929 | $\begin{aligned} & \mathrm{L} 929 \\ & (\mathrm{C} 3 \mathrm{H} / \mathrm{An}) \end{aligned}$ | $\begin{aligned} & \mathrm{L} 929 \\ & (\mathrm{C} 3 \mathrm{H} / \mathrm{An}) \end{aligned}$ | Wt | N/A |
| FG-23.1 | $\begin{aligned} & \mathrm{L} 929 \\ & (\mathrm{C} 3 \mathrm{H} / \mathrm{An}) \end{aligned}$ | $\begin{aligned} & \mathrm{L} 929 \\ & (\mathrm{C} 3 \mathrm{H} / \mathrm{An}) \end{aligned}$ | $\begin{aligned} & \text { mt-ND6 } \\ & \text { (iC13887) } \end{aligned}$ | Complex I |
| FBalbc/J | $\begin{aligned} & \mathrm{L} 929 \\ & (\mathrm{C} 3 \mathrm{H} / \mathrm{An}) \end{aligned}$ | Balb/cJ | WT | N/A |
| mB77 | $\begin{aligned} & \text { L929 } \\ & (\mathrm{C} 3 \mathrm{H} / \mathrm{An}) \end{aligned}$ | Balb/cJ | $\begin{aligned} & \text { mt-tRNA Ile } \\ & (\mathrm{G} 3739 \mathrm{GA}) \end{aligned}$ | $\begin{aligned} & \text { tRNA Ile, more } \\ & \text { ROS } \end{aligned}$ |
|  | L929 |  | mt-ATP6 |  |
| Bc-TMP-II | (C3H/An) | Balb/cJ | (A8414G) | Complex V |
| FBalb/cJ- <br> ShFp | $\begin{aligned} & \mathrm{L} 929 \\ & (\mathrm{C} 3 \mathrm{H} / \mathrm{An}) \end{aligned}$ | Balb/cJ | ShRNA-FpSDH | Complex II |
| FMI. 12 | $\begin{aligned} & \mathrm{L} 929 \\ & (\mathrm{C} 3 \mathrm{H} / \mathrm{An}) \end{aligned}$ | NIH.3T3 | mt-ND4 <br> (delA10227) | Complex I |
| FMI. 5 | $\begin{aligned} & \mathrm{L} 929 \\ & (\mathrm{C} 3 \mathrm{H} / \mathrm{An}) \end{aligned}$ | NIH.3T3 | $\begin{aligned} & \text { mt-ND6 } \\ & \text { (delC13887) } \end{aligned}$ | Complex I |
| F3T3.6 | $\begin{aligned} & \text { L929 } \\ & (\mathrm{C} 3 \mathrm{H} / \mathrm{An}) \end{aligned}$ | NIH.3T3 | Wt | N/A |

Mice Fibroblasts with different mitochondrial mutation

Sup. Table 1

### 8.3. The PDK1 Inhibitor Dichloroacetate Controls Cholesterol Homeostasis Through the ERK5/MEF2 Pathway

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#### Abstract

Controlling cholesterol levels is a major challenge in human health, since hypercholesterolemia can lead to serious cardiovascular disease. Drugs that target glucidic metabolism can also modify lipid metabolism and hence cholesterol plasma levels. In this sense, dichloroacetate (DCA), a pyruvate dehydrogenase kinase (PDK) inhibitor, augments pyruvate usage in the mitochondria increasing oxidative phosphorylation (OXPHOS), but, also, decreases cholesterol and triglycerides in animal models. Thus, DCA was used in the 70 s to treat diabetes mellitus, hyperlipoproteinemia and hypercholesterolemia with satisfactory results. The mechanism of action was unknown and we describe it here. DCA, by promoting OXPHOS, induces expression of the MAPK ERK5 that turns on the transcription factor MEF2. This induces de novo LDLR expression, which sequesters cholesterol into cells in several cell lines and in primary human hepatocytes, as well as in two animal models. ERK5/MEF2 pathway offers an interesting target for pharmacology development.


## Introduction

Elevated levels of low-density lipoprotein (LDL) in blood is a predominant risk component for atherosclerosis, a large cause of mortality (Mozaffarian et al., 2016). Control of plasma cholesterol levels largely resides in low-density lipoprotein receptor (LDLR), which mediates the endocytosis of cholesterol-rich LDL, This process takes place mainly in the liver, which intakes close to $70 \%$ of circulating LDL. LDL is degraded in lysosomes and cholesterol made available for repression of microsomal enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, the rate-limiting step in cholesterol synthesis. Hence, LDLR regulates intracellular and extracellular cholesterol homeostasis and is involved in atherosclerosis due to accumulation of LDL-cholesterol in blood (G. W. Go \& A. Mani, 2012).

Lipid and glucid metabolic pathways are interconnected and targeting glucidic metabolism may result in altered cholesterol levels. The pyruvate dehydrogenase (PDH) kinase (PDK) inhibitor dichloroacetate (DCA) inhibits glycolysis and induces oxidative phosphorylation (OXPHOS) in human and mouse cell lines as well as in animal models (N. Allende-Vega et al., 2015; S. Charni et al., 2010; Khan et al., 2016). DCA decreases plasma triglyceride and cholesterol in animal models and in humans (G. Ribes, Valette, \& Loubatieres-Mariani, 1979; P. W. Stacpoole, G. W. Moore, \& D. M. Kornhauser, 1978). The effect was probably secondary to the decrease on plasma very-low-density lipoproteins (VLDL) by stimulation of triglyceride oxidation (P. W. Stacpoole \& Greene, 1992; P. W. Stacpoole et al., 1978), although the mechanism remains unknown. Following these findings there was an attempt to use DCA to treat two cases of familial hypercholesterolemia (FH) (G. W. Moore et al., 1979). DCA reduced circulating cholesterol levels in both patients by decreasing LDL cholesterol (G. W. Moore et al., 1979). Unfortunately, DCA must be discontinued following neuropathological effects and this precluded its use to treat high cholesterol levels. The biological mechanism underlining DCA effects on cholesterol was unknown and to the best of our knowledge, no further studies were conducted to investigate it. Nowadays the most accepted explanation would be that the change in glucidic metabolism alters lipid metabolism. In this sense, DCA, by inhibiting glycolysis, activates AMPK (N. Allende-Vega et al., 2015), which inhibits cholesterol synthesis (Y. Li et al., 2011; S. Liu et al., 2015) leading to LDLR expression (J. M. Brusq et al., 2006; Y. X. Wang et al., 2012). Enhanced LDLR expression is mediated by a MAPKK because the MAPKK inhibitors PD98059 (J. M. Brusq et al., 2006) and U0126 (P. Abidi, Y. Zhou, J. D. Jiang, \& J. Liu, 2005; W. Kong et al., 2004) restrain it.

This supports that DCA has a similar mechanism of action than the alkaloid berberine or its analogs (J. M. Brusq et al., 2006; Y. X. Wang et al., 2012). However, while testing this hypothesis, we found that a different mechanism was involved. We have previously observed that DCA increased expression of the MAPK ERK5, which mediated several of the physiological effects of DCA through activation of the MEF2 family of transcription factors (S. Charni et al., 2010; Khan et al., 2016; N. Lopez-Royuela et al., 2014; M. G. Rathore et al., 2012; M. Villalba et al., 2014; M. Villalba et al., 2013). We found by transcriptome analysis that $L D L R$ was one of the most downregulated genes in hematopoietic cells expressing a small hairpin RNA for ERK5 (shERK5) and one of the most upregulated after DCA treatment. In fact, DCA activates multiple genes with promoters containing MEF2 binding sites. Genomic analysis using the UCSC genome browser (http://genome.ucsc.edu/) shows that $L D L R$ is one of such genes because its promoter contains several binding sites for at least two MEF2 proteins, MEF2A and MEF2C, which have been validated in several cell lines by Chromatin Immunoprecipitation (ChIP; (http://genome.ucsc.edu/).

## Results

## DCA enhanced LDLR expression

We first confirmed that DCA increased LDLR mRNA in hematopoietic cells (Fig. 1A, left panel). Since liver is the main organ that removes LDL-cholesterol from blood, we tested the effect of DCA in two hepatic cell lines, finding that LDLR mRNA levels were also increased (Fig. 1A, right panel). Elevated LDLR mRNA correlated with increased LDLR protein expression in plasma membrane (Fig. 1B and supplemental Fig. 1A). We then tested the functional consequence of this enhanced expression by incubating control or DCA-treated cells with fluorescently labeled LDL. DCA increased LDL transport in these cell lines (Fig. 1C and supplemental Fig. 1B).

## Cells performing OXPHOS increased LDLR activity

DCA induces OXPHOS in leukemic cells (N. Allende-Vega et al., 2015; Catalán, Charni, Jaime, et al., 2015; S. Charni et al., 2010; Khan et al., 2016; N. Lopez-Royuela et al., 2014; M. Villalba et al., 2014). To investigate if the metabolic switch from aerobic glycolysis to OXPHOS mediated DCA effect on LDLR expression, we used a glucose-free culture medium containing a final glutamine concentration of 4 mM and 10 mM galactose. Glutamine is used to drive mitochondria to utilize OXPHOS and galactose allows cells to synthesize nucleic acids through the pentose phosphate pathway (S. Charni et al., 2010; N. Lopez-Royuela et al., 2014; L. J. Reitzer et al., 1979; R. Rossignol et al., 2004). We called it 'OXPHOS medium', because it forced leukemic cells to use OXPHOS as primary ATP source (N. Allende-Vega et al., 2015; S. Charni et al., 2010; M. G. Rathore et al., 2012). We observed that acute myeloid leukemia (AML) OCI-AML3 cells growing in OXPHOS medium for 2 weeks, like those treated with DCA, presented increased ERK5 and LDLR mRNA (supplemental Figure 2A), LDLR protein and LDL intake (Fig. 1D), suggesting that, as expected, DCA effects were due to a metabolic switch. DCA and OXPHOS also increased LDLR mRNA and protein and LDL intake in primary lymphoma cells derived from a B cell lymphoma patient (BCL-P2; Fig. 1A, supplemental Fig. 2B and Fig. 1E). We found similar results in the hepatic cell lines HepG2C3A and HuH7, with OXPHOS media increasing LDLR protein and uptake (supplemental Fig. 3).

In primary human hepatocytes, DCA also induced LDLR expression 6 and 24 h post treatment (Fig. 2A). However, effects disappeared at 48 and 72 h with a net decrease (Fig.

2A). This is likely due to the short DCA half-life, since LDLR mRNA was kept high if fresh DCA was added to the medium every 24 h (Fig. 2B).

## ROS did not mediate DCA-induced LDLR expression

The cellular oxidative state can regulate LDLR expression (X. Zheng et al., 2002) and DCA induces ROS production in some hematopoietic cell lines, although not in all ( N . AllendeVega et al., 2015; Khan et al., 2016). This was equally observed in hepatic cell lines in which HepG2-C3A did not increase ROS and Huh7 did (Fig. 3A). In contrast, all cell lines increased LDLR expression, suggesting that ROS production was not essential (Figs. 1A and 3B). Next, we incubated both cell lines with the antioxidant N -acetyl-cysteine (NAC). This efficiently blocked DCA-induced ROS production in HuH7 cells (Fig. 3A); however, it failed to significantly affect DCA-induced LDLR mRNA (Fig. 3B) or plasma membrane protein (Fig. 3C). To definitively exclude that ROS played any role in LDLR induction, we incubated primary hepatocytes with DCA in presence or absence of NAC. Fig. 3D showed that NAC did not inhibit and in fact increased LDLR mRNA expression. These results excluded a major role for de novo ROS production in LDLR expression after DCA treatment.

## DCA induced LDLR in vivo

We next verified that DCA induced LDLR in vivo. We engrafted human AML primary cells in non-obese diabetic/severe combined immunodeficient (NOD/SCID)-interleukin-2 receptor $\gamma$ null (NSG) mice, as previously described (N. Allende-Vega et al., 2015; Khan et al., 2016). Mice with established tumors (day 80 post-graft) were treated daily with DCA (Fig. 4A). The treatment was not toxic and did not show any notable effect on mice survival ( N . Allende-Vega et al., 2015). Human tumor AML cells gather in murine spleen and bone marrow, hence we isolated mRNA from these organs. We used human-specific primers and observed that DCA significantly increased expression of LDLR mRNA (Fig. 4A).
DCA also induced mouse LDLR mRNA in liver and spleen in a separate experiment in which wt mice were treated daily for 1 and up to 3 days with DCA (Fig. 4B). The effect was first observed in hematopoietic cells gathering in spleen and, later, in liver. Thus, DCA induced LDLR expression in multiple cell populations in vivo. This could, at least partially, explain the reduction in cholesterol levels after DCA treatment in vivo in several species including humans (G. W. Moore et al., 1979; G. Ribes et al., 1979; P. W. Stacpoole \& Greene, 1992; P. W. Stacpoole, Harwood, \& Varnado, 1983; P. W. Stacpoole et al., 1978).

## ERK5 regulated LDLR expression

We further investigated the underlining mechanism of DCA-induced LDLR expression and the role of the ERK5/MEF2 pathway, which is activated by DCA (S. Charni et al., 2010; Khan et al., 2016; N. Lopez-Royuela et al., 2014; M. G. Rathore et al., 2012). To this end, we targeted ERK5 utilizing a small hairpin RNA (shERK5). Reducing expression of ERK5 resulted in decreased LDLR mRNA levels in hematopoietic cells under resting conditions (Fig. 5A). We could not investigate DCA effects on cells expressing shERK5 because ERK5 is essential for cells to perform OXPHOS and hence DCA is highly toxic on cells with reduced ERK5 levels (S. Charni et al., 2010; Khan et al., 2016; N. Lopez-Royuela et al., 2014; M. G. Rathore et al., 2012). Conversely, overexpression of ERK5 increased LDLR mRNA (Fig. 5A). Decreasing ERK5 levels with small interference RNA for ERK5 (siERK5) also impaired LDLR expression in primary hepatocytes (Fig. 5B) or in HuH7 hepatic cells (supplemental Fig. 4A). Overexpression of ERK5 in Jurkat cells augmented LDLR protein and enhanced LDL uptake (Fig. 5C). The MAPKK MEK5 activates ERK5 in several physiological contexts (B. A. Drew, M. E. Burow, \& B. S. Beckman, 2012). Thus, we next used the MEK5 inhibitor BIX02189 and showed that it decreased LDLR protein and LDL uptake in Jurkat cells (Fig. 5D). We validated these findings in primary tumor cells derived from a BCL patient. In these cells both, the ERK5 inhibitor XMD8-92 and BIX02189, decreased LDLR protein (supplemental Fig. 5) Taken together, these results indicate that ERK5 is essential for LDLR expression and function in multiple cell lines.

## AMPK did not mediate DCA-induced LDLR expression

DCA induces AMPK activation (Allende-Vega et al.), suggesting that AMPK, by blocking de novo cholesterol production (Y. Li et al., 2011; S. Liu et al., 2015), could mediate LDLR expression as it has been observed with berberine or its analogs (J. M. Brusq et al., 2006; Y. X. Wang et al., 2012). In fact, a PD98059 (J. M. Brusq et al., 2006) and U0126 (P. Abidi et al., 2005; W. Kong et al., 2004) sensible pathway mediated berberine effect. Although these MAPKK inhibitors were initially described as specific MEK1 inhibitors, they also inhibit the ERK5 upstream kinase MEK5 (Kamakura, Moriguchi, \& Nishida, 1999). This indicates that DCA may have a similar mechanism of action than berberine. To test this hypothesis, we used metformin, which stimulates AMPK in Jurkat and OCI-AML cells (Allende-Vega et al.). Surprisingly, metformin did not increase, but rather decreased, LDLR mRNA (Fig. 6A), protein (Fig. 6B) and LDL intake (Fig. 6C) in two hematopoietic cell lines. It also decreased LDL uptake in HEPG2-C3A cells (Fig. 6D). Moreover, blocking expression of the catalytic
subunit of AMPK, AMPK $\alpha$, with two different siRNA that effectively decrease AMPK $\alpha$ levels (Allende-Vega, Dias, Milne, \& Meek, 2005), did not statistically decrease LDLR mRNA. In summary, we uncovered a totally new pathway that controls LDLR expression.

## LDLR expression required MEF2

ERK5 mediates part of its functions in metabolism through the MEF2 family of transcription factors (S. Charni et al., 2010; Khan et al., 2016; N. Lopez-Royuela et al., 2014; M. G. Rathore et al., 2012). Interestingly, LDLR promoter contains predicted binding sites for MEF2A and C that have been validated in several cell lines (http://genome.ucsc.edu/). Therefore, we treated OCI-AML3 cells with siRNA targeting both of them. This halved the expression of both transcription factors and was sufficient to significantly decrease LDLR mRNA levels (Fig. 7A). Finally, we investigated whether DCA activated $L D L R$ promoter. We used H3 acetylation on lysine 27 and observed that DCA significantly increased this modification linked to promoter activation (Fig. 7B).

## The ERK5/MEF2 pathway also controlled expression of the LDL receptor-adapter protein 1 (LDLRAP1)

The LDL receptor-adapter protein 1 (LDLRAP1) is a cytosolic protein that interacts with the cytoplasmic tail of LDLR. LDLRAP1 promoter contains MEF2 binding sites (http://genome.ucsc.edu/), suggesting that it may share with LDLR the same regulation pathway. Consequently with this hypothesis, DCA enhanced LDLRAP1 expression in hepatic cell lines and primary hepatocytes (Fig. 8A). OCI-AML3 and primary tumor B cells also increased LDLRAP1 mRNA after DCA treatment or after incubation in OXPHOS medium (Fig. 8B). Under resting conditions, siERK5 reduced LDLRAP1 mRNA in primary hepatocytes or in Huh7 cells or primary tumor cells (Fig. 8C (left), supplementary Fig. 4C). Similarly OCI-AML3 cells transfected with siMEF2 repressed the expression of LDLRAP1 mRNA (Fig. 8C (right)). In summary, cells performing OXPHOS increased the expression of an additional protein involved in LDLR activity.

## Discussion

Glucidic and lipid metabolism are intrinsically bound and their dysfunction play a major role in cardiovascular disease. Diabetes is typically associated to dyslipidemia, but vice versa, lipid changes also disturb glucose metabolism (Parhofer, 2015). DCA, by stimulating PDH activity, decreases glucose catabolism and stimulates OXPHOS. To fuel it, cells could rely on fatty acid oxidation (FAO), suggesting that DCA could increase lipid catabolism. LDL particles transport cholesterol and triglycerides; hence an increase in LDLR should allow cells to increase fat availability. We propose that the avidity for fat which leads DCA-treated cells to increase LDLR expression and, subsequently, to an increase in cholesterol cell uptake. This is in agreement with our current results that forcing cells to perform OXPHOS in vitro reproduces DCA effects on LDLR. Whereas other groups have suggested this hypothesis, we have identified the mechanism ERK5, which directs the choice of catabolic substrates (S. Charni et al., 2010; Khan et al., 2016; N. Lopez-Royuela et al., 2014; M. G. Rathore et al., 2012; M. Villalba et al., 2014; M. Villalba et al., 2013), activates MEF2-dependent promoters leading to LDLR expression. This new pathway, which modulates cholesterol levels, could be a new pharmaceutical target to treat hypercholesterolemia.

DCA clinical concentration in DCA-treated patient is unclear because the initial half-life of DCA is less than 1 hour and it is not detectable in patients during the initial treatment that can last the first 2 to 3 months (E. D. Michelakis et al., 2010; P. W. Stacpoole, Kurtz, Han, \& Langaee, 2008). However, DCA inhibits its own metabolism and serum concentrations increase, eventually reaching a plateau, with plasma concentrations around $0.3 \mathrm{mM}(\mathrm{P} . \mathrm{W}$. Stacpoole et al., 2008). Michelakis et al gave $50 \mathrm{mg} / \mathrm{Kg} /$ day of DCA to patients and obtained similar values: $0.44 \pm 0.16 \mathrm{mM}$ (E. D. Michelakis et al., 2010). On average, this amount of DCA should give a blood concentration of 4.6 mM , i.e. by considering $70 \mathrm{Kg} /$ patient and a total of 5 L of blood. However, the ultimate destination of the DCA that was not in blood was unknown. In this manuscript, we have observed that in primary hepatocytes fresh DCA should be daily added to media to keep physiological effects. Hence, and as expected, hepatocytes probably metabolize DCA faster than other cell types (P. W. Stacpoole, 1989). We have mainly used high ( 10 mM ) DCA concentrations for acute responses and "physiological" concentrations ( 1 to 5 mM ) for chronic treatments. These last values are in the range of those found in DCA-treated patients (E. D. Michelakis et al., 2010; P. W. Stacpoole et al., 2008).

DCA decreases cholesterol plasma levels in several animal models and humans (G. W. Moore et al., 1979; G. Ribes et al., 1979; P. W. Stacpoole \& Greene, 1992; P. W. Stacpoole et al., 1978). There was an attempt to use DCA for treating hypercholesterolemia (G. W. Moore et al., 1979). DCA reduced circulating cholesterol levels in two patients through a mechanism involving a reduction in LDL cholesterol (G. W. Moore et al., 1979), although both patients initially showed low LDLR surface activity. However, DCA was halted due to its neuropathological effects and this precluded its use to treat high cholesterol. These pathological effects have been observed in other clinical contexts, e.g. lactic acidosis and stroke-like episodes (MELAS) (P. Kaufmann et al., 2006). Hence, uncovering the mechanism induced by DCA could facilitate the development of compounds that could decrease cholesterol levels without inducing neuropathology.
We demonstrate in multiple cell lines and in vivo models that DCA induced LDLR expression. We cannot exclude that multiple mechanisms underline our observations. For example, DCA inhibits HMG CoA reductase activity in liver and leukocytes (P. W. Stacpoole, 1989). This could lead to an even higher demand on exogenous cholesterol and subsequently to an increase in LDLR levels. We have excluded that ROS levels or AMPK activation play a major role in this process. In contrast, the MAPK ERK5 is essential. Berberine activates a MAPK pathway sensitive to PD98059 (J. M. Brusq et al., 2006) and U0126 (P. Abidi et al., 2005; W. Kong et al., 2004), two inhibitors that block the ERK5 pathway (Kamakura et al., 1999). Then ERK5 could also partly mediate berberine effects. In addition, we have also recently shown that the MAPK ERK5 targets Sirt1 (N. Lopez-Royuela et al., 2014) that also stabilizes LDLR protein (M. X. Miranda et al., 2015). Therefore, ERK5 could target LDLR function in multiple ways and some of them independently of MEF2 family.
In this work, our original results utilizing hematological cells have been confirmed in hepatic cells, including primary human hepatocytes, which are main regulators of cholesterol levels. Finally we also confirmed our results in the HCT116 human colon cancer cells and in two in vivo models. This highlights the generic context of our observations.

## Material and Methods

## Ethical statement

Experimental procedures were conducted according to the European guidelines for animal welfare (2010/63/EU). Protocols were approved by the Animal Care and Use Committee "Languedoc-Roussillon" (approval number: CEEA-LR-12163).

## In vivo mouse experiments

In vivo experiments were carried out using 6 to 8 weeks/old male NSG mice. Mice were bred and housed in pathogen-free conditions in the animal facility of the European Institute of Oncology-Italian Foundation for Cancer Research (FIRC), Institute of Molecular Oncology (Milan, Italy). For engraftment of human cells, 1 million AML cells were injected intravenously (i.v.) through the lateral tail vein in non-irradiated mice. NSG mice with established human AML tumors (day 80 post-graft) were treated with DCA ( $50 \mathrm{mg} / \mathrm{kg}, 1$ dose/day by gavage, starting at day 1 for 16 consecutive days). Human tumor AML cells gather in mouse spleen and bone marrow, hence we isolated mRNA from these organs. We used human-specific primers to visualize expression of human LDLR mRNA. In a different experiment B6 wt mice were treated with a daily single dose of DCA ( $50 \mathrm{mg} / \mathrm{kg} / \mathrm{day}$ ) intraperitoneally and mouse LDLR mRNA was analyzed in spleen and liver after different times.

## Cell lines and culture conditions

The leukemic human cell lines T Jurkat TAg and OCI-AML3 were grown in RPMI 1640Glutamax (GIBCO) supplemented with 5\% (Jurkat) or 10\% (OCI) FBS (Khan et al., 2016; N. Lopez-Royuela et al., 2014). Primary cells from a lymphoma B cell patient (BCL-P2) were grown in the same medium with $10 \%$ FBS. In certain experiments cells were grown in RPMI 1640 without glucose (GIBCO 11879) with the addition of 2 mM glutamine and 10 mM galactose (OXPHOS medium). The Jurkat TAg cells carry the SV40 large T Ag to facilitate cell transfection. HepG2-C3A and HuH7 cells were grown in MEM and DMEM respectively supplemented with FBS, sodium pyruvate, glutamine, penicillin and streptomycin. The HCT116 human colon cancer cells were cultured in low glucose ( 5 mM ) DMEM medium supplemented with $10 \%$ FBS. Cellular confluence during experiments was between $80-85 \%$.

## Human liver samples and preparation of PHHs cultures

Liver samples were obtained from liver resections performed in adult patients for medical reasons. The use of human specimens for scientific purposes was approved by the French National Ethics Committee. Written informed consent was obtained from each patient prior to
surgery. Human hepatocytes isolation and culture were performed as described previously (Pichard et al., 2006). Briefly, after liver perfusion, hepatocytes were counted and cell viability was assessed by trypan blue exclusion test. A suspension of $1 \times 10^{6}$ cells $/ \mathrm{mL}$ per well was added in 12-well plates pre-coated with type I collagen (Beckton Dickinson) and cells were allowed to attach for 12 h . Then, the supernatant containing dead cells and debris was carefully removed and replaced with 1 mL of serum-free long-term culture medium (Lanford medium, LNF). The number of confluent attached cells was estimated at $\sim 1.5 \times 10^{5}$ cells $/ \mathrm{cm}^{2}$.

## Reagents and antibodies

DCA was from Santa Cruz Technologies. Galactose and glutamine were from GIBCO. Human anti-LDLR-PE and IgG were from BD Biosciences and 7AAD from Beckman. The MEK5 inhibitor BIX02189 and the ERK5 inhibitor XMD8-92 were from Selleck.

## Transient transfection

Jurkat cells in logarithmic growth phase were transfected with the indicated amounts of plasmid by electroporation (J. Garaude et al., 2006; J. Garaude et al., 2008). In each experiment, cells were transfected with the same total amount of DNA by supplementing with empty vector. Cells were incubated for 10 min at RT with the DNA mix and electroporated using the Gene Pulser Xcell ${ }^{\mathrm{TM}}$ Electroporation system (Bio-Rad) at $260 \mathrm{mV}, 960 \mathrm{mF}$ in 400 $\mu 1$ of RPMI 1640. Expression of the different proteins was confirmed by western blot. The transfection efficiency in Jurkat TAg cells is between 60 and $80 \%$. OC-AML- 3 cells were transfected using Amaxa ${ }^{\text {TM }}$ D-Nucleofector ${ }^{\text {TM }}$ Lonza Kit according to manufactured protocol. In HuH7 and HCT116 cells, transfection of $30-50 \mathrm{nM}$ siRNAs was carried out using Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM (Invitrogen), according to the manufacturer's instructions. Primary hepatocytes were transfected twice at day first and third post-seeding. Cells were harvested 48 to 96 h post-transfection.

## Plasmids

The expression vectors for ERK5, the pSUPER expression vector for GFP alone or GFP plus shERK5 and the pSiren-retroQ-puro (BD Biosciences) retroviral vectors for shERK5 and control have been previously described (J. Garaude et al., 2006). Control, MEF2A and C and ERK5 siRNA were ON-TARGETplus SMARTpools (mixture of 4 siRNA) from Dharmacon.

## Counting and determination of cell viability.

Cell number, viability and cell death was analyzed with the Muse Cell Analyzer (Millipore) by incubating cells with Muse Count \& Viability and Annexin V and Dead Cell kits respectively, following manufacturer's instructions.

## ROS measurement

Cells lines were plated at 300,000 cells $/ \mathrm{ml}$ and treated with DCA for the indicated times, harvested and counted to perform further analysis. To evaluate ROS levels, we labeled cells with CellROX® Deep Red Reagent or with CH-H2DCFDA (Life Technologies) for 30 minutes and analyzed them by FACs following manufacturer's instructions.

## RT-PCR and DNA sequencing

Total RNA was extracted using NucleoSpin RNA isolation columns (Macherey-Nagel), reverse transcription was carried out using iScript ${ }^{\mathrm{TM}}$ cDNA Synthesis Kit (Biorad). Quantitative PCR was performed with KAPA SYBR Green qPCR SuperMix (Cliniscience) and a CFX Connect ${ }^{\mathrm{TM}}$ Real-Time qPCR machine (Biorad) with LDLR, LDLRAP1, ERK5 and actin primers (supplemented Fig. 6). All samples were normalized to $\beta$-actin mRNA levels. Results are expressed relative to control values arbitrarily set at 100 . Supplemental Figure 6 shows all primers used in this study.

## LDL Intake

After treatment cells were incubated with BODIPY FL LDL (Invitrogen) in PBS with 2\% FBS and incubated at $37^{\circ} \mathrm{C}$ for 30 min . Cells were then washed and suspended in 200-250 $\mu \mathrm{l}$ PBS 2\% FBS and analyzed using a Gallios flow cytometer (Beckman) and the Kaluza software.

## Flow Cytometry

Briefly, $1 \times 10^{6}$ cells were stained with antibody in PBS with $2 \% \mathrm{FBS}$ and incubated at $37^{\circ} \mathrm{C}$ for 30 min . Cells were then washed and suspended in 200-250 $\mu \mathrm{I}$ PBS $2 \%$ FBS and staining was analyzed using a Gallios flow cytometer (Beckman) and the Kaluza software.

ChIP analysis

## ChIP experiment

OCI-AML3 cells were treated with 10 mM DCA for 72 h . Ten million cells were centrifuged ( $5 \mathrm{~min} ; 1200 \mathrm{rpm}$ ) and the pellet was washed two times in 1X phosphate-buffered saline (PBS) at room temperature and suspended in 10 mL of 1 X PBS. Cells were fixed in $1 \%$ paraformaldehyde (Electron Microscopy Sciences) at room temperature for 5 min . Fixation wa lysed in 1 ml of cell lysis buffer ( 5 mM PIPES, $85 \mathrm{mM} \mathrm{KCl}, 0.5 \% \mathrm{NP} 40$, Na Butyrate $10 \mathrm{mM}+2 \mathrm{X}$ protease inhibitor cocktail (Halt ${ }^{\mathrm{TM}}$ Protease Inhibitor Cocktail, EDTA-Free (100X), Thermofischer)) at $0^{\circ} \mathrm{C}$ for 10 min . Nuclei were recovered by centrifugation ( 10 min , 5000 rpm ) at $4^{\circ} \mathrm{C}$ and lysed in $250 \mu \mathrm{l}$ nuclei lysis buffer ( 50 mM Tris- $\mathrm{HCl} \mathrm{pH} 7.5,1 \%$ SDS, 10 mM EDTA, Na Butyrate $10 \mathrm{mM}+$ Halt $^{\text {TM }}$ Protease Inhibitor Cocktail (3X)) at $4^{\circ} \mathrm{C}$ for at least 2 hours. $250 \mu \mathrm{l}$ of each sample were then sonicated 2 times for $5 \mathrm{~min}(30 \mathrm{~s}$ on $/ \mathrm{off})$ at $4{ }^{\circ} \mathrm{C}$ using a Bioruptor (Diagenode). After sonication, absorbances at 280 nm (A280) of 1/100
diluted samples were measured and A280nm and was adjusted to 0.133 with nuclei lysis buffer. One hundred microliter were used for ChIP experiments in a final volume of 1 ml . Samples were incubated under gentle agitation at $4^{\circ} \mathrm{C}$ overnight in the presence of $3 \mu \mathrm{~g}$ of either a specific antibody or a negative control. Antibodies (anti-K27Ac Ab4729 (Abcam) and negative control IgG (Diagenode)) were previously bound to DYNA Beads Protein G Novex (Life Technology) according to the supplier's recommendations. Dynabeads-bound immunoprecipitates were sequentially washed once with a low salt buffer ( 50 mM Tris- HCl pH 7.5, $150 \mathrm{mM} \mathrm{NaCl}, 1 \%$ triton, $0.1 \%$ SDS, 1 mM EDTA, 1 mM Na Butyrate + Halt ${ }^{\text {TM }}$ Protease Inhibitor Cocktail (1X)), a high-salt buffer ( 50 mM Tris- $\mathrm{HCl} \mathrm{pH} 7.5,500 \mathrm{mM} \mathrm{NaCl}$, $1 \%$ triton, $0.1 \%$ SDS, 1 mM EDTA, 1 mM Na Butyrate) and a LiCl-containing buffer ( 20 mM Tris-HCl $\mathrm{pH} 7.5,250 \mathrm{mM} \mathrm{LiCl}, 1 \% \mathrm{NP} 40,1 \% \mathrm{Na}$ deoxycholate, 1 mM EDTA, 1 mM Na Butyrate) and, then, twice with a TE buffer ( 10 mM Tris- $\mathrm{HCl} \mathrm{pH} 7.5,1 \mathrm{mM}$ EDTA, Tween $200.02 \%$ ). Samples were then eluted in $250 \mu \mathrm{~L}$ of elution buffer ( $100 \mathrm{mM} \mathrm{NaHCO} 3,1 \%$ SDS), and DNA-protein complexes were incubated at $65^{\circ} \mathrm{C}$ for 5 hours to reverse crosslinks. Samples were then treated with $100 \mathrm{mg} / \mathrm{ml}$ proteinase K and $100 \mathrm{mg} / \mathrm{ml}$ RNAse A at $45^{\circ} \mathrm{C}$ for 2 hours to digest proteins and contaminating RNA. DNA was purified with an extraction kit (NucleoSpin Gel and PCR clean-up, Macherey-Nagel) according to the manufacturer's recommendations and qPCR analysis was performed using the Roche LightCycler 480 realtime PCR system. The data were normalized with inputs taken from samples before the immunoprecipitation and treated under the same conditions. The primers used to amplify various regions of LDLR gene promoter.

## Statistical analysis

The statistical analysis of the difference between means of paired samples was performed using the paired t test. The results are given as the confidence interval ( $*: \mathrm{p}<0.05,{ }^{* *}$ : $\mathrm{p}<0.01$, ***: $\mathrm{p}<0.005$ ). All the experiments described in the figures with a quantitative analysis have been performed at least three times in duplicate. Other experiments were performed three times with similar results.

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## Figure Legends

Figure 1. OXPHOS induced LDLR expression and LDL uptake. A) The hematopoietic cell lines Jurkat and OCI-AML3 and primary cells from a BCL patient (BCL-P2) as well as HepG2-C3A and Huh7 hepatic cell lines were treated with 10mM DCA for 24 h and LDLR mRNA was analyzed by RT-qPCR. B) two hepatic cell lines were treated for 72 h with 5 mM DCA and LDLR protein in plasma membrane was analyzed by FACs. C) Cells were treated as in (B) and fluorescent LDL intake analyzed by FACs. D) OCI-AML3 cells were grown in OXPHOS medium for 2 weeks and LDLR expression (left) and LDL intake (right) were analyzed by FACs. E) BCL-P2 cells were treated with 5 mM DCA for 1 week (left) or were grown in OXPHOS medium for 2 weeks (center) and LDLR protein in plasma membrane analyzed by FACs. LDL intake (right) was analyzed in cells growing in OXPHOS. The bar graphs represent means $\pm \mathrm{SD}$ of 3 independent experiments performed in triplicate; * $\mathrm{p}<0.05$, ** $\mathrm{p}<0.01,{ }^{* * *} \mathrm{p}<0.005$ student t -test compare to control cells.

Figure 2. DCA induced LDLR expression in primary human hepatocytes. A) Primary hepatocytes were treated with the indicated concentrations of DCA for the indicated times. B) Cells were treated at time 0 with DCA and some were treated every 24 h before harvesting as indicated. LDLR mRNA was analyzed by RT-qPCR. The bar graphs represent means $\pm$ SD of 3 independent donors performed in triplicate; * $\mathrm{p}<0.05,{ }^{* *} \mathrm{p}<0.01,{ }^{* * *} \mathrm{p}<0.005$ student t -test compare to control cells or as depicted in the graphic.

Figure 3. Increase in ROS levels was not required for LDLR expression. A) Both hepatic cell lines were treated with 2 mM NAC 1 h before adding DCA ( 10 mM ) for 24 h . Cells were labeled with CH-H2DCFDA and analyzed by FACs for ROS production. LDLR mRNA (B) or protein (C) from these cells was analyzed as described in Fig. 1. D) Primary hepatocytes from 2 independent donors were treated for 6 and 24 h as in (A) but with two different DCA concentrations before analyzing LDLR mRNA expression. Results represent the means $\pm$ SD of these donors with experiments performed in triplicate. The data represent means $\pm \mathrm{SD}$; * $\mathrm{p}<0.05,{ }^{* *} \mathrm{p}<0.01,{ }^{* * *} \mathrm{p}<0.005$ student t -test compare to cells non treated with DCA.

Figure 4. DCA induced LDLR expression in vivo. A) NSG mice were engrafted with primary human AML cells. At day 80 post-graft, they were treated with DCA $(\mathrm{n}=4)$ or leave untreated $(\mathrm{n}=4)$. At day 140 , mRNA from bone marrow or spleen was isolated and human

LDLR mRNA expression was quantified by qPCR. B) B6 wt mice ( $\mathrm{n}=4 / 5$ per group) were treated with a dose of DCA ( $50 \mathrm{mg} / \mathrm{kg}$ ) everyday intraperitoneally and mouse LDLR mRNA was analyzed in spleen and liver at different times. The data represent means $\pm$ SD; * $\mathrm{p}<0.05$, ** $\mathrm{p}<0.01, * * * \mathrm{p}<0.005$ student t -test compare to non treated mice.

Figure 5. ERK5 controled LDLR expression and LDL uptake. A) $10^{7}$ Jurkat-TAg cells were transfected with $5 \mu \mathrm{~g}$ of the empty pSUPER Neo vector (control) or with this vector containing a small hairpin RNA for ERK5 (shERK5) or with a pcDNA vector expressing ERK5 (ERK5). Forty-eight hours later mRNA expression of the whole population was analyzed by qPCR and represented as the $\%$ of mRNA compared to cells transfected with the empty vector. B) Primary hepatocytes were transfected with control siRNA (control) or with siRNA against ERK5 (siERK5). 96 h later mRNA was collected and ERK5 and LDLR mRNA expression was analyzed by qPCR. C) Jurkat cells were transfected with ERK5 as described in (A) and LDLR plasma membrane protein (left) and LDL intake (right) were analyzed by FACs. D) Jurkat (left and center) and OCI-AML3 (right) cells were treated with $5 \mu \mathrm{M}$ of the MEK5 inhibitor BIX02189 for 24 h and LDLR protein (left) or LDL intake (center and right) were analyzed by FACs. Bar graphs represent means $\pm \mathrm{SD} ;{ }^{*} \mathrm{p}<0.05$, ** $\mathrm{p}<0.01,{ }^{* * *} \mathrm{p}<0.005$ student t -test compare to empty vector transfected cells (control).

Figure 6. AMPK did not regulate DCA-induced LDLR expression and LDL uptake. Two different hematopoietic cell lines were treated with 5 mM metformin for 24 h and LDLR mRNA (A), protein (B) and LDL uptake (C) were analyzed. D) HepG2-C3A cells were treated as in (A) and LDL uptake was measured. E) HCT116 cells were transfected with 2 small interference RNA (siRNA) for AMPKa or with control siRNA and treated with 20 mM DCA for 6 h before mRNA analysis.

Figure 7. DCA required the transcription factor MEF2 to target LDLR promoter. A) OCI-AML3 cells were transfected with 40 nM siRNA control or with 20 nM siRNA for each MEF2A and MEF2C (siMEF2). Twenty-four hours later cells were incubated for 24 h with 10 mM DCA. mRNA expression was analyzed by qPCR and represented as the $\%$ of mRNA compared to cells transfected with the empty vector. B) OCI-AML3 cells were incubated for 72 h with 10 mM DCA. Cells were prepared for ChIP analysis using an antibody against H3 acetylation on lysine 27. Acetylation was revealed at different points of the LDLR promoter
by using specific oligonucleotides. Bar graphs represent means $\pm \mathrm{SD} ; * \mathrm{p}<0.05$, ${ }^{* *} \mathrm{p}<0.01$, *** $\mathrm{p}<0.005$ student t -test compare to empty vector transfected cells (control).

Figure 8. DCA induced LDLRAP1 expression. A) Both hepatic cell lines or primary hepatocytes were treated with DCA as in Fig. 1 and 2 and LDLRAP1 mRNA was analyzed. B) OCI-AML3 cells (left) and primary cells from a BCL patient (BCL-P2; rigth) were treated with 5 mM DCA or grown in OXPHOS medium for 2 weeks and LDLRAP1 mRNA was measured. C) Primary hepatocytes were transfected as in Fig. 5 and expression of LDLRAP1 was analyzed by q-PCR. The bar graphs represent means $\pm$ SD of 3 independent experiments performed in triplicate; * $\mathrm{p}<0.05,{ }^{* *} \mathrm{p}<0.01,{ }^{* * *} \mathrm{p}<0.005$ student t -test compare to control cells.







FIG 1



FIG 2



FIG 3


FIG 4


FIG 5





Supplemental Figure 1. A) Jurkat cells were treated with 10 mM DCA for 24 h and LDLR protein was analyzed by FACs.
B) HepG2 cells were treated with 10 mM DCA for 72 h and LDL intake was analyzed by FACs.


Supplemental Figure 2. (A) OCI- AML3 or (B)BCL-P2 cells were grown in OXPHOS medium for 2 weeks before mRNA analysis.


Supplemental Figure 3. HepG2 and HuH 7 cells were grown in OXPHOS medium for 2 weeks before analyzing LDLR protein (upper panel) and LDL intake (lower panel) by FACs.


Supplemental Figure 4. A) Huh7 cells were transfected with control siRNA or with siRNA against ERK5 (siERK5). 36 h later mRNA was collected and ERK5 and LDLR mRNA expression was analyzed by qPCR
B) HepG2C cells were transfected with control siRNA or with siRNA against MEF2A and MEF2C (siMEF2A/C). 36 h later mRNA was collected and MEF2A/C and LDLR mRNA expression was analyzed by qPCR
C) LDLRAP1 mRNA was analyzed from Huh7 cells as in A


Supplemental Figure 5. Primary BCL-P2 cells were treated with $5 \mu \mathrm{M}$ of the MEK5 inhibitor (BIX02189) or the ERK5 inhibitor(XMD8-92) for 48 h and LDLR protein was analyzed by Facs.

## L'inhibiteur PDK1 «Le Dichloroacétate » contrôle l'homéostasie du cholestérol à travers la voie de signalisation ERK5 / MEF2

L'athérosclérose est la première cause de mortalité au niveau mondial, et à l'origine de la plupart des maladies cardio-vasculaires. L'accumulation du cholestérol dérivé de lipoprotéines de faible densité, ou LDL, constitue l'un des éléments clés de l'athérosclérose. Environ 70\% du cholestérol LDL est éliminé par le foie par l'intermédiaire des récepteurs de lipoprotéines de faible densité (LDLR). Ces récepteurs permettent l'endocytose du LDL afin qu'il soit dégradé ultérieurement par des enzymes microsomales. Le métabolisme du glucose régulerait également le métabolisme du cholestérol d'une manière indirecte. Le dichloroacétate (DCA) inhibe la glycolyse et induit une phosphorylation oxydante (OXPHOS) et diminue le cholestérol plasmatique dans plusieurs modèles animaux et humains. Le DCA a été utilisé en cliniques, mais a été interrompu suite à des effets neuropathologiques.

Ici, nous rapportons le mécanisme d'action. Le DCA a augmenté l'expression de MAPK ERK5 par l'activation du facteur de transcription MEF2. Le promoteur de LDLR contient plusieurs sites de liaison MEF2. En outre, l'analyse transcriptomique révèle que ce gène était parmi les plus régulé dans les cellules exprimant un petit ARN en épingle à cheveux pour ERK5 (shERK5).

Nous avons observé dans plusieurs cellules hématopoiiétiques et hépatiques que le DCA augmente l'expression de l'ARNm et la protéine LDLR. Un effet similaire a été observé lorsque des cellules ont été cultivées dans un milieu sans glucose mais contenant du galactose et de la glutamine (milieu OXPHOS). Un effet fonctionnel a été également observé grâce à une augmentation de l'absorption du cholestérol LDL fluorescent. De même, les hépatocytes primaires humains montrent sous le traitement DCA une augmentation d'expression de l'ARNm du LDLR. Puisque le DCA régule l'état oxydatif de la cellule en entraînant une production de ROS. Nous avons donc étudié le rôle des ROS dans la régulation du récepteur LDL et observé que ce mécanisme était indépendant de ROS. Toutefois, nous confirmons in vivo dans deux modèles de souris que le traitement DCA augmente l'expression de l'ARNm du LDLR.

Une étude plus approfondie du ces mécanismes a révélé que ERK5 régule l'expression du LDLR et que l'inhibition chimique ou par siRNA de ERK diminue l'expression de LDLR et par conséquence l'absorption du LDL. En outre, la régulation du LDLR par ERK5 est médiée par MEF2 alors que ce mécanisme est indépendant de la voie MAP kinase. Nous avons également observé qu'une voie similaire régule l'expression d'une protéine adaptatrice nécessaire à l'internalisation du récepteur aux LDL 1 (LDLRAP1) qui interagit avec la queue cytoplasmique du LDLR.

En résumé, nous démontrons dans de diverses lignées cellulaires ainsi que dans des modèles in vivo que le DCA induit une expression du LDLR. Nous mettons aussi l'accent sur l'importance de la voie de signalisation ERK5-MEF2 dans les futures recherches concernant le métabolisme lipidique.

## 9. Discussion

Cells respond to extra cellular stimuli via activation of special signaling pathways, which are essential in proliferation, development and cellular response to the environment. Any dysregulation of these intracellular signaling pathways can initiate tumor-genesis enabling tumor cells to overcome the pro-apoptotic signals, modify metabolism and escape the immune system resulting in excessive proliferation. Complete understanding of these pathways and their role in tumor development could be vital to improve cancer therapies.

MAP Kinase pathways are activated by growth factors or stress and regulate many cellular responses such as proliferation, differentiation and survival. The ERK5 pathway has been identified twenty-two years ago and its role has been intensively studied. Our group has investigated the ERK5 signaling pathway in detail in recent past (Charni et al., 2009; Johan Garaude et al., 2006; Khan et al., 2016; Nuria Lopez-Royuela et al., 2014; Moeez G Rathore et al., 2012).

Metabolism in most types of tumor cells is different than in normal cells since they have high glucose consumption for glycolysis. Normal cells use glucose as primary energy source and produce ATP through oxidative phosphorylation (respiration) but tumor cells process glucose by fermentation despite the presence of ample oxygen, a process first discovered by Otto Warburg and termed as Warburg Effect (Warburg et al., 1927). The amount of energy produced by fermentation is less yet this metabolic alteration offers selective advantages to tumor cells (Martin Villalba et al., 2013). This change of metabolic pathways has attained high attention in recent times and is considerably discussed.

Our lab established the relationship between energy metabolism and ERK5 (Moeez G Rathore et al., 2012). These metabolic changes are directly connected to MHC-I expression, a molecules involved in immune surveillance (Charni et al., 2009). Those tumor cells that escape immune attack continue to proliferate thus, immune system selects tumor cell metabolism. Our results show that regular expression of ERK5 is essential for normal metabolism. OXPHOS induces expression of ERK5 in different leukemia cell lines while MHC-I expression also increased on tumor cell surfaces when they perform OXPHOS. Thus the ERK5 pathway connects tumor metabolism to immune surveillance (Charni et al., 2009; Khan et al., 2016; Martin Villalba et al., 2013).

Cells require ERK5 for survival and proper biological functions because Erk5 -/- mice show abnormal organ development and are prone to early death (Sohn et al., 2002). Similarly, leukemic cells expressing shERK5 have defective mitochondrial activity and in OXPHOS condition Jurkat cells expressing shERK5 display a high rate of cell death (Moeez G Rathore et al., 2012).

Tumor metabolism is a dynamic process and during tumorigenesis a series of metabolic remodeling occurs where tumor cells sometimes increase OXPHOS (Jose \& Rossignol, 2013; Martin Villalba et al., 2013). But how these cells protect themselves from deleterious effects of ROS produced from mitochondria during the process of OXPHOS is unknown. These cells must acquire an anti ROS mechanism for their survival. Mitochondrial activity is the main source of ROS production but also involved in protection against ROS. In first part of my PhD we explained the mechanism of cellular antioxidant response under mitochondrial activity. We observed that in shERK5 expressing cells, KEAP1 is strongly down regulated. KEAP1 is a protein that is responsible for ubiquitinational degradation of NRF2, a master regulator of the cellular antioxidant mechanism. Our results show that when cells are forced to perform OXPHOS they increased ERK5 expression. ERK5 activates MEF2 family of TFs, which binds and triggers miR23a promoter (Moeez G Rathore et al., 2012). This newly synthesized miR23a suppresses the KEAP1 protein by targeting 3'UTR of KEAP1 mRNA.

The down regulation of KEAP1 renders NRF2 free in cytoplasm which translocates to nucleus and binds to the antioxidant response element (ARE) present in the upstream promoter region of many genes responsible for antioxidative response and thus initiates their transcription (Itoh et al., 1997; Itoh et al., 2004). Mitochondrial activity leads to ROS production depending on cell type (Nerea Allende-Vega et al., 2015) and ROS can activate NRF2 (Kensler \& Wakabayashi, 2010) so we further investigated the role of ROS on this pathway. Interestingly treatment of N-acetyl-L-cysteine (NAC), a ROS scavenger molecule, did not affect this mechanism. This shows that ROS is not involved in this process, which is already shown in AML cells (Stuart A Rushworth et al., 2012). Thus we observed that cells performing OXPHOS initiate an anti oxidant response mediated by ERK5-MEF2 pathway and this phenomenon is independent of ROS production (Khan et al., 2016).

Additionally during this we observed that human NRF2 gene promoter has many binding sites for the MEF2 family and further investigation reveled that ERK5-MEF2 pathway can directly regulate de novo expression of NRF2. Consequently, ERK5 controls cell metabolism and anti
oxidant response in human cell lines through the expression of NRF2 and the ERK5 substrate MEF2 acts as an intermediary molecule in the regulation of antioxidant response. ERK5 and NFR2 are redox sensitive proteins and their expression is induced under $\mathrm{H}_{2} \mathrm{O}_{2}$-induced oxidative stress (Johan Garaude et al., 2006; Khan et al., 2016). Therefor, our results showed that ERK5 mediated activation of NRF2 protect cells from oxidative stress (Khan et al., 2016) and ERK5 provides protection against oxidative stress directly and indirectly via activation of the cellular antioxidant response (Khan et al., 2016; Nuria Lopez-Royuela et al., 2014). This can explain that down regulation of ERK5 in leukemic cells increase oxidative stress-induce apoptosis (Moeez G Rathore et al., 2012).

Dichloroacetate (DCA), a small molecule activates pyruvate dehydrogenase (PDH) complex by inhibiting pyruvate dehydrogenase kinase1 (PDK1) thus directs pyruvate to enter the Krebs's cycle to obtain metabolic energy through OXPHOS in tumor cells. DCA has already been shown to have strong anti-tumor effects (Bonnet et al., 2007; Flavin, 2010; E. Michelakis et al., 2010). Our lab previously showed that DCA increases MHC-I expression by switching metabolism moreover it also sensitizes cells to chemotherapy agents via AMPK pathway depending of p53 status (Nerea Allende-Vega et al., 2015; Seyma Charni et al., 2010). This small molecule has many other physiological effects including in lipid metabolism since it was used in the past to lower plasma cholesterol (P. W. Stacpoole, 2011; P. W. Stacpoole et al., 1983). DCA decreases glucose catabolism and stimulates OXPHOS through mitochondrial activity. Cells in this case could rely on fatty acid oxidation, which suggest that DCA could increase lipid catabolism.

High level of plasma lipids, particularly low-density lipoprotein (LDL), causes various types of cardiovascular diseases including atherosclerosis resulting in heart attacks. Liver mainly clears plasma cholesterol through expression of low-density lipoprotein receptors (LDLR). Increased expression of LDLR improves the liver efficacy directly (Weijia Kong et al., 2004; Melroy X Miranda et al., 2015). These LDLR receptors are involved in receptor-mediated endocytosis of LDL cholesterol, which are transported inside the cells for further metabolism through lysosomal degradation for production of energy. The expression of LDLR is negatively regulated by intracellular cholesterol level. Under cholesterol depletion these receptors are transcriptionally activated by sterol regulatory element-binding protein (SREBP), which binds to sterol regulatory element present in the promoter region of LDLR gene. When cholesterol level within cells is augmented LDLR is degraded by proprotein
convertase subtilisin/kexin type 9 (PCSK9), which inhibits its recycling (Dong et al., 2015), thus cholesterol influx is withdrawn.

DCA was used in past as cholesterol lowering drug with promising results in various animal models (Gérard Ribes et al., 1979; Peter W Stacpoole et al., 1978). It was also used in clinics to treat patients suffering from familial hypercholesterolemia (FH) and plasma cholesterol level was drastically reduced in these patients but was discontinued due to development of neuropathological effects (George W Moore et al., 1979). The underlying mechanism was unknown and the second part of my PhD thesis described the mechanism of action. We performed a transcriptome analysis in various cell lines treated with DCA and observed that $L D L R$ was one of the most upregulated genes. We also observed that $L D L R$ gene is down regulated in hematopoietic cells expressing a small hairpin RNA for ERK5 (shERK5). Using genomic browser UCSC revealed many MEF2 binding sites on promoter region of this gene.

We started testing this hypothesis and observed that DCA up regulate LDLR expression in hematopoietic as well as in hepatic cells, including human primary hepatocytes. To observe the physiological effects we incubated cells after DCA treatment with fluorescent LDL and found that DCA treatment upsurges LDL uptake compared to non-treated cells. Blocking ERK5 activity either chemically or by siRNA against ERK5 suppresses LDLR expression and reduced LDL uptake. Further investigation disclosed that ERK5 mediated regulation of LDLR required MEF2 signaling. Since DCA treatment produces ROS depending of cell type, we investigated the role of ROS and observed that LDLR regulation by ERK5 was independent of de novo ROS production.

Berberine, an extract from Chinese herbs was shown to regulate LDLR expression through the AMPK pathway (Weijia Kong et al., 2004) and DCA activated this pathway in hematopoietic cells (Nerea Allende-Vega et al., 2015). So we investigated the role of AMPK in DCA mediated LDLR expression and observed that this pathway was not responsible for LDLR regulation in our case. Firstly, metformin activates AMPK while in our case it decreases LDLR expression and LDL cholesterol uptake. Similarly blocking AMPK pathway via siRNA did not inhibit LDLR up regulation after DCA treatment. These results showed that DCA mediated cellular OXPHOS leads to activation of ERK5 MEF2 pathway that triggers transcriptional activation of $L D L R$ gene. To further prove this idea we checked the promoter activity and observed that DCA treatment significantly stimulate the promotor of

LDLR gene. Correspondingly when cells were grown in OXPHOS medium they also exhibited upregulation of LDLR expression and LDL uptake.

Interestingly, DCA regulates SIRT1 expression (Nuria Lopez-Royuela et al., 2014), which results in PCSK9 reduction (Melroy X Miranda et al., 2015) thus inhibiting LDLR degradation. Similarly, statins, which are used intensively for regulating plasma cholesterol through inhibition of cellular cholesterol synthesis, also activate ERK5 (Chu, Duellman, Weaver, Tao, \& Yang, 2015; K. Wu, Tian, Zhou, \& Wu, 2013). Moreover, decreasing ERK5 or MEF2 with siRNA statistically decreased LDLR expression. Thus these results showed that ERK5-MEF2 pathway regulates lipid metabolism through distinctive paths and could be an interesting tool to focus for developing new lipid lowering drugs.

During this work we also realized that the ERK5 pathway regulates various other genes involved in lipid metabolism. Various fatty acids transporters like CD36, CD36L1 and LRP1 are at least partly regulated by ERK5. Moreover our preliminary data show that proteins involved in fatty acid esterification (ACSLs), mitochondrial transportation (CPT) and betaoxidation (ACADVL) are also regulated by ERK-MEF2 pathway.

Tumor cells rely on lipids metabolism in order to meet extra energy requirement and inhibiting lipid metabolism inhibit tumor growth. Suppression of genes involved in lipid metabolism results in inhibited cancer progression (Currie et al., 2013). Moreover it is also recently shown that CD36 is involved in tumor metastasis and its inhibition impairs metastasis (Pascual et al., 2017). This has opened a new window in developing strategies against cancer therapies since lipid metabolism and cancer development along with tumor progression are linked in various ways. Thus ERK MEF2 pathway offers additional intensive investigations for complete understandings before clinical implementation.

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