UNIVERSITÀ DEGLI STUDI DI TRIESTE

XXX CICLO DEL DOTTORATO DI RICERCA IN

BIOMEDICINA MOLECOLARE

DEPDC1A, a novel SREBP1 cofactor, regulates fatty acid metabolism in breast cancer

Settore scientifico-disciplinare: **BIO/13**

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To my family

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ABSTRACT

Breast cancer (BC) figures as the most frequently diagnosed and the leading cause of cancer-related deaths among women worldwide. Despite considerable progress has been made in cancer detection and therapy assignment, several BCs become resistant to therapy and, moreover, a considerable proportion of patients develops metastasis during therapy or experiences relapse.

Growing body of evidence indicates that alterations in tumour metabolism are linked to therapeutic resistance, tumour relapse and dissemination. These altered metabolic traits are caused by genetic alterations and environmental factors.

The most frequently mutated gene in BC is the tumor suppressor *TP53,* a well-characterized transcription factor, which plays a central role in cellular homeostasis and prevention of tumour growth. In BC missense mutations occur very often in its DNA binding domain, providing neomorphic mutant p53 proteins that lose the wildtype onco-suppressive functions and acquire instead new oncogenic properties (Gain-of-Function). Indeed, mutant p53 proteins establish aberrant interactions with different transcription factors, thus inducing oncogenic transcriptional programs and metabolic reprogramming.

Our previous work outlined a mutant p53 driven signature that promotes aggressiveness in BC in which *DEP domain containing 1A* (*DEPDC1A)* emerged as an important mediator of migration and invasiveness (Girardini et al., 2011). DEPDC1A expression is almost undetectable in normal cells, but it is overexpressed in different cancers and its overexpression is associated with poor prognosis. DEPDC1A is a transcriptional cofactor that exists in two different splice variants V1 and V2, but its role in oncogenesis, as well as in a physiological context, remains elusive.

Here we show, through a high-throughput transcriptional analysis, that DEPDC1A is able to impinge on lipid metabolism. In particular we observed that mRNAs belonging to the fatty acids biosynthesis pathway genes *ATP-Citrate Lyase* (*ACLY*), *Stearoyl-CoA Desaturase 1* (*SCD1*) and *Elongation Of Very Long Chain Fatty Acids 6* (*ELOVL6*) were consistently downregulated upon *DEPDC1A* silencing suggesting a key role for this factor in controlling fatty acid metabolism in cancer cells. Indeed, ablation of

DEDPC1A caused a significant decrease of lipid droplets content and fatty acid desaturation in MDA-MB-231 cell line.

ACLY, *SCD1* and *ELOVL6*, as a part of fatty acids biosynthesis pathway, are specific targets of Sterol Regulatory Element Binding Protein (SREBP) transcription factors, master regulators of lipid metabolism. Interestingly, protein co-immunoprecipitation and Chromatin Immunoprecipitation assays demonstrated that DEPDC1A physically interacts with SREBP1 and that it is required for an efficient transcriptional activation of this particular subset of genes, thus acting as transcriptional cofactor of SREBP1.

Finally, we showed that DEPDC1A, through SCD1 upregulation, is able to promote aggressive phenotypes, such as migration, and that DEPDC1A overexpression in normal cells is sufficient to induce sensitization toward SCD1 inhibition.

This study unveils a novel oncogenic transcriptional program induced by the aberrant interaction between DEPDC1A and SREBP1 transcription factor that is able to induce fatty acid biosynthesis and desaturation in cancer cells and to establish a metabolic addiction that can be potentially exploited in cancer therapy.

INTRODUCTION

1.1 Breast cancer: current knowledge and challenges

Among all cancer types, breast cancer figures as the most frequently diagnosed and the leading cause of cancer-related deaths among women worldwide (Torre et al., 2015). Breast cancer is considered a heterogeneous disease composed by several subtypes that differ at the level of molecular landscape, disease progression and response to therapy (Curtis et al., 2012).

Currently, in clinical practice, diagnosis and treatment decisions are based on molecular classification that takes into account the hormone receptor status and grossly subdivides breast cancers into six major subtypes: luminal A, luminal B, Her2, claudin low, basal-like breast cancer and normal-like (Perou et al. 2000; Prat et al. 2010). Although these molecular and clinico-pathological parameters can stratify breast cancers according to their aggressiveness and metastatic proclivity, in many cases they are not sufficient to predict their outcome and response to therapy. In fact, on one hand, several breast cancers are unresponsive to targeted- and chemotherapy and, on the other, a considerable proportion of patients develops metastatic breast cancer during therapy or experiences relapse (Mego et al., 2010). In both cases, this indicates that, despite advances in detection and therapy assignment, there are still molecular underpinnings that are not fully understood and that therapies tailored on the primary tumour are not effective in treating metastatic disease. The main limitation is that the therapeutic choices do not take into account the heterogeneity of the primary tumour. Moreover, little is known about the biological features of those cells that are able to metastasize or that remain dormant but still retain the ability to drive tumour recurrence (Mego et al., 2010). For these reasons, it is becoming clear that current histopathological classification, although useful, has to be integrated with molecular data derived from dedicated research and genomic, transcriptomic and proteomic analyses of metastatic breast cancers in order gather molecular and phenotypic information to be translated into more personalized and precise treatment choices (Curtis et al., 2012; Norum and Sorlie, 2014).

In this vein, recent studies indicate that metabolic reprogramming is crucial for the persistence of a small population of breast cancer cells that survives therapeutic intervention and that gives rise to tumour recurrence (Minimal Residual Disease).

Indeed, altered metabolic traits are observed to occur generally across many types of cancer cells. Environmental cues and cell intrinsic features of cancer cells have both shown to be at the basis of the metabolic reprogramming (Lyssiotis and Kimmelman, 2017), which, in turn, is linked to drug resistance and relapse. The inhibition of metabolic pathways *in vivo* has been shown to decrease breast cancer reappearance and metastatic growth (Sounni et al., 2014; Havas et al., 2017; Pascual et al., 2017), indicating that hitting tumour metabolism can be a valid opportunity to target residual breast cancer and metastasis.

1.2 Metabolic rewiring in cancer, an overview

Metabolism is a complex network of multi-enzymatic pathways that work co-ordinately to: a) obtain energy through the degradation of complex molecules (nutrients) and b) transform simple molecules into cellular structural and functional components (e.g. membrane lipids, nucleotides, proteins etc.). -

Tumour cells reprogram metabolic pathways to sustain their bio-energetic and biosynthetic needs according to both internal and external cues with a remarkable plasticity. This metabolic rewiring is instrumental for tumour formation and metastatic ability. In particular, altered metabolism, i.e. upregulated or suppressed conventional metabolic pathways upon tumorigenic mutations, sustains anabolic growth in nutrient-replete environment and catabolic pathways are hijacked to support cell survival in adverse conditions (DeBerardinis and Chandel 2016).

The first metabolic pathway that was shown to be altered, is glycolysis. It is known from early observations of the german scientist Otto von Warburg that quiescent and differentiated cells display a metabolism in which the glucose is fully degraded in CO2 by glycolysis coupled to the Tri-Carboxylic-Acid Cycle (TCA). On the contrary, rapidly proliferating cells, like cancer cells, switch to aerobic glycolysis in which the glucose is fermented to lactate even when oxygen is present (Warburg effect) (Warburg et al., 1925; Warburg, 1956) (Figure 1).

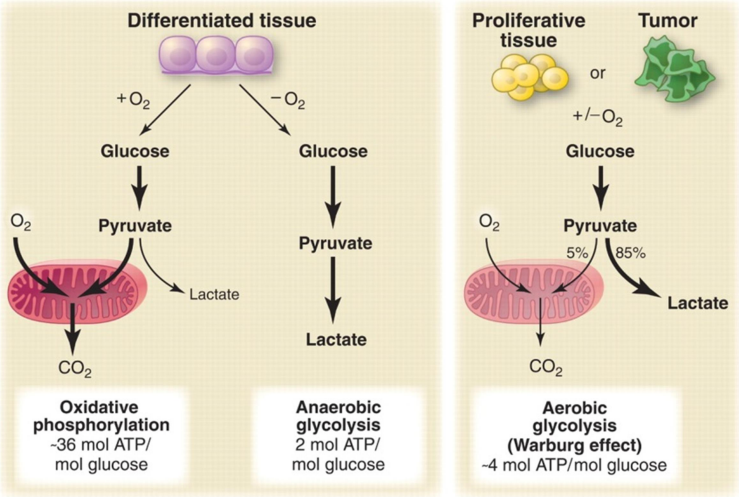


Figure 1. The Warburg Effect. Differences between oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (Warburg effect). Non proliferating (differentiated) tissues metabolize glucose to pyruvate via glycolysis and then completely oxidize most of that pyruvate in the mitochondria to CO_2. When oxygen is limiting, cells can ferment pyruvate in lactate (anaerobic glycolysis). In contrast, cancer cells tend to convert most glucose to lactate regardless of whether oxygen is present (aerobic glycolysis). This property is common to normal proliferative tissues. (Vander Heiden et al., 2009).

The reasons why proliferating cells prefer metabolism with a lower energy yield (2 *versus* 36 ATP for each glucose molecule) when the energy demand is the highest (such as during mitosis) puzzled the scientists for decades.

The first hypothesis for this *conundrum* was that the mitochondria of cancer cells were not properly functioning, but this was disproved by the evidence that cancer cells do not have defects in oxidative metabolism (Frezza and Gottlieb, 2009); now it is clear that the reduced energy amount derived from aerobic glycolysis is compensated by an increased glucose absorption, which in many cases is sustained by the overexpression of the glucose transporters in cells membranes (GLUTs), and the overexpression of many glycolytic enzymes such as Hexokinase 1 and 2 (HKs), Glyceraldeyde-3-Phosphate-Dehydrogenase (GAPDH) and Lactate Dehydrogenase (LDH) (Macheda et al., 2005; Marin-Hernandez et al., 2006).

Moreover aerobic glycolysis provides proliferating cells with several advantages, such as a shorter and faster way to produce ATP and the production of intermediates for many biosynthetic pathways. Moreover it allows the cells to use the mitochondria as functional biosynthetic organelles rather than ATP factories (Ward and Thompson, 2012), as for instance glycerol and citrate in the mitochondria are converted into lipids, oxaloacetate and pyruvate are used for the biosynthesis of non-essential amino acids,

ribose sugar for nucleotide biosynthesis, and finally the pentose phosphate pathway for NADPH production. Aerobic glycolysis furthermore eliminates the dependency of energy production from oxygen concentration, which, in the case of rapidly proliferating tumours, would be a limiting factor (Cairns et al., 2011). Finally, the lactic acid, which is massively produced as a waste of aerobic glycolysis, is secreted in the microenvironment, which becomes acidic, thus boosting tumour invasion. Lactate can also be taken up by adjacent stromal cells and used as an energy substrate to support growth or to generate and secrete pyruvate, which is then taken up by the cancer cells in a paracrine loop (Romero-Garcia et al., 2016).

Since the seminal discovery of Warburg, many other observations have been made to confirm and extend the concept that proliferating cells have a different metabolism comparing to the quiescent ones, and that proliferating cancer cells display altered metabolic pathways (Figure 2). Indeed, alterations in lipid homeostasis have been shown to be required for tumour growth and progression. In particular, it has been demonstrated that cancer cells display an increased lipid uptake and, unlike non transformed cells, activate *de novo* lipid biosynthesis for the production of fatty acids and cholesterol (Santos and Schultze 2012; Luo et al., 2017); lipids are known to be required for biomass generation, signalling regulation and energy production and hence they are crucial for proliferating cells. Interestingly, recent findings revealed that cancer stem cells, although not rapidly proliferating, are addicted to specific lipid transporters and lipid modifications (Pascual et al., 2017; Li et al., 2017; Noto et al., 2017).

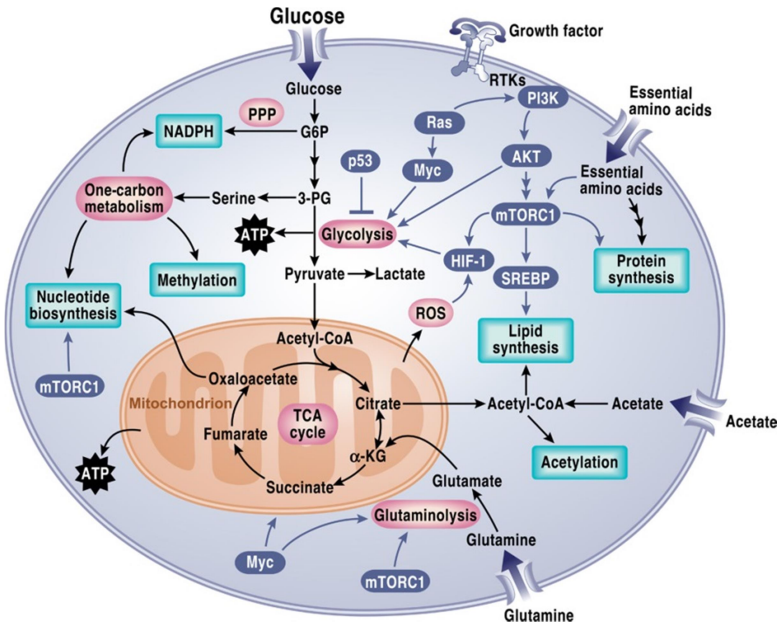


Figure 2. Signalling pathways regulating cancer metabolism. Schematic representation of the signalling pathways that regulate cancer metabolism. Alterations of the PI3K/Akt/mTOR, p53, MYC or HIF1 signalling pathways induce the Warburg effect, anabolic growth, and cancer progression (DeBerardinis and Chandel, 2016).

Metabolic reprogramming discriminates cancer cells from the surrounding cells and from most other non-transformed cells in the body. Hence the major goal is to exploit this diversity to selectively eradicate the disease. Metabolic characterization of cancer cells will help in understanding those processes without which tumour growth and dissemination would be impaired; in particular, the enzymes that belong to those metabolic pathways can be potentially inhibited in order to deprive cancer cells of crucial metabolites without affecting normal tissues. In this line many efforts are ongoing in this field to inhibit the Warburg effect (targeting tumor specific glycolytic enzymes or glucose transporters) or the lipid biosynthetic pathway (Hay 2016; Roehrig and Schulze 2016).

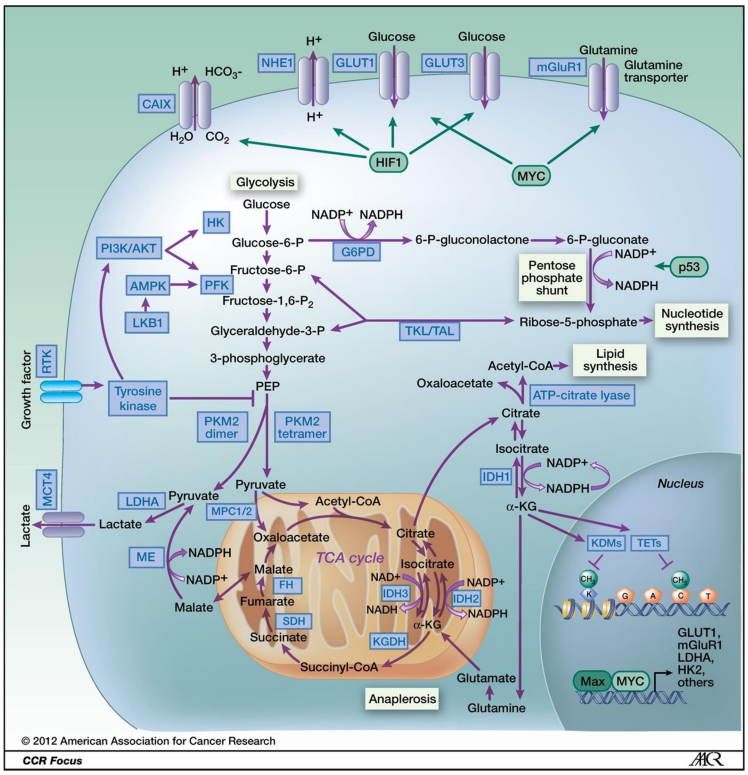


Figure 3. Targeting cancer metabolism. Schematic illustration of metabolic pathways that can be targeted in malignant cells. Blue boxes indicate enzymes and transporters that may be useful therapeutic targets in cancer. Green ovals represent transcription factors that alter metabolic pathways. (Teicher et al., 2012).

1.2.1 Molecular underpinnings of breast cancer metabolic reprogramming.

The molecular mechanisms that underlie metabolic shifts in cancer cells are complex and still understudied. Many breast cancers harbour activating mutations in genes like growth factor receptors (RTKs), G-proteins (RAS), and kinases (PI3K, AKT), or inactivating mutations in negative regulators of proliferation and metabolism like PTEN, TP53, VHL, RB. These genetic lesions cause the constitutive activation of growth factor signalling pathways that lead to aberrant cell proliferation and cancer-associated metabolic reprogramming (Nik-Zainal et al., 2016).

Hyper-activation of the PI3K/AKT/mTOR axis is one of the most frequent events in cancer (Hay 2016) and, alone, it can trigger the Warburg effect also in non-transformed cells (DeBerardinis et al., 2007). Indeed, in breast cancer it leads to i) an increased glucose uptake through the over-expression of glucose transporters (GLUT1-4) on cell membranes (Godoy et al., 2006; Garrido et al., 2013); ii) induction of glutamine uptake and catabolism; iii) fatty acids biosynthesis in breast cancer through the upregulation of ATP-Citrate Lyase (ACLY), Acetyl-CoA Carboxylase (ACC), and Fatty Acid Synthase (FASN) enzymes; iv) cholesterol biosynthesis through deregulation of downstream SREBP2 transcription factors (Freed-Pastor et al., 2012).

To induce the metabolic reprogramming a broad change in gene expression is also required, which can be accomplished through the alteration of the activity of many transcription factors; in breast cancer, as in many other neoplasms, MYC, HIF1 and p53 are central regulators of cell metabolism and their dysregulation is responsible for cancer associated metabolic rewiring (Teicher et al., 2012).

MYC belongs to a family of transcription factors (c-MYC, L-MYC, s-MYC, and N-MYC) and it is required for cell proliferation. In breast cancer MYC drives glucose metabolism by suppressing the expression of thioredoxin-interacting protein (TXNIP), a potent negative regulator of glucose uptake and glycolysis (Shen et al., 2015). Moreover, MYC activation is also able to rewire the glutamine metabolism through the activation of glutaminase 1 and 2, which are important enzymes in the glutaminolysis pathway and fundamental to produce anabolic substrates (i.e. for lipogenesis) (Shajahan et al., 2014).

The *c-MYC* locus is frequently amplified in breast cancer and it can be activated as a downstream target of many oncogenic signalling pathways such as RAS-MAP-ERK cascade or mTOR complexes (Long et al., 2016). Interestingly, a MYC-associated

signature has been linked with TNBC (Fallah et al., 2017), possibly reflecting its role in mediating breast cancer aggressiveness.

HIF1 (Hypoxia inducible factor) is a transcription factor, which is activated by a variety of stress conditions like hypoxia, inflammation, and oxidative stress. It is controlled by the coordinate action of prolyl hydroxylases and the ubiquitin ligase and tumor suppressor Von Hippel-Lindau (VHL). Several studies link HIF1 activity to breast cancer aggressiveness; in particular, high HIF1 expression levels negatively correlate with patients' survival and, more important, HIF1 was found overexpressed in circulating tumour cells of breast cancer patients and in breast cancer metastatic lesions (Gilkes and Semenza 2013). After its activation, HIF1 is able to induce the Warburg effect by upregulating GLUT1, HK1, HK2, and LDHA, as well as the lactate-extruding enzyme monocarboxylate transporter 4 (MCT4) (Pouyssegur et al., 2006; Semenza, 2007). HIF1 is also able to dampen oxidative phosphorylation by inhibiting the conversion of pyruvate to acetyl-coA through pyruvate dehydrogenase (PDH).

The importance of abnormal HIF1 activation in tumours relies in its ability to promote metabolic adaptation of the cell in response to the hostile cancer microenvironment.

1.3 The role of TP53 in breast cancer and metabolism

The discussion about the major players in the regulation of breast cancer metabolism wouldn't be exhaustive without describing the tumor suppressor protein p53. p53 is a potent transcription factor, encoded by the *TP53* gene, that plays a central role in cellular homeostasis and prevention of tumor growth. Indeed, p53 is activated by different cellular insults, that could threaten genomic stability, and triggers a complex and coordinated response that, depending on the inputs, can lead to cell cycle arrest, senescence or cell death, thus acting as onco-suppressor (Kaiser and Attardi, 2017).

In normal conditions p53 protein is kept at low levels by its physiological degradation through the interaction with the ubiquitin ligases MDM2 and MDMX and the proteasome machinery. Under stress conditions (DNA damage, irradiation, hypoxia, nutrient starvation, activated proto-oncogenes), p53 is post-translationally modified and the interaction with MDM2/MDMX is inhibited, thus leading to the accumulation of the protein and activation of its functions (Bieging et al., 2014).

As an onco-suppressor, p53 has an important impact also on cellular metabolism; p53 is able to dampen glycolysis by direct transcriptional repression of GLUT1 and GLUT4 (Schwartzenberg-Bar-Yoseph et al., 2004), and the induction of TIGAR (TP53-induced

glycolysis and apoptosis regulator) a p53 target gene able to lower fructose-2,6-bisphosphate levels in cells, thus inhibiting glicolysis (Bensaad et al., 2006). Moreover, p53 induces the expression of IGFBP3 and PTEN to negatively regulate AKT signalling, and induces the expression of AMPK-β and TSC2 by inhibiting mTOR (Feng and Levine, 2010). Simultaneously, wild type p53 is able to inhibit accumulation of lipids (Yahagi et al., 2003; Jang et al., 2011), to induce oxidative phosphorylation and maintain the mitochondrial mass and activity (Gottlieb and Vousden, 2010). In this way, p53 can oppose to the instauration of the Warburg effect and other metabolic signatures, which are associated with active proliferation and that, together with the loss of apoptosis induction and increased genomic instability, can ultimately lead to malignant transformation.

Mutations in *TP53* are the most frequent genetic lesions in cancer (Kandoth et al., 2013) with different rates across different cancers, ranging from 10% of all cases in hematopoietic malignancies to 90% in ovarian serous carcinomas (Rivlin et al., 2011). In addition, mutations in *TP53* have been associated to poor prognosis in breast cancer patients and are among the most frequent mutations in metastatic breast cancers (Robinson et al., 2017).

The majority of TP53 mutations (>70%) are missense mutations that most frequently hit the region encoding for the core DNA binding domain (Mantovani et al., 2017). There is enrichment in the specific residues R175, R248, R249, R273, R282, and G245, which are named hot-spot mutations (Brosh and Rotter, 2009) (Figure 4).

Mutations can lead either to the complete abrogation of protein expression or to the expression of truncated or full length mutated proteins that abrogate the wild type (WT) functions of the transcription factor. Moreover, in the case of missense mutants, the mutated protein can exert a dominant negative (DN) effect on the remaining wild type allele (if the mutation is restricted to one allele), leaving the cells without a crucial checkpoint (Parrales and Iwakuma, 2015).

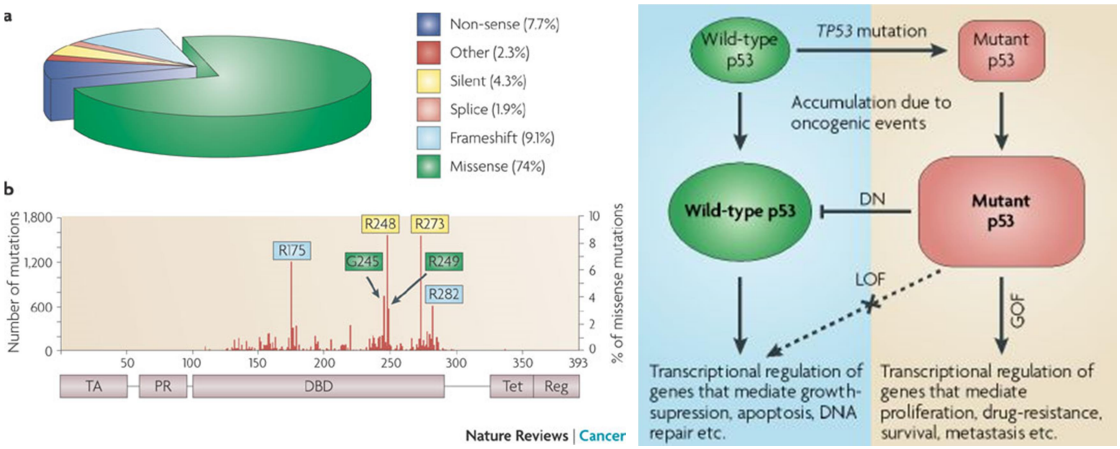


Figure 4. Missense mutations in *TP53* locus and their effect on cancer **(a)** Pie chart representing the different tumour-derived mutation types reported in the IARC TP53 Mutation Database. (b) The distribution of reported missense mutations along the aminoacid sequence of p53. The six most common hotspot mutations are highlighted in yellow for DNA-contact mutations, green for locally distorted mutants and blue for globally denatured mutants. (Brosh and Rotter, 2009). (c) Phenotypic effects of TP53 missense mutations (Brosh and Rotter, 2009).

Hotspot mutations in p53 are traditionally classified in two groups: *conformational mutants*, in which the folding of the core domain of p53 is altered, thus impeding the p53 protein to bind the promoters of its target genes, and the *DNA contact mutants,* with those residues mutated that are responsible for directly binding DNA, but that still retain a near-native core domain structure (Figure 4) (Bullock et al. 2000; Cho et al. 1994; Joerger et al., 2008). In addition, mutant p53 is stabilized and activated in cancer cells in a context-dependent manner (Kim and Lozano, 2018).

Beyond loosing WT properties and exerting DN activity, hotspot mutants are endowed with neomorphic features (Gain-of-function, GOF), which foster cancer growth and progression (Soussi and Wiman, 2015). In fact, although the protein has lost the ability to bind DNA, it can nevertheless impact on gene transcription by establishing aberrant interactions with other transcription factors diverting their physiological activity and eliciting oncogenic effects (Muller and Vousden, 2013). In cancer, the effects of mutant p53 GOF on cellular homeostasis are pervasive: it can directly inactivate p73/p63 proteins, which are members of the p53 family with onco-suppressive functions partially overlapping with p53 and promote tumorigenesis (Strano et al., 2000; Strano et al., 2002; Adorno et al., 2009). In addition, it impacts on the chromatin structure fostering genomic instability and aneuploidy (Murphy et al., 2000; Jong et al., 2004; Polotskaia et al., 2015), it promotes cell survival exerting a negative effect on apoptosis

(Vegran et al., 2007; Lim et al., 2009), it increases cell proliferation by cell cycle genes transactivation (Di Agostino et al., 2006; Liu et al., 2001), it induces cell migration and invasion through integrins and Epidermal Growth Factor Receptor (EGFR) recycling (Muller et al., 2009) and it triggers neo-angiogenesis and inflammation (Fontemaggi et al., 2009; Weisz et al., 2007; Yeudall et al., 2012). Furthermore, work performed in our laboratory elucidated how mutant p53 increases breast cancer aggressiveness through induction of a specific transcriptional program (Girardini et al., 2011), and inhibition of various oncosuppressive pathways through the upregulation of the proteasome machinery (Walerych et al., 2016).

From the viewpoint of metabolism, one of the most striking observations is that mutant p53 in breast cancer is able to actively bind to the SREBP2 transcription factor, inducing the expression of many genes in the mevalonate pathway, promoting cholesterol and isoprenoid synthesis in TNBC (Freed-Pastor et al., 2012). Building on this, our laboratory demonstrated that one of the major consequences of the aberrantly activated mevalonate pathway is the RhoA-dependent activation of YAP and TAZ (Sorrentino et al., 2014), two oncogenes, which are important regulators of organ growth and regeneration (Piccolo et al., 2014) and for BC metastasis (Kim et al., 2015).

Moreover, mutant p53 induces the expression of genes involved in fatty acid synthesis, such as FASN (Freed-Pastor et al., 2012) and promotes glycolysis and the Warburg effect in breast cancer cell lines through the induction of glucose uptake and glycolytic flux (Zhang et al., 2013). Moreover recent evidence indicates that in addition to glycolysis, mutant p53 is able to induce oxidative phosphorylation in breast cancer cell lines, although there is no general consensus about the ability of all the mutants to induce these phenotypes to the same extent (Eriksson et al., 2017). Mutant p53 is also involved in promoting Insulin signalling and AKT1 activation by binding and inactivating the oncosuppressor DAB2IP (Valentino et al., 2017).

A recent study shows that mutant p53 is also able to decrease AMPK activity; AMPK is a metabolic sensor and it is activated in energy deficiency conditions (Low ATP/high AMP); when activated, it phosphorylates a wide range of targets in order to maximize ATP production, through the increase of catabolic processes, and to downregulate anabolic pathways, thus inhibiting cell growth; in presence of mutant p53 proteins, the activation of AMPK in response to metabolic stress conditions (glucose deprivation, serum starvation) was reduced, indicating that mutant p53 is able to impair an important tumour suppressor metabolic checkpoint (Zhou et al., 2014).

1.4 The Sterol Regulatory Element Binding Proteins (SREBPs) and lipid metabolism.

Lipid metabolism has been found connected in many ways to cancer progression and metastasis, thus, understanding how the control on lipid homeostasis is exerted, is of crucial importance. The master regulators of lipid metabolism are Sterol Regulatory Element Binding Proteins (SREBP), which are ER membrane embedded transcription factors responsible for the transcription of all the enzymes required for the lipid biosynthesis and scavenging (Brown and Goldstein, 1997; Hua et al., 1996; Radhakrishnan et al., 2004).

SREBP proteins are mainly regulated by a negative feedback mechanism exerted by cholesterol. They are associated with SCAP (SREBP-Cleavage Activating Protein), a cholesterol sensing ER transmembrane protein; when cholesterol levels are high, SCAP interacts with INSIGs proteins (Insulin Induced Genes), which exerts an inhibitory control over SCAP/SREBPs complex by retaining it in the endoplasmic reticulum; when the levels of cholesterol are low, the SCAP/SREBPs complex detaches from INSIG allowing the transport of the complex to the Golgi apparatus via COPII vescicles. At the Golgi level two proteases (S1P and S2P) cleave SREBP into an active fragment (corresponding to the N-terminal of the protein) that migrates into the nucleus and activates the transcription of target genes. The transcriptional program activated by SREBPs in response to the low levels of cholesterol induces the biosynthesis and the uptake of this important metabolite in order to restore the physiological intracellular concentration (Figure 5) (Goldstein and Brown, 2006).

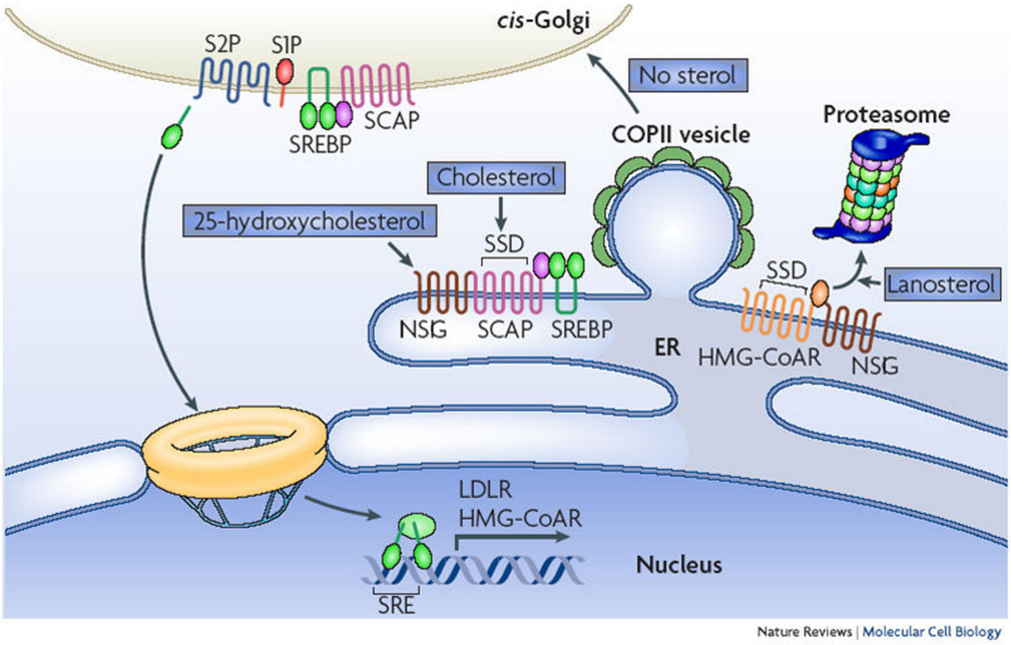


Figure 5. Regulation of lipid metabolism by Sterol regulatory element binding proteins (SREBPs). Under conditions of copious sterol in the endoplasmic reticulum (ER), the ER retention protein INSIG prevents entry of the SREBP–SCAP (SREBP cleavage activating protein) complex to COPII-coated vesicles. Transport of SREBP to the Golgi is needed for proteolytic release of the transcription factor, which is then transported to the nucleus to activate sterol-regulated genes (Ikonen 2008).

There are two genes encoding for two different SREBPs: *SREBF1* and *SREBF2* from which SREBP1 and SREBP2 proteins are generated. From *SREBF1* two different isoforms are produced, SREBP1a and SREBP1c, using a different TSS (Ikonen, 2008). SREBP1 and SREBP2 have a different tissue distribution and a distinct but partially overlapping transcriptional program. While the former controls the fatty acids biosynthesis, the second is a master regulator of cholesterol metabolism. SREBP1a and SREBP1c have similar functions but a different expression pattern: SREBP1c is the predominant isoform in most tissues and it is activated by nutritional stimuli like insulin signalling (Shimomura et al., 1998), while SREBP1a expression is specific for intestinal epithelium, heart, bone marrow and dendritic cells (Im et al., 2011).

1.4.1 Role of SREBP transcription factors in cancer

Enhanced lipogenesis and lipid uptake have been observed in many rapidly proliferating cancer cells, and SREBPs, although never mutated, are found often overexpressed in cancer cells and several SREBPs activation mechanisms have been evidenced. For example, in glioblastoma, SCAP has been found to be glycosylated; this modification

diminishes its affinity for INSIG1 with consequent increased trafficking of SCAP/SREBPs complex to the Golgi (Cheng et al., 2015).

In tumours with hyperactivated PI3K/Akt/mTOR pathway SREBPs are significantly more active and couple the enhanced glycolytic flux in cancer cells to lipogenesis and lipid uptake in order to meet the increasing lipid demand of rapidly proliferating cells. SREBPs have been found to be hijacked by mutant p53 to increase the mevalonate pathway and fatty acid biosynthesis genes, as described before (Freed-Pastor et al., 2012).

Although lipogenesis and SREBPs hyperactivity are crucial for cancer progression, these transcription factors do not seem to be promising therapeutic targets; indeed, even if inhibitors of SREBPs, such as fatostatin, are available, their effect on cancer progression might be counterproductive; inhibition of SREBPs increases Thyroid C Cell cancer aggressiveness and proliferation and, consistently, overexpression of SREBP1a causes G1 arrest by inducing cell cycle inhibitors (Nakakuki et al., 2007). Moreover, genetic ablation of both SREBPs causes apoptosis in untransformed cells (Griffiths et al., 2013).

On the contrary, targeting downstream lipogenic enzymes might be a more appropriate and effective strategy. In particular, growing interest is directed on SCD1 (Stearoyl-CoA Desaturase 1), an enzyme responsible for the mono-unsaturation of fatty acids. SCD1 generates oleic acid (C18:1) from stearic acid (C18:0) and has been found overexpressed in multiple cancers, in particular in hypoxic conditions. Genetic ablation or pharmacological inhibition cause cancer cell death and arrest cell migration (Fritz et al., 2010; Peck et al., 2016). Moreover, a recent paper showed that SCD1 inhibition causes the death of Cancer Stem Cells in lung and ovarian cancer (Noto et al., 2017; Li et al., 2017) and another work indicates that SCD1 is requested for stemness maintenance (Ben-David et al., 2013).

Mono and poly-unsaturated fatty acids (MUFAs and PUFAs) are important for several biological processes: they regulate membrane fluidity and they are precursors of many signalling molecules such as lysophosphatidic acid. Moreover MUFAs and PUFAs are required for post-translational modifications of proteins, and, in this way, they regulate signalling pathways such as WNT (Rios-Esteves et al., 2013; Noto et al., 2017).

In vitro studies reveal that SCD1 inhibition is deleterious only in absence of exogenous lipids, but *in vivo* experiments show that silencing of SCD1 reduces the growth of lung, prostate and liver cancers (Scaglia et al., 2008; Budhu et al., 2013; Fritz et al., 2010).

Also inhibition of Fatty Acid Elongase 6 (ELOVL6) has shown to reduce lung squamous cells carcinoma growth *in vivo* (Marien et al., 2016). Inhibition of SCD1 and ELOVL6 as a strategy to alter the quantity and the quality of the fatty acids, may therefore have a deep impact on cancer cell biology.

1.5 DEPDC1A and the DEP Domain Containing Proteins

A work published by our laboratory identified a mutant p53-driven transcriptional signature, associated with breast cancer aggressiveness and poor survival of BC patients (Girardini et al., 2011), that includes 10 genes, such as BUB1, C21ORF45, CENPA, CYCLIN E2, CPSF6, DEPDC1A, EPB41L4B, FAM64A, NCAPH and WDR67.

In particular we focused our attention on DEPDC1A (*DEP Domain Containing 1A*), because, among all signature genes, it was the strongest inducer of tumour aggressiveness and invasion (Girardini et al., 2011) and because its tumorigenic activity was linked to important processes like inflammation (Harada et al., 2010). Importantly, DEPDC1A overexpression almost completely rescued the decrease of cell migration associated with mutant p53 knock-down (Girardini et al., 2011), revealing that it promotes aggressive phenotypes also independently from mutant p53.

Few reports are available about DEPDC1A functions in health and disease but, importantly, its overexpression has been associated with cancer progression. It has been reported as overexpressed in human bladder cancer tissues and cell lines, while its mRNA was not detected in normal adult tissues and cells, with the only exception of testis (Kanehira et al., 2007). Other works showed that it is overexpressed also in breast cancer (Kretschmer et al., 2011), multiple myeloma (Kassambara et al., 2013), lung cancer (Okayama et al., 2012), prostate cancer (Huang et al., 2017) and nasopharyngeal carcinoma (Feng et al., 2017).

Harada and colleagues have shown that DEPDC1A promotes tumorigenesis through the activation of the NF-kB pathway by the transcriptional repression of the A20 gene, a known inhibitor of NF-kB activity (Shembade et al., 2010); since DEPDC1A does not have any recognized DNA binding domain, the transcriptional repression is achieved through the interaction with the ZNF224 transcription factor, revealing for the first time a role for DEPDC1A as a transcriptional co-factor. Moreover, the treatment with a cell-permeable dominant negative peptide (11R-DEP: 611-628) that interferes with the DEPDC1A-ZNF224 complex induces growth arrest and apoptosis in bladder cancer

cells, making this complex a promising therapeutic target for treatment in bladder cancer (Harada et al., 2010).

An alternative role was suggested by Mi and colleagues, indicating DEPDC1A as a cell cycle regulator (Mi et al., 2015). They showed that DEPDC1A is strongly expressed during the mitotic phase in cancer cells and that its genetic ablation with short interfering RNAs leads to mitotic arrest and defects, while in another paper it is shown that the effect of DEPDC1A on mitosis is due to its localization on centrosomes (Chen et al., 2017).

Although the functions of DEPDC1A both in physiological and neoplastic conditions are not fully understood, all these data point out an important role of DEPDC1A in tumorigenesis in a broad spectrum of cancers and suggest that its function is amenable for therapeutic interventions. In this perspective, different efforts have been made to synthetize immunogenic epitopes by us and by others, in order to generate DEPDC1A-based anti-tumour vaccines (Obara et al., 2012; Tosi et al., 2017).

The *DEPDC1A* gene is located on chromosome 1 and it encodes two main protein variants, annotated as DEPDC1A isoform 1 (DEPDC1A-V1: GeneBank Accession AB281187), consisting of 12 exons that encode an 811 amino-acid protein, and DEPDC1A isoform 2 (DEPDC1A-V2: GeneBank Accession AB281274), consisting of 11 exons that encode a 527 amino-acid protein. These two splice-variants differ by a 284 amino acid sequence encoded by exon 8 present only in isoform 1 (Figure 6); it is not clear how the expression of these two isoforms is regulated, but our unpublished data indicate that the variant 1 is mainly expressed in cancer cell lines, while the variant 2 is expressed also in non-transformed cell lines. Both isoforms possess a Nuclear Localization Signal (NLS), and are nucleo-cytosolic proteins, in line with the roles of DEPDC1A in the regulation of the mitotic spindle and in transcriptional modulation (Kanehira et al., 2007).

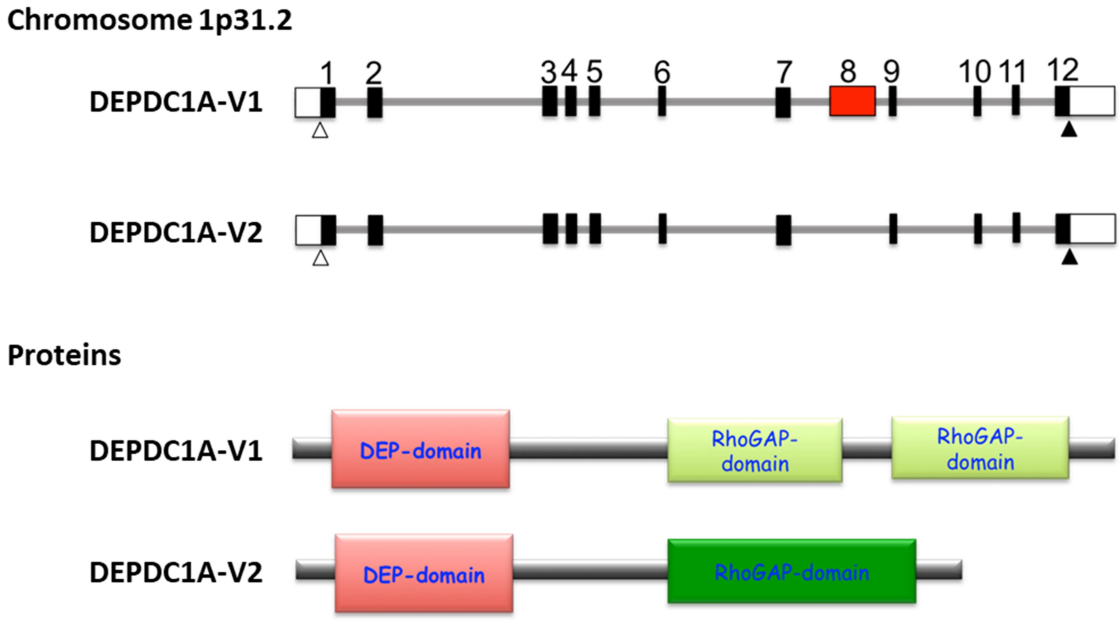


Figure 6. *DEPDC1A* isoforms and protein domain prediction. Upper panel: schematic representation of the human *DEPDC1A* gene organization, and the differences between the two variants. Bottom panel: DEPDC1A domain prediction in both isoforms.

DEPDC1A is highly conserved among a wide range of species from *C. elegans* to humans. The nematode ortholog let-99 acts upstream to the heterotrimeric G protein alpha subunit GPA-11 to control the activation of JNK-1 and induce apoptosis after vincristine treatment (Sendoel et al., 2014). In humans there is a paralog named DEPDC1B, which acts as a mitosis promoting factor, coordinating de-adhesion events by RhoA inhibition, thus inducing the entry in M-phase (Marchesi et al., 2014).

DEPDC1A belongs to the DEP domain containing family of proteins, which share in their N-terminus a conserved DEP domain, a motif of about 80-100 amino acids, named after the three proteins it was initially found in (Dishevelled, Egl-10, Pleckstrin) (Ponting et al., 1996; Karrath et al., 1998). Further analysis of the sequence of DEPDC1A reveals also two predicted RhoGAP (Rho GTPase Activating Protein) domains in V1 isoform and only one in V2.

1.5.1 DEP domain

The DEP Domain is a globular protein domain conserved among all the eukaryotes (Civera et al., 2005) and it is present, as a single copy or in tandem, in 64 human proteins (Ponting et al., 1996). DEP domains display a unique alpha helix/beta sheet fold and have been implicated in membrane binding utilizing different mechanisms

(Ballon et al., 2006; Wong et al., 2000) and it is involved in signal transduction (Figure 7).

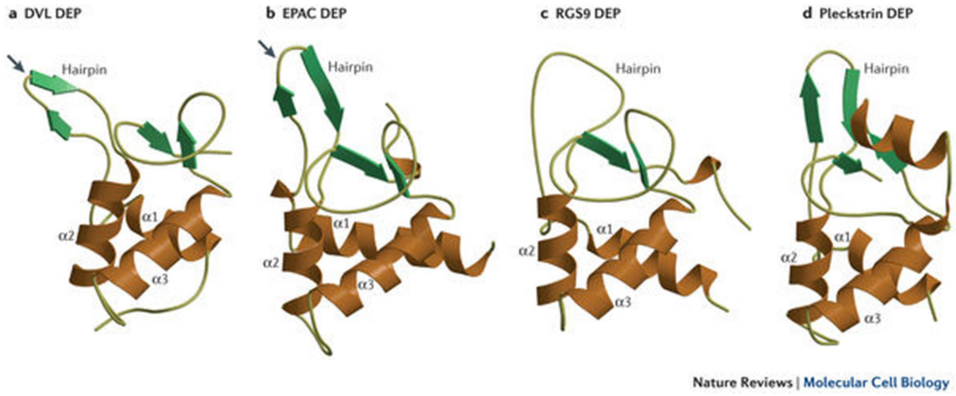


Figure 7. DEP domain structures. Cartoon structures of Dishevelled, EGL-10 and pleckstrin (DEP) domains that show their characteristic αβ fold. DEP domains comprise a conserved helical core, which consists of three helices and a protruding β-hairpin arm between the helices α1 and α2. This helical core is highly conserved in all DEP domains (Consonni et al., 2014).

Multiple sequence alignments and using neighbour-joining phylogenetic trees allowed to identify 6 distinct subfamilies of the DEP domain containing proteins: i) Dishevelled (Dsh) subfamily, ii) RGS (Regulator of G protein Signalling) proteins, iii) Epac family (Exchange Protein directly Activated by cAMP), iv) Pleckstrin proteins, v) the FYVE containing kinases (proteins with the FYVE domain, from first letter of the first four proteins in which it was found Fab1p, YOTB, Vac1p and EEA1) and vi) yeast proteins with divergent DEP domains (Civera et al., 2005).

For some of these proteins, the role of the DEP domain has been studied; in dishevelled protein, the DEP domain is important for the activation of c-Jun N-terminal kinase (JNK), the upregulation of Beta-catenin activity, and the stimulation of Lef-1 mediated transcription (Warton et al., 2003; Boutros et al., 1998). Moreover the DEP domain of a photoreceptor-specific signalling protein, RGS9 (Regulator of G protein Signalling 9), plays an essential role in its own intracellular delivery interacting with R9AP, a member of the extended SNARE protein family (Martemyanov et al., 2003).

In Pleckstrin proteins the DEP domain represents a novel and distinct subfamily and shares important structural features with the DEP domains of Dishevelled. Interestingly, the Pleckstrin DEP domain does not seem to be directly involved in membrane localization of the protein, and its molecular function remains unknown (Civera et al., 2005).

In Epac2, the DEP domain is crucial for membrane localization. Epac is a target of cyclic AMP, an important second messenger in signal transduction and functions as a guanine nucleotide exchange factor for the Ras-like small GTPase Rap (De Rooij et al., 2000; Qiao et al., 2002).

1.5.2 The RhoGAP domain

The GAP domain mediates GTP hydrolysis in GDP in Rho GTPase proteins (22 members in mammals (Ridley 2006) enhancing their GTPase activity, thus inducing the inactive state of the Rho proteins.

The small GTPase proteins are members of the Ras superfamily and contain over 150 members, divided into five major branches on the basis of sequence and functional similarities, named Ras, Rho, Rab, Ran and Arf (Wennenberg et al., 2005). They share a ~20 kDa core G domain (corresponding to Ras residues 4–166) involved in GTP binding and hydrolysis (Vigil et al., 2010). In addition to the GAP proteins, the GTPase activity is modulated also by GEFs (Guanine nucleotide Exchange Factors), which activate GTPases by promoting the GTP-bound state, and GDIs (Guanine nucleotide Dissociation Inhibitors), which sequester the GTPases in their GDP-bound state (Figure 8).

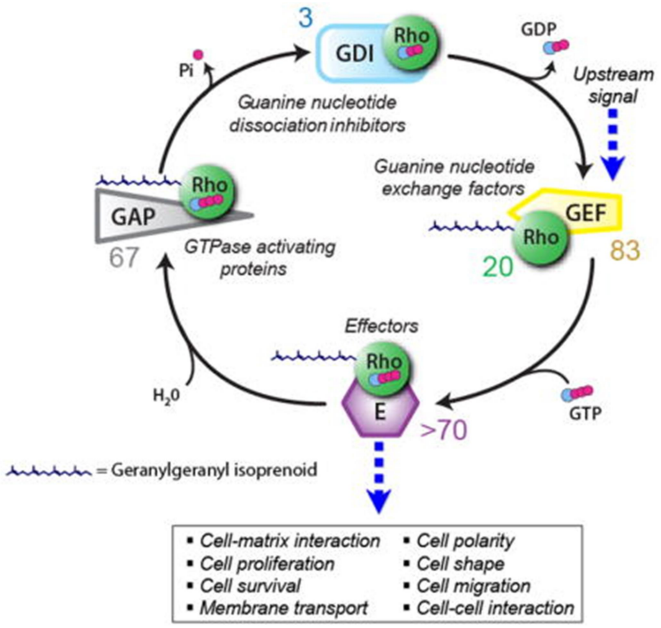


Figure 8. The GDP-GTP cycle of G proteins. Ras superfamily proteins possess intrinsic guanine nucleotide exchange and GTP hydrolysis activities. However, these activities are too low to allow efficient and rapid cycling between their active GTP-bound and inactive GDP-bound states. GEFs and GAPs accelerate and regulate these intrinsic activities (Vigil et al., 2010).

With the relevant exception of RAS, GTPase proteins are not commonly mutated in cancer, but deregulated expression or protein function of GEFs and GAPs have been found to have a role in tumorigenesis (Vigil et al., 2010).

In regulating small RhoGTPases, the RhoGAP activity works as molecular switch, coupling extracellular inputs to intracellular signal transduction pathways. The RhoGAP domain is a motif of approximately 190 amino acids shared by ~70 proteins, which presents a conserved arginine residue termed the "arginine finger" which is essential for the catalytic activity (Amin et al., 2016).

A structural study reveals that DEPDC1A Rho-GAP domain is probably not functioning because it lacks the arginine finger residue in position 242 which, as above-mentioned, is crucial for its activity (Amin et al., 2016). For this reason its role remains unclear. Nevertheless, RhoGAP proteins may play a role in biological processes independently from their RhoGAP activity; for example, α1-chimaerin, a RhoGAP protein lacking of a GAP activity, is able to bind GTPases such as Rac1 and Cdc42 and in this way cooperates within actin cytoskeleton remodelling processes (Kozma et al., 1996). Interestingly, also DEPDC1B possesses an inactive RhoGAP domain, but still is able to bind and inhibit RhoA (Marchesi et al., 2014). These observations therefore suggest a possible non-canonical role also for the RhoGAP domain of DEPDC1A.

1.5.3 DEPDC1A as promoter of aggressive phenotypes in breast cancer.

Unpublished work from our laboratory reveals that DEPDC1A is involved in multiple steps of breast cancer progression. Moreover, bioinformatic analysis reveals that DEPDC1A expression is significantly higher in breast cancers compared to normal tissues and that high DEPDC1A expression levels positively correlate with a poor prognosis in breast cancer patients.

Importantly, the relevance of DEPDC1A in tumour progression and metastasis formation *in vivo* has been confirmed in a mouse mammary xenograft model, where cells depleted of DEPDC1A are not able to form primary tumours and metastases in immunocompromised mice.

Interestingly, *in vitro* experiments showed that of the two isoforms, only the V1 seems to positively correlate with tumorigenesis, since its expression is detectable only in cancer cell lines while the V2 seems to have a broader spectrum of expression. Moreover, only overexpression of the DEPDC1A-V1 isoform is able to induce

proliferation, migration, anchorage-independent growth, change in cell polarity and stemness features in a normal mammary epithelial cell line MCF-10A, while the V2 only weakly induces some of these traits when overexpressed in these cells.

All these results together suggest a robust oncogenic potential of DEPDC1A-V1, as also observed by others, which is able to induce tumour-like features in non-transformed cells and is required for tumour growth and metastasis formation *in vivo*.

AIM OF THE THESIS

Metastatic breast cancer is usually an incurable disease. Modern therapeutic interventions have only palliative effects and are not able to avoid dissemination and cancer progression. The reasons of this failure have been mainly ascribed to tumour plasticity and heterogeneity, which still remain underestimated and, consequently, make it difficult to correctly stratify the patients according to outcome and metastatic risk. Moreover, such variability limits the effectiveness of therapeutic regimens, mainly based on the diagnosis of the primary tumour and which are not able to hit aggressive/metastatic tumour cells.

It is becoming clear that the current methods of breast cancer diagnosis and classification, although useful, are not sufficient to embrace this growing complexity, thus making it necessary to integrate this knowledge with information derived from genetic, transcriptomic, epigenomic and metabolomic analyses of both primary tumours and disseminated cells, in order to find tumour dependencies that could be exploited to the specific detriment of metastatic cancer cells.

In this perspective, work performed in our laboratory evidenced that DEPDC1A, a mutant p53 target gene, is a strong inducer of breast cancer progression *in vitro* and its abrogation strongly impairs metastasis formation *in vivo.* Moreover, data from literature evidenced that DEPDC1A is found overexpressed in many other tumours and associated to poor prognosis in patients, indicating that it might have a role in a broad spectrum of aggressive cancers. The fact that DEPDC1A is barely detected in normal cells and becomes overexpressed only in cancer further justifies the interest in this protein - and its molecular network - as potential therapeutic targets.

Starting from these premises, we hypothesized that DEPDC1A could be relevant for metastasis formation and, therefore, the aim of this thesis was to: 1) understand, by using high-throughput transcriptomic analysis, the biological pathways that are deregulated in breast cancer through DEPDC1A and that could be relevant for cancer cell aggressiveness, 2) elucidate the molecular mechanisms by which DEPDC1A induces its oncogenic program, 3) find potential vulnerabilities that could be pharmacologically targeted.

RESULTS

2.1 DEPDC1A transcriptionally regulates genes of the fatty acid biosynthesis

DEPDC1A is poorly detectable in non-transformed cells, while it was shown to be overexpressed in many cancers from different tissue origin, and mainly associated to poor patients' outcome (Kanehira et al., 2007). In addition, our previous unpublished results have unveiled that overexpression of DEPDC1A in a non-transformed breast cell line is able to induce malignant phenotypes, such as proliferation, migration, loss of cell polarity. Since the cellular functions of DEPDC1A are still poorly defined, we asked how DEPDC1A might impact on cancer cells' biology. Based on its role as a transcriptional co-factor of ZNF224 and E2F1 (Harada et al., 2010; Huang et al., 2017), we performed a high-throughput transcriptomic analysis by RNA-seq of the highly metastatic MDA-MB-231 TNBC cells upon DEPDC1A silencing in order to gain novel insights into DEPDC1A functions.

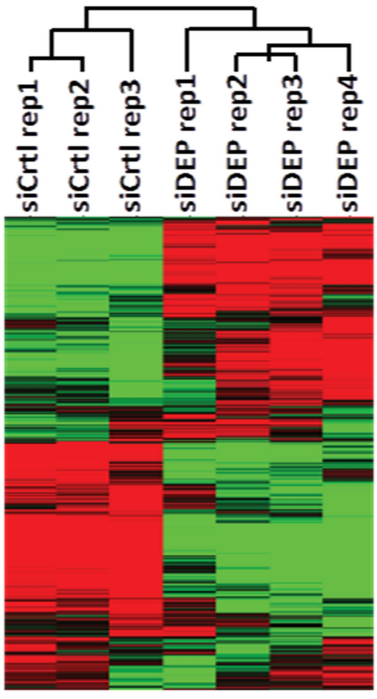


Figure 9. RNAseq analysis of total UP and DOWN regulated gene upon DEPDC1A silencing. Heat map representing the clustering of up-regulated and down-regulated genes upon control or DEPDC1A RNA interference, as indicated. Three samples for control and four for DEPDC1A silencing were analysed.

Compared to control silencing, 1362 genes were differentially expressed in DEPDC1A siRNA treated samples in a statistically significant manner by applying a cut off of logFC 0.75 and a p-value < 0.05. An unbiased analysis of functional annotation (AFA) of genes with significantly changed expression levels (by >0.5-fold) performed with the Ingenuity Pathway Analysis (IPA) software revealed that a great number of important biological processes were perturbed by DEPDC1A silencing. We chose to consider only those processes that were robustly downregulated according to their negative z-score.

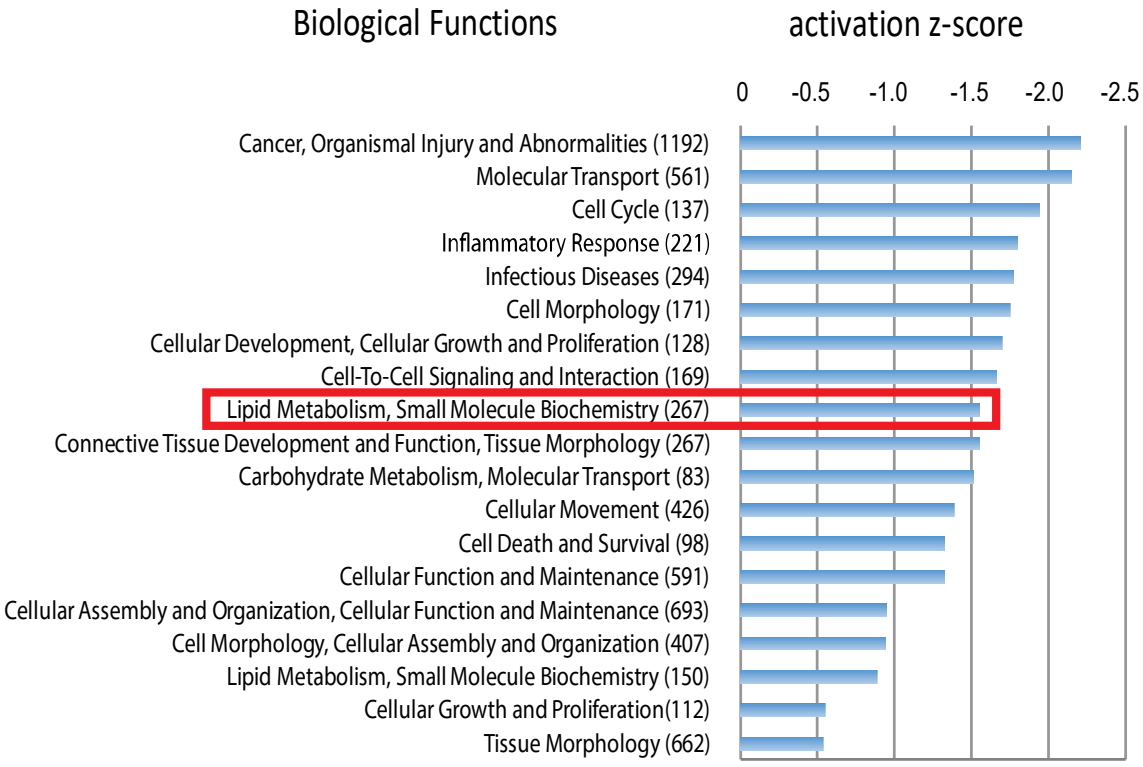


Figure 10. IPA Functional Annotation of DEPDC1A-modulated genes. Ingenuity pathway analysis of the genes with greater than 1.5 fold change in expression of MDA-MB-231 cells upon silencing of DEPDC1A with respect to control silenced cells.

In line with previous publications and our data, the biological functions of inflammation, cell cycle, cell growth, proliferation and cellular movement appeared significantly enriched and downregulated. Notably, also lipid metabolism was significantly downregulated and represented an interesting novelty. In particular, we observed that several mRNAs all belonging to fatty acid biosynthesis pathway genes like *ATP-Citrate Lyase* (*ACLY*), *Stearoyl-CoA Desaturase 1* (*SCD1*) and *Elongation Of Very Long Chain Fatty Acids 6* (*ELOVL6*) were consistently downregulated, suggesting

a role for DEPDC1A in controlling fatty acid metabolism in cancer cells. The downregulation of these enzymes was validated by qRT-PCR in MDA-MB-231 cells by using two independent siRNAs against DEPDC1A (Fig. 11).

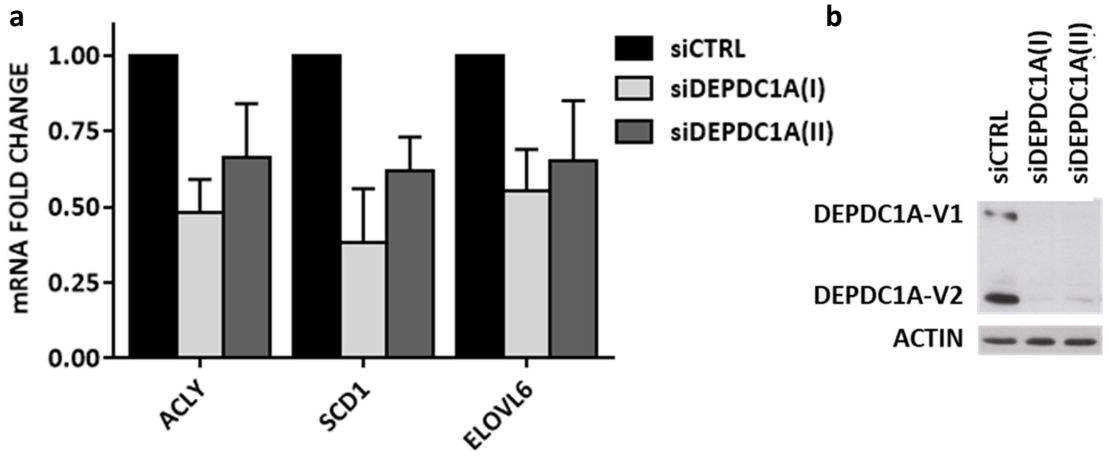
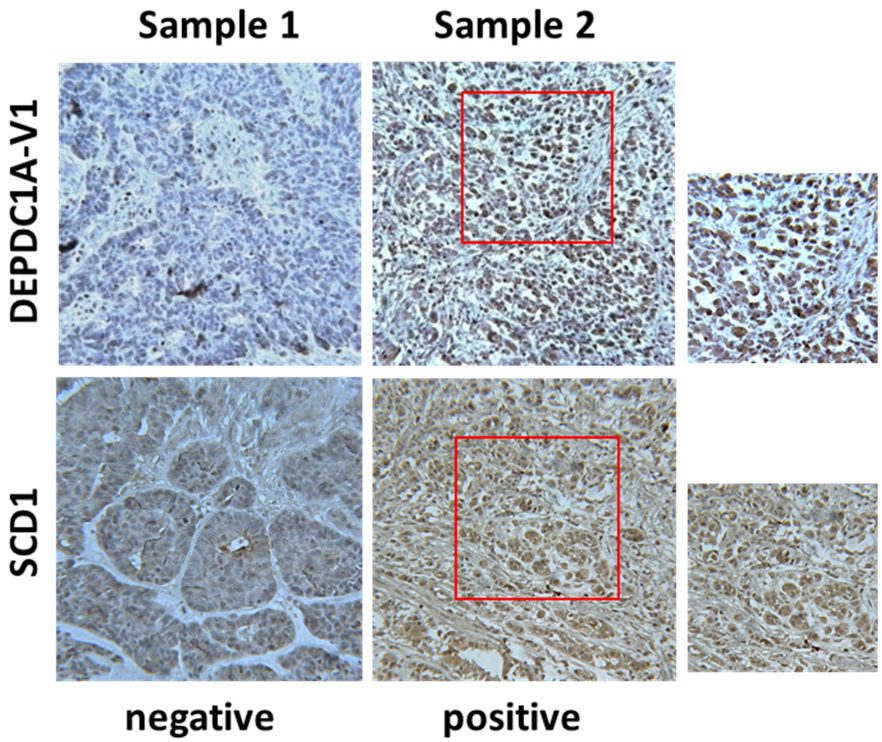


Figure 11. Validation of RNAseq results. (a): qRT-PCR validation of selected DEPDC1A induced genes. Changes in gene expression are indicated as fold change upon depletion by two independent DEPDC1A targeting siRNAs in MDA-MB-231 cells, relative to cells transfected with control siRNA. Error bars indicate s.d of at least 3 replicates, asterisk indicates p-value <0.05. (b) Western blot analysis of the cellular lysates of the experiments in (a). DEPDC1A protein isoforms V1 and V2 upon control and DEPDC1A (I) or (II) silencing are indicated; Actin levels are shown as a loading control.

This result demonstrated that DEPDC1A positively regulates the expression of these genes in MDA-MB-231 breast cancer cell line. To corroborate our findings we performed an immunohistochemical analysis for SCD1 and DEPDC1A-V1 proteins in serial sections of a panel of human breast cancer biopsies. Here we show immunohistochemical staining of representative samples, where positive DEPDC1A-V1 sample shows also high SCD1 protein levels, while DEPDC1A-V1 negative sample is negative also for SCD1; a statistical analysis performed taking into account all the examined samples shows a significant correlation between the two proteins (pvalue = 0.0083) (Figure 12) .

a



b

DEPDC1A-V1	SCD1	SAMPLES
+	+	4
-	-	10
-	+	3
TOT		17
P value = 0.0083 (Barnard unconditional test for superiority)		

Figure 12. Immunohistochemical staining for DEPDC1A-V1 and SCD1 in human breast cancer biopsies. (a): immunohistochemical staining for DEPDC1A-V1 and SCD1 in serial sections of 17 human breast cancer biopsies (representative samples are shown). **(b):** table showing the Immunohistochemical staining patterns of DEPDC1A-V1 and SCD1 in the breast cancer samples analysed.

Considering that the number of these samples was limited, and therefore not sufficient to strongly corroborate the data, we wanted extend our analysis by interrogating the Breast Invasive Carcinoma gene expression dataset of TCGA (https://cancergenome.nih.gov/) which contains RNA-seq data relative to 1100 Breast cancer samples. We analysed the mRNA levels of ACLY, SCD1 and ELOVL6 in the

samples with high levels of DEPDC1A and, although it was not possible to find a strong correlation (Pearson Score <0.3), a positive trend was evidenced (data not shown).

These data all together indicates that DEPDC1A regulates expression levels of fatty acid biosynthesis in breast cancer and let us hypothesize that overexpression of DEPDC1A in cancer cells could impact on *de novo* fatty acid biosynthesis and prompted us to investigate this possibility.

Fatty acids biosynthesis is a multistep process that entails the condensation of Acetyl-CoA molecules and NADPH consumption to produce straight carbon chains of different length. The pathway starts with a molecule of acetyl-CoA, that in mammals is produced mostly by carbohydrate metabolism, derived from pyruvate by pyruvate dehydrogenase (PDH) in the mitochondrion, which cannot be exported to the cytoplasm where the fatty acid biosynthesis occurs; therefore a molecule of citrate is diverted from the TCA cycle and translocated in the cytosol, where it is used by ACLY to produce acetyl-CoA and oxaloacetate, accomplishing the first lipogenic step; the TCA is not the only source of citrate, which can be retrieved also from glutaminolysis; ACLY is thus the enzyme that connects the carbohydrate and amino acid metabolism to the lipid biosynthesis pathway. To synthetize the fatty acid carbon chain, the acetyl-CoA is transformed in malonyl-CoA by acetyl-CoA carboxylase (ACC) and then, by the activity of FASN, other acetyl-CoAs are added until a 16 carbon atoms long fatty acid is obtained (palmitic acid). At this point the fatty acid molecule can be elongated, through elongase enzymes, like ELOVL6, and desaturated, through desaturases, such as SCD1, to obtain a pool of different fatty acids.

Free fatty acids are extremely toxic for the cell, and, once they are synthetized, they are immediately incorporated in other molecules or employed in several processes, among which there are beta-oxidation, phospholipids formation and signal transduction; fatty acids can also be stored as triglycerides in lipid droplets.

Lipid droplets (LDs) are subcellular organelles of neutral lipids storage (triglycerides and cholesterol esters) surrounded by a phospholipid monolayer. LDs are more than simple lipids repositories, in fact they participate in the maintenance of lipid homeostasis, they avoid lipo-toxicity, and they serve as an important buffer for oxidative stress (Walther and Farese, 2012). LDs are indicators of fatty acids and triglycerides biosynthesis and uptake; normally, apart from liver, adipose tissue and breast during lactation, cells do not form many droplets, but transformed cells have been

found to produce them in large quantity, probably due to an excessive lipid biosynthesis and uptake that would otherwise be toxic; in this way they were described to have an active role in promoting cell survival and tumorigenesis (Bozza and Viola, 2010).

2.2 DEPDC1A induces lipid droplets formation and fatty acid desaturation.

To understand if DEPDC1A is able to influence fatty acid synthesis we decided to measure the content of lipid droplets in MDA-MB-231 cells upon DEPDC1A silencing as a read out of lipid accumulation.

First, to stain lipid droplets we employed a lipophilic fluorescent dye, named LD540, specific for neutral lipids (Spandl et al., 2009); then, in order to measure only the lipid biosynthesis process and avoid the contribution of lipid uptake, we measured the lipid droplets in cells grown in media supplemented with lipid free serum. As shown in Figure 13, upon treatment of MDA-MB-231 cells with two independent siRNAs for DEPDC1A, the content of lipid droplets was significantly reduced with respect to control silenced cells, suggesting that that DEPDC1A is able to impinge on both lipid synthesis and accumulation.

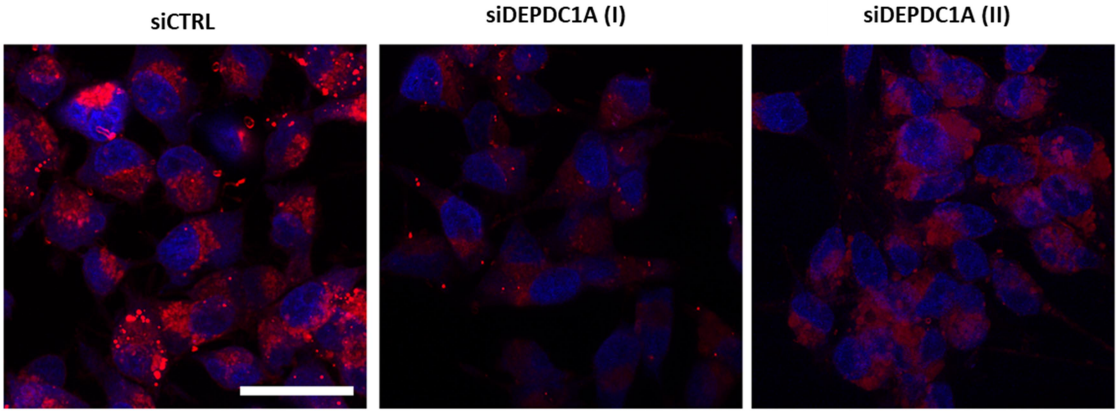


Figure 13. Lipid Droplets staining in DEPDC1A depleted MDA-MB-231 cells. Images of lipid droplets stained with LD540 fluorescent dye in control (siCTRL) or DEPDC1A (I) and (II) siRNA (siDEPDC1A (I) and (II)) transfected MDA-MB-231 cells. Nuclei are stained with DAPI. The scale bar is 20um.

To confirm and extend the observation that DEPDC1A influences the lipid droplets content and to quantify the phenomenon, we knocked down DEPDC1A in different breast cell lines and measured fluorescently labelled lipid droplets by FACS analysis (Fig. 14). Intriguingly, while in the breast cancer cell lines MDA-MB-231, MDA-MB-

468 and MCF-7 the fluorescence intensity was significantly reduced following DEPDC1A silencing, in normal mammary epithelium cells (MCF-10A), which have undetectable levels of DEPDC1A-V1, we did not observe any difference, thus confirming the specificity of our findings.

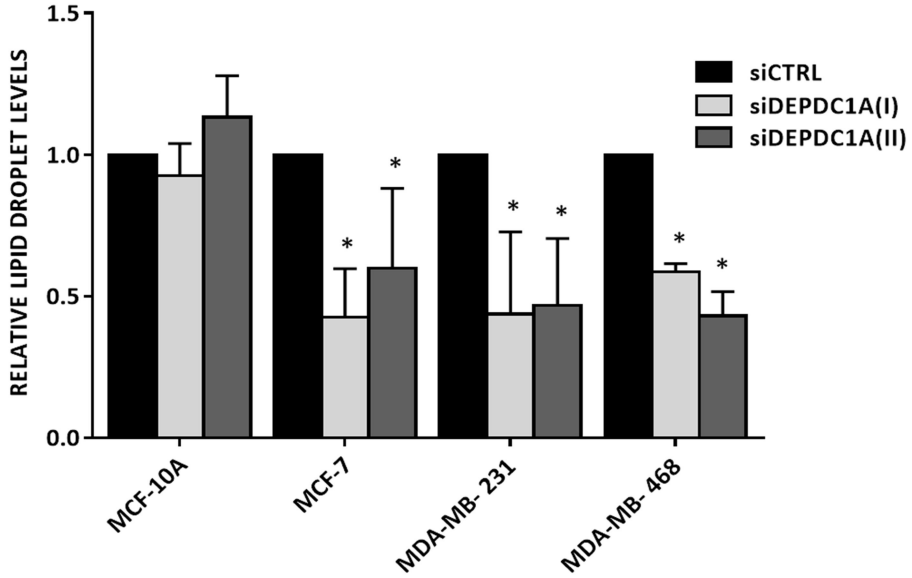


Figure 14. FACS based quantification of lipid droplets following DEPDC1A depletion in a panel of breast cell lines. Quantification by FACS analysis of fluorescently-labeled lipid droplets in different breast cancer (MDA-MB-231, MDA-MB-468, MCF-7) and normal (MCF-10A) cell lines upon control or two independent DEPDC1A siRNAs. The levels of lipid droplets are expressed as fold change with respect to the control siRNA. Error bars indicate s.d of at least three replicates, asterisks indicate p-values <0.05.

To formally demonstrate that the modulation of lipid droplets was DEPDC1A dependent, we generated stable MDA-MB-231 clones in which endogenous DEPDC1A was constitutively knocked down by a short hairpin RNA and an exogenous shRNA-resistant DEPDC1A-V1 cDNA was stably overexpressed. Notably, as shown in Figure 15, re-overexpression of DEPDC1A-V1 was sufficient to rescue the reduction of lipid droplets displayed in shDEPDC1A expressing cells, thus confirming our hypothesis.

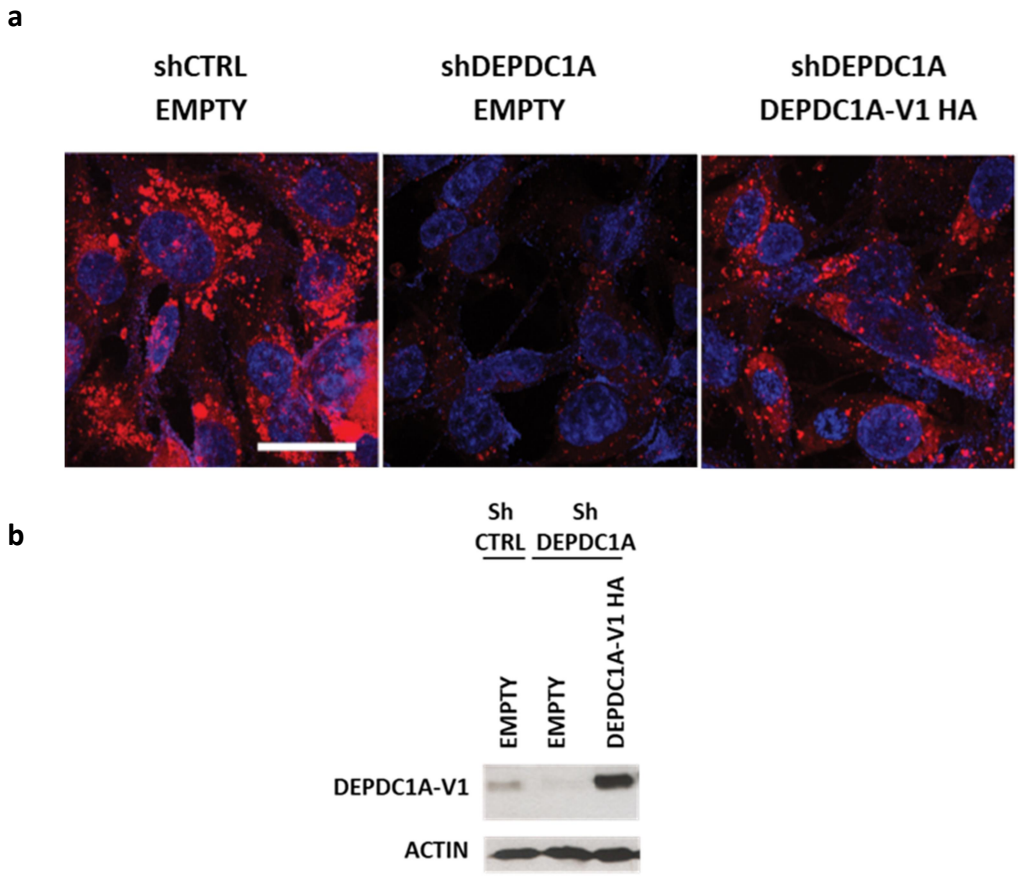


Figure 15. Overexpression of a siRNA resistant DEPDC1A in MDA-MB-231 depleted of the endogenous protein rescues lipid droplet formation. Images of lipid droplets stained with LD540 fluorescent dye in MDA-MB-231 stably transduced with shControl, shDEPDC1A and with re-overexpression of a shRNA-resistant DEPDC1A-V1 isoform. Nuclei are stained with DAPI. The scale bar is 20um. (b) Western blot analysis of the cellular lysates of the experiments in (a). DEPDC1A protein isoform V1 of cells transduced with CTRL or DEPDC1A short hairpin RNA and with an shRNA-resistant DEPDC1A re-overexpression is indicated; Actin levels are shown as a loading control.

Next, to understand if the effect exerted by DEPDC1A-V1 on lipid droplets accumulation was dependent on induction of its target genes, we employed MCF-10A cells stably overexpressing HA-tagged DEPDC1A-V1 and analysed their lipid droplets content by FACS. We were are able to observe an increased content of lipid droplets in DEPDC1A overexpressing cells (Figure 16a) and also increased SCD1 protein levels (Figure 16b), compared to the empty vector infected cells; in these same conditions we silenced SCD1 and we obtained a strong reduction of the phenotype (Figure 16a), indicating that the lipid-related transcriptional program activated by DEPDC1A is instrumental for the increased content of lipid droplets.

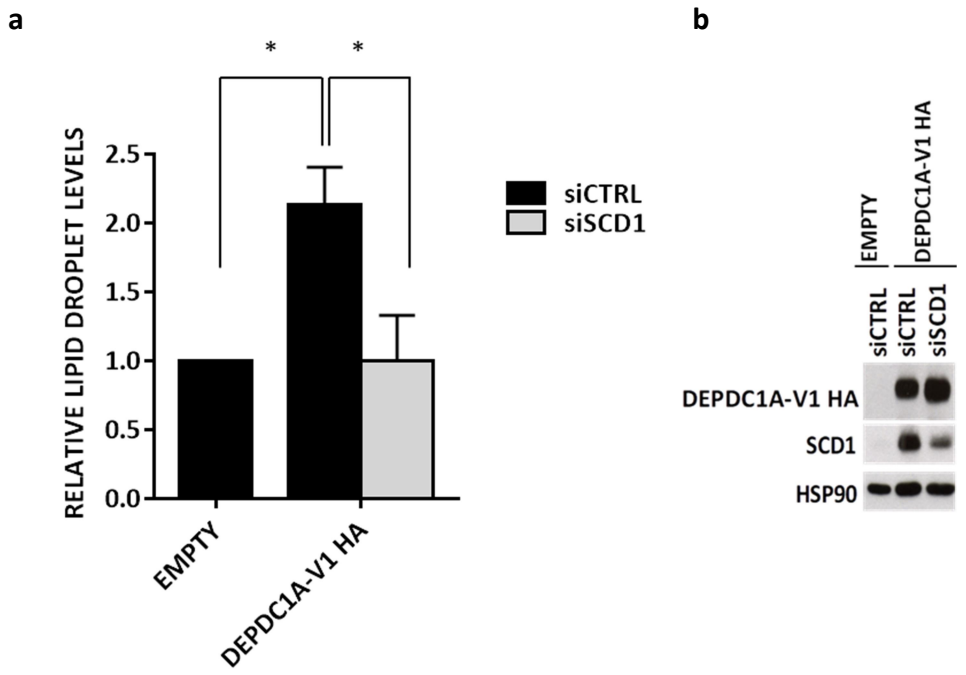


Figure 16. FACS based quantification of lipid droplets in MCF-10A cells following modulation of genes of the DEPDC1A/lipid axis. Quantification by FACS analysis of fluorescently-labeled lipid droplets in MCF-10A cells stably transduced with EMPTY or HA-tagged DEPDC1A-V1 vector upon control or SCD1 silencing. The levels of lipid droplets are expressed as fold change with respect to the EMPTY vector siCTRL. Error bars indicate s.d of at least three replicates, asterisks indicate p-value <0.05. (b) Western blot analysis of the cellular lysates of the experiments in (a). HA tagged DEPDC1A-V1 and SCD1 proteins isoforms of MCF-10A cells transduced with EMPTY or DEPDC1A-V1 overexpressing vectors upon CTRL or SCD1 silencing are indicated; HSP90 levels are shown as a loading control.

To implement our findings in an *in vivo* setting, we took advantage of primary tumour samples derived from a mammary xenograft experiment of shCTRL or shDEPDC1A MDA-MB-231 cells. Since it was not possible to directly stain lipid droplets on those samples, we decided to verify the levels of specific markers of lipid droplets biogenesis, by controlling perilipin 2 (PLIN2) protein levels. PLIN2, is an ubiquitously expressed protein associated with LDs, which is crucial for both their formation and maintenance. Indeed, absence of PLIN2 has been shown to impair LDs formation (Sztalryd and Kimmel, 2014). As shown in Figure 17, in the shDEPDC1A samples we observed a strong reduction of SCD1 protein levels and, in parallel, a complete abrogation of PLIN2 protein expression, thus indicating a very likely reduction of LDs content. Moreover, this result indicates that also *in vivo*, DEPDC1A might be able to impinge on fatty acid metabolism by enhancing both fatty acid biosynthesis and LDs formation.

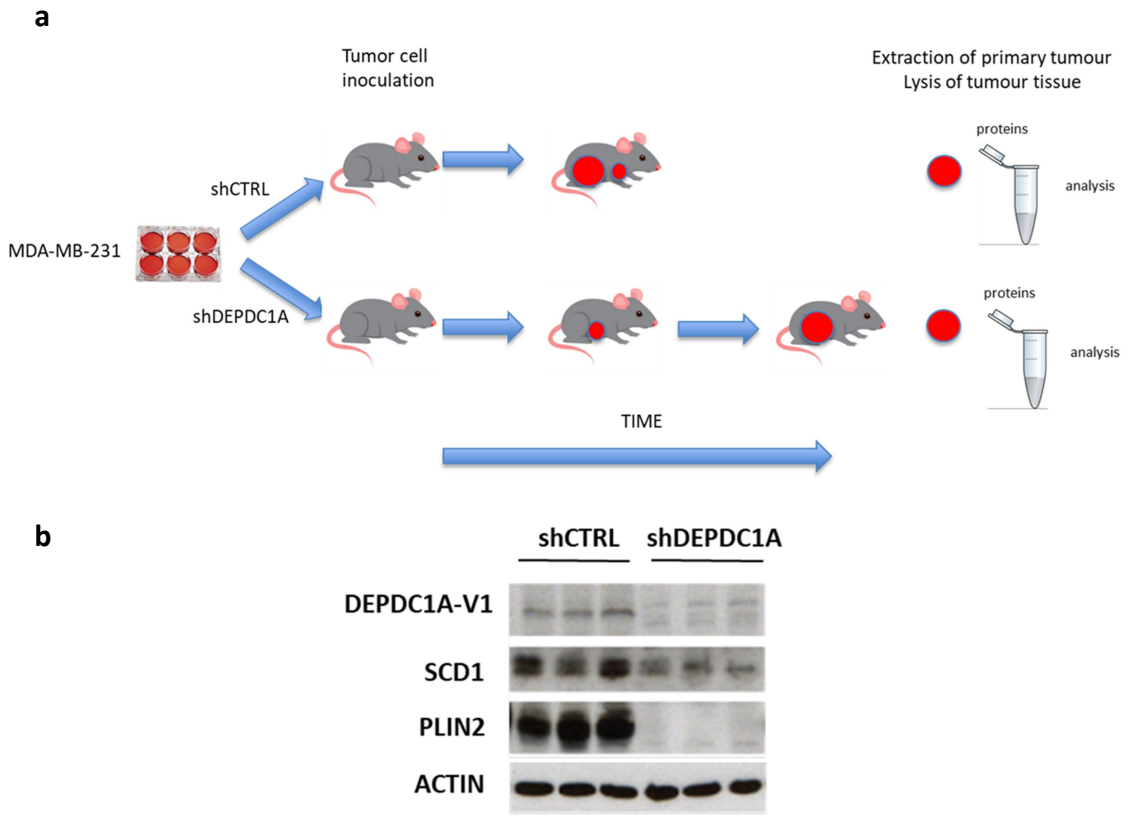


Figure 17. DEPDC1A silencing impairs fatty acid metabolism *in vivo*. (a) Scheme of the xenograft experiments with metastatic MDA-MB-231 cells, stably transduced with a lentiviral vector encoding for the firefly luciferase reporter gene and co-transduced with specific short hairpin RNAs targeting DEPDC1A or mock lentiviral vector, which were injected into mammary fat pad of nude mice. Tumour and lung metastatic growth were followed by caliper and bioluminescence measurements, respectively. After 31 days Control mice (shCtrl) had outgrown tumours and metatases, while shDEPDC1A mice had reduced tumour growth and only limited metastasis. Tumours for both shCTRL and shDEPDC1A were extracted when they had reached the same dimension, lysed and used for western blot analysis. (b) Western blot of DEPDC1A, SCD1 and PLIN2 in frozen primary tumours samples derived from mouse mammary xenograft experiment shown in (a); Actin levels are shown as loading control.

Apart from lipid accumulation, we next asked if, through SCD1 regulation, DEPDC1A was able to modify also desaturation levels of the fatty acids. To do so, we took advantage of gas chromatography/mass spectrometry analysis of Fatty Acid Methyl Esters (FAME) obtained through the saponification of lipids. This technique allows discriminating different fatty acids and their desaturation status. As shown in Figure 18, DEPDC1A-silenced MDA-MB-231 displayed a slight, nevertheless significant,

reduction of the of unsaturated fatty acid pool, with an effect comparable to that exerted by SCD1 silencing.

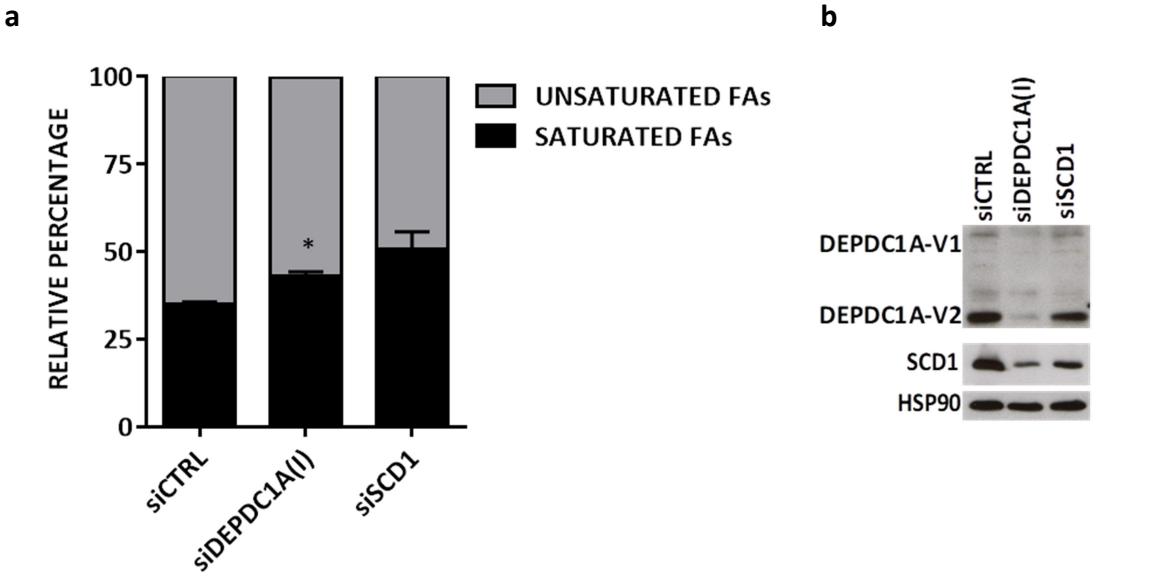


Figure 18. Impact of DEPDC1A silencing on fatty acid saturation in MDA-MB-231 cells. (a) Quantification by FAME-LC/MS of the total saturated and unsaturated fatty acids in MDA-MB-231 cells upon DEPDC1A and SCD1 silencing; the values are expressed as relative percentage of the total fatty acid content. Error bars indicate s.d of 3 replicates, asterisk indicates p-value <0.05. (b) Western blot analysis of the cellular lysates of the experiments in (a). V1 and V2 isoforms of DEPDC1A protein and SCD1 of cells transfected with CTRL, DEPDC1A (I) and SCD1 siRNAs are indicated; HSP90 levels are shown as a loading control.

All together these data show that DEPDC1A is able to increase fatty acids accumulation and desaturation, with an important effect on cancer associated lipid metabolism rewiring, thus offering a possible mechanism for DEPDC1A-induced cancer associated phenotypes.

2.3 SCD1 inhibition impairs cell migration and viability.

Our previous published and unpublished data indicate that DEPDC1A-V1 is able to induce many oncogenic phenotypes in non-transformed MCF-10A cells. In particular, we have shown that DEPDC1A is a strong promoter of migration and invasiveness. In order to understand if the lipid biosynthesis and unsaturation are instrumental for DEPDC1A-V1 activity, we took advantage of MCF-10A cells stably transduced with EMPTY- or HA-tagged DEPDC1A-V1 overexpressing vectors and we measured their migratory ability in a trans-well assay upon control- or SCD1 silencing. In line with our

results, DEPDC1A-V1 overexpression strongly induced cell migration, but, as evidenced in Figure 19, SCD1 silencing strongly impaired this phenotype, indicating that SCD1 activity is crucial for DEPDC1A-V1 induced migration.

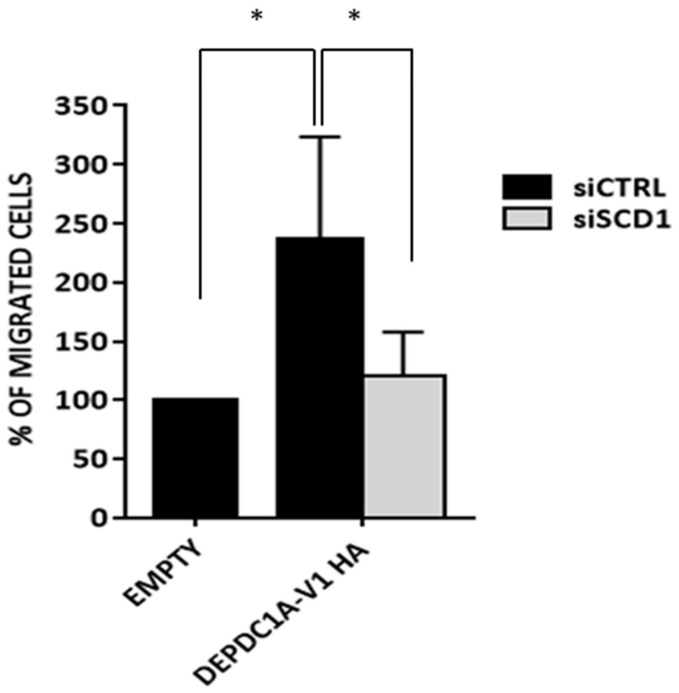


Figure 19. SCD1 silencing impairs DEPDC1A-V1 induced migration in MCF-10A cells. Migration of MCF-10A cells stably transduced with EMPTY- or HA-tagged DEPDC1A-V1 overexpressing vector upon control (siCTRL) or SCD1 silencing (siSCD1) by transwelling assays. The number of migrated cells is expressed as fold change with respect to the EMPTY vector siCTRL treated cells. Error bars indicate s.d of at least three replicates. Asterisks indicate p-value <0.05.

Considering that cancer cells become dependent to oncogene-induced metabolic rewiring and that DEPDC1A may indeed contribute to it, we asked whether DEPDC1A overexpressing cells might be sensitive to SCD1 inhibition. To this aim we measured cell viability of EMPTY-vector or DEPDC1A overexpressing MCF-10A cells left untreated or treated with DMSO or the selective SCD1 inhibitor MF-438. As shown in Figure 20, while EMPTY MCF-10A cells were not affected by MF-438 treatment, the viability of DEPDC1A-V1overexpressing cells was significantly lower, showing that DEPDC1A-V1 overexpression is sufficient to induce a sensitization toward this metabolic inhibitor.

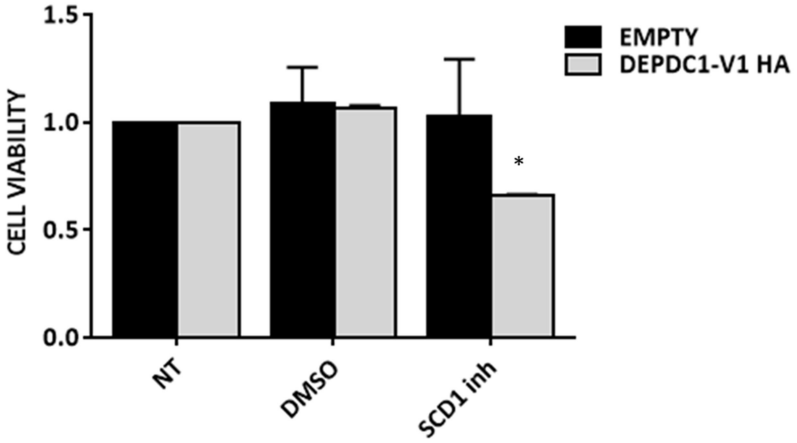


Figure 20. DEPDC1A overexpression induces sensitization toward SCD1 inhibition in MCF-10A cells. ATP-lite assays were performed in MCF-10A cells stably transduced with EMPTY or DEPDC1A overexpressing vector left untreated (NT) or treated with DMSO or the SCD1 specific inhibitor MF-438 (2uM). Cell viability was measured with a multi-plate reader after 48h. The values are expressed in fold change with respect to the NT cells. Error bars indicate s.d of at least three replicates. Asterisk indicates p-value <0.05.

2.4 DEPDC1A is a transcriptional cofactor of SREBP1.

Based on DEPDC1A acting as a transcriptional co-factor, we next asked if the transcriptional regulation of the fatty acid biosynthesis genes was exerted at the level of their promoters. We chose to analyse the promoter of endogenous *SCD1*, as this was the most effectively regulated gene by DEPDC1A, and we performed a Chromatin Immunoprecipitation (ChIP) assay using an anti-DEPDC1A antibody or non-related IgGs as a negative control (Figure 21a). Three different regions of the *SCD1* promoter and one in the first intron were analysed by qRT-PCR on the immunoprecipitated chromatin, (Fig. 21b) to determine the binding enrichment of DEPDC1A.

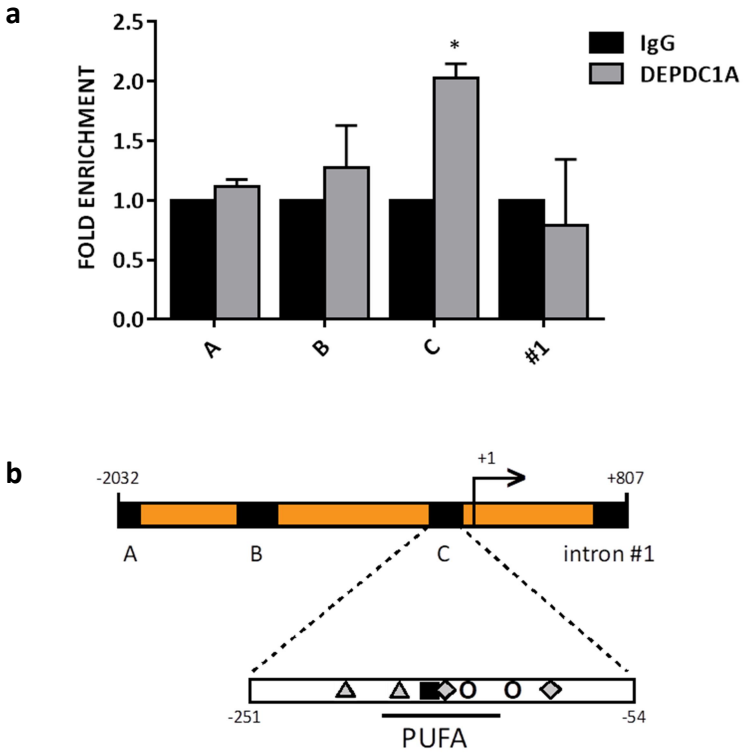


Figure 21. DEPDC1A binds to the endogenous *SCD1* promoter. ChIP assay of DEPDC1A or IgG antibodies on endogenous *SCD1* promoter in MDA-MB-231 cells; three different regions of the *SCD1* promoter and the first intron were analysed by qRT-PCR; the enrichment of immunoprecipitated chromatin is expressed as fold change relative to the negative control IgG. Error bars indicate s.d of at least three replicates. Asterisk indicates p-value<0.05. (b) scheme representing the human *SCD1* promoter with the analysed regions represented with respect to the transcription start site (+1). Enlargement of region C indicates presence of different TF response elements as indicated by diamonds, circles, triangles, and in particular a SRE element (black square).

Interestingly, we observed a two-fold binding enrichment in region C, corresponding to a fragment located -251/-54 nucleotides upstream of the TSS (+1) (Figure 21a). This region has been reported to contain a poly-unsaturated fatty acid response element (PUFA), a binding region for the liver-x-receptor (LXR) transcription factor and a consensus sequence for SREBPs (Benè et al., 2001). This result together with the notion that SREBPs are master regulators of lipid metabolism and known regulators of ACLY, SCD1 and ELOVL6, prompted us to determine if DEPDC1A could exert its transcriptional activity through interaction with SREBPs. To this aim, we first demonstrated with co-immunoprecipitation assays that these proteins interact with each other, in particular we saw that the endogenous DEPDC1A protein interacts with the 125 kDa full length form of SREBP1 in MDA-MB-231 cells (Figure 22).

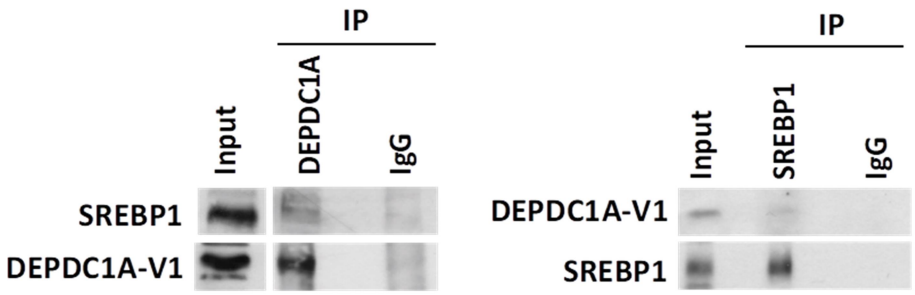


Figure 22. DEPDC1A interacts with SREBP1 transcription factor. Co-immunoprecipitation assay between endogenous DEPDC1A and SREBP1 in MDA-MB-231.

Furthermore, to understand which SREBP1 isoform was involved and if DEPDC1A could bind also SREBP2, we overexpressed separately in HEK293T cells the nuclear and active form of SREBP1a, SREBP1c and SREBP2 proteins with an N-terminal flag epitope together with an HA-tagged DEPDC1A-V1. Co-immunoprecipitation experiments allowed us to observe that DEPDC1A is able to bind SREBP1a and to a lesser extent also SREBP2, but not to SREBP1c (Figure 23).

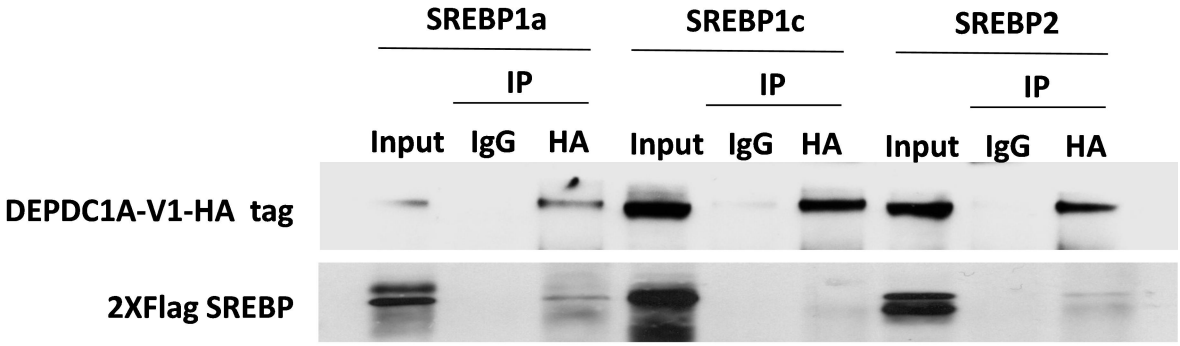


Figure 23. DEPDC1A interacts with SREBP1a and SREBP2. Co-immunoprecipitation assays between DEPDC1A and the nuclear form of SREBP1a, SREBP1c and SREBP2 proteins overexpressed in HEK293T cells.

To formally demonstrate that DEPDC1A is recruited to chromatin by SREBP1, we performed a ChIP assay using an anti-HA antibody or non-related antibody (IgG) in MDA-MB-231 cells overexpressing HA-tagged DEPDC1A-V1, upon Control or SREBP1 silencing. Region C of the *SCD1* promoter, in which we previously observed DEPDC1A binding, was analysed by qRT-PCR and the result showed that, without

SREBP1, region C is not any more enriched in the chromatin immunoprecipitated by the anti-HA antibody (Figure 24), indicating that the ability of DEPDC1A to bind to the *SCD1* promoter is lost in absence of SREBP1.

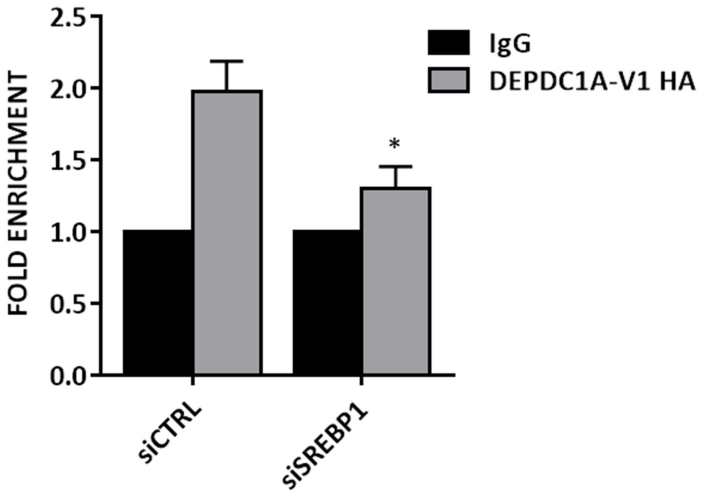


Figure 24. DEPDC1A binds to the human *SCD1* promoter through SREBP1. ChIP assay of DEPDC1A-V1 HA or IgG antibodies on endogenous *SCD1* promoter in MDA-MB-231 cells upon control (siCtrl) or SREBP1 (siSREBP) silencing; region C was analysed by qRT-PCR and the enrichment of immunoprecipitated chromatin is expressed as fold change with respect to the negative control IgG. Error bars indicate s.d of at least three replicates. Asterisk indicates p-value<0.05.

Next we asked whether the DEPDC1A-SREBP1 interaction is functional for the transcriptional activity of SREBP1. To this aim, we measured the binding ability of SREBP1 on the *SCD1* promoter in cells with or without DEPDC1A siRNA by ChIP experiments. As shown in Figure 24, DEPDC1A depletion had a little effect on SREBP1 chromatin binding on *SCD1* promoter, indicating that the reduction in SCD1 expression upon DEPDC1A silencing is not entirely due to a less efficient SREBP1 chromatin localization (Figure 24).

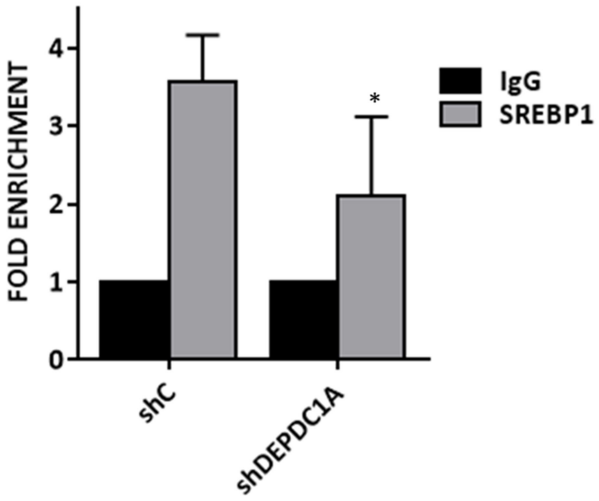


Figure 24. SREBP1 binding on the *SCD1* promoter upon DEPDC1A knock-down. Chromatin Immunoprecipitation assay of SREBP1 or IgG antibodies on *SCD1* promoter in MDA-MB-231 cell line upon control or DEPDC1A silencing; the C region was analysed by qRT-PCR and the enrichment of immunoprecipitated chromatin is expressed as fold change with respect to the negative control IgG. Error bars indicate s.d of at least three replicates. Asterisk indicates p-value<0.05.

Since DEPDC1A silencing seems to have only a weak effect on the SREBP1 binding on *SCD1* promoter, we wanted to investigate the effect of DEPDC1A silencing on the SREBP1 transcriptional activity. To answer this question, we performed a luciferase reporter assay in MDA-MB-231 cells with a human *SCD1*-promoter luciferase reporter vector whose promoter sequence contains region C; to activate the SREBP1 transcriptional activity we cultured the cells with media supplemented with either 10% Fetal Bovine Serum or 1% Lipid Depleted Serum and we measured the luciferase activity upon control or DEPDC1A silencing; as shown in Figure 25 and in line with literature data, when the cells were grown in lipid-free serum supplemented medium, we induced a SREBP1 activation and a consequent increase of the luciferase signal with respect to cells grown in full serum containing medium. Interestingly, when in the same conditions we silenced DEPDC1A, SREBP1 activation was abrogated to the same extent of SREBP1 silencing.

These data point toward a role of DEPDC1A in regulating SREBP1 transcriptional activity on *SCD1* promoter even without affecting its chromatin binding.

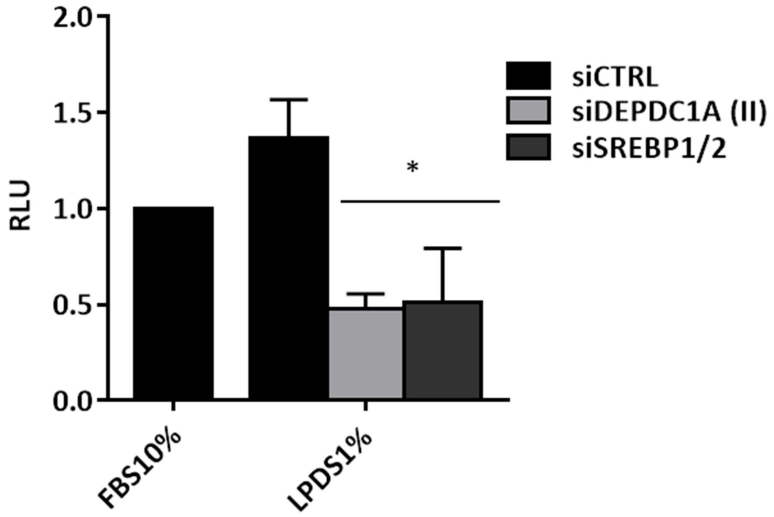


Figure 25. DEPDC1A is required for the transcriptional activation of the *SCD1* promoter by SREBP1. Luciferase assay using *SCD1*-luc reporter vector in MDA-MB-231cell line treated with the indicated siRNAs; cells were cultured in DMEM medium supplemented with FBS 10% or Lipid Depleted Serum (LPDS) 1% for 12h and the difference is expressed in fold change with respect to the siCTRL FBS 10%. The firefly luciferase (luc) signal is normalized on luminescence levels resulting from coexpressed Renilla luciferase and is indicated in relative light units (RLU). Error bars indicate s.d of at least three replicates. Asterisk indicates p-value <0.05.

To verify that this effect was not due to reduced SREBP1 protein levels, we monitored their variation in MDA-MB-231 cells in the different experimental conditions as above by Western blot analysis. As shown in Figure 26, the analysis indicates that SREBP1 cleavage increases in cells grown with lipid-free serum, with a corresponding increase in SCD1 protein levels. Upon DEPDC1A silencing, the levels of SREBP1 remained unchanged, while SCD1 strongly decreased to levels comparable to those in full serum conditions. The silencing of SREBP1 effectively reduced both precursor and cleaved SREBP1 protein and impaired SCD1 expression as well.

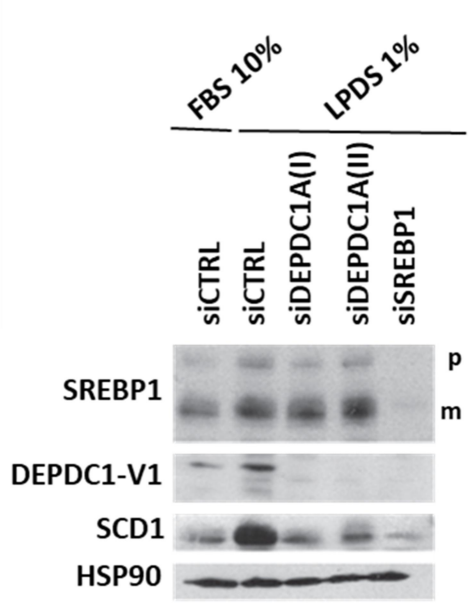


Figure 26. DEPDC1A silencing impairs SREBP1 transcriptional activity. Western blot analysis of SREBP1, DEPDC1A-V1 and SCD1 in lysates of MDA-MB-231 cultured in media supplemented with FBS 10% or Lipid Depleted Serum (LPDS) 1% for 12h upon control, DEPDC1A or SREBP1 silencing. HSP90 is shown as loading control.

All these data indicates a role of DEPDC1A as transcriptional co-factor of SREBP1; it is required for SREBP1 chromatin occupancy, and its absence strongly impairs the lipid starvation-induced transcriptional activation of its target genes without modifying its protein levels.

2.5 DEPDC1A is a transcriptional target of SREBPs transcription factor.

Looking to the Western blot of Figure 26, it did not escape to our attention that lipid depleted serum-induced activation and subsequent silencing of SREBP1, respectively increased and abolished DEPDC1A protein levels. This could be explained by the fact that DEPDC1A might itself be a SREBP transcriptional target. To test this hypothesis, we measured transcript levels of DEPDC1A upon different siRNAs against SREBP1 and SREBP2 with qRT-PCR. Interestingly, genetic ablation of both SREBPs significantly downregulated *DEPDC1A* messenger RNA, indicating that a transcriptional regulation by SREBPs exists (Figure 27a). Accordingly, also DEPDC1A protein levels were decreased following SREBPs RNA interference (Figure 27b).

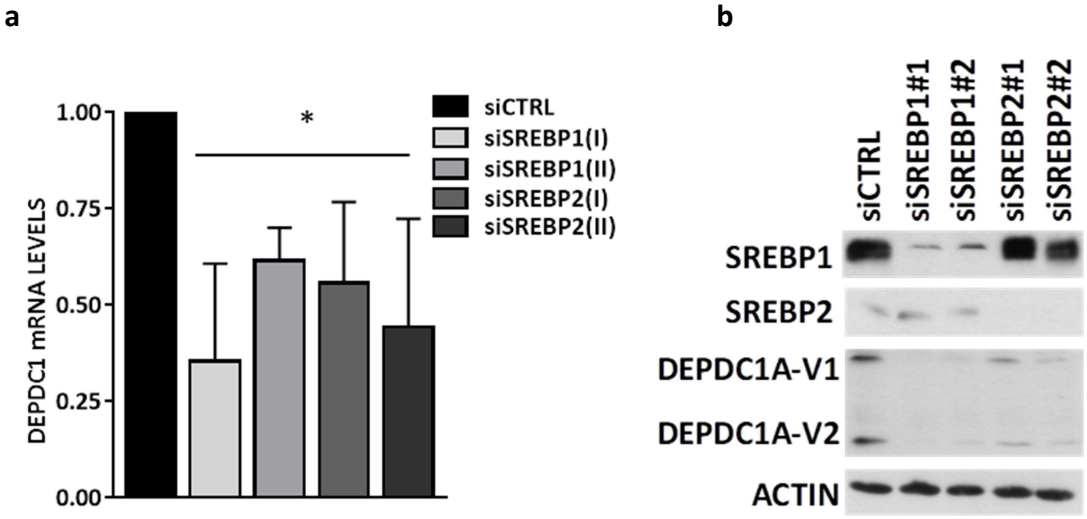


Figure 28. DEPDC1A is transcriptionally regulated by SREBP proteins. (a) qRT-PCR analysis of DEPDC1A transcript in MDA-MB-231 cells treated with the indicated siRNAs. Changes in gene expression are indicated as fold change with respect to siCtrl. Error bars indicate s.d of at least three replicates. Asterisk indicates p-value <0.05. (b) Western blot analysis of the cellular lysates of the experiments in (a); SREBP1, SREBP2 and DEPDC1A proteins in MDA-MB-231 cells treated with the indicated siRNAs are shown. Actin is shown as loading control.

Next, we sought to determine whether this is a direct transcriptional control, in other terms, if SREBP transcription factors directly bind to the *DEPDC1A* promoter. First, using a transcription factor binding site predictor software (Transfac®) we analysed the *DEPDC1A* promoter in order to verify the presence of a consensus sequence for SREBPs, and indeed we found a SRE element between -157/146nt upstream the TSS. Then, we performed ChIP experiments using anti-SREBP1, anti-SREBP2 or non-related IgG antibodies on the *DEPDC1A* promoter and indeed we obtained a strong binding enrichment in the region containing the SRE element, showing that SREBP transcription factors can modulate DEPDC1A expression directly by binding to its promoter (Figure 28). Altogether, the results of Figures 25-29 demonstrate that, in cancer cells, SREBPs induce their own transcriptional co-factor DEPDC1A, to enhance their transcriptional activity.

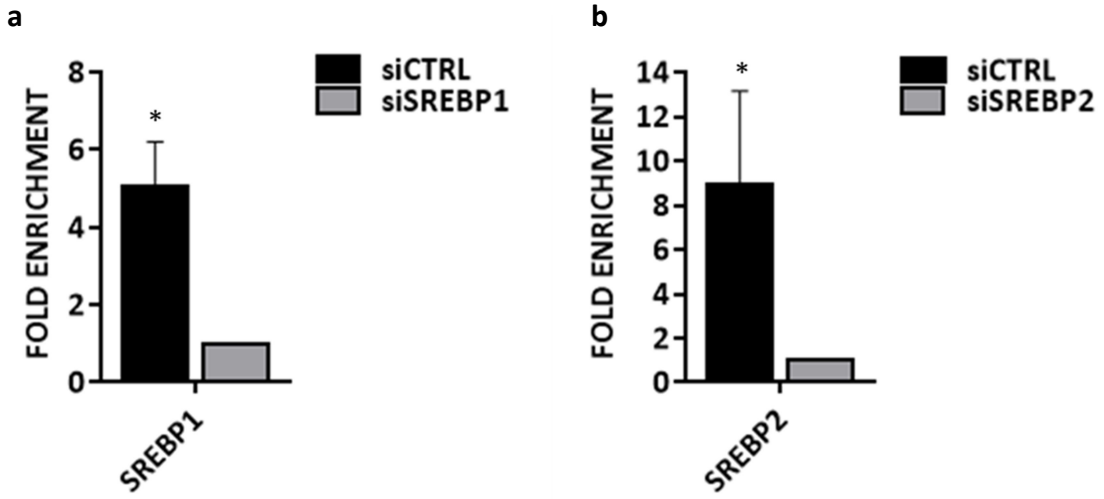


Figure 28. SREBP transcription factors bind to the human *DEPDC1A* promoter. ChIP assay of SREBP1, SREBP2 or IgG antibodies on *DEPDC1A* promoter in MDA-MB-231 cells upon control, SREBP1 or SREBP2 silencing; The region with the predicted SREBPs binding site was analysed by qRT-PCR and the enrichment of immunoprecipitated chromatin is expressed as fold change with respect to the negative control IgG. Error bars indicate s.d of at least three replicates. Asterisks indicate p-value<0.05.

DISCUSSION

Currently only ~20% of patients with metastatic breast cancers respond to therapy ,a fact mainly ascribed to the intrinsic heterogeneity and plasticity that cancer cells display. This complexity, whose molecular underpinnings are only partially understood, is believed to underlie imprecise diagnoses and definition of metastatic risk, often leading to therapeutic decisions that fail to effectively tackle the disease (Santa-Maria and Gradishar, 2015). In addition, even after a primary response, metastatic breast cancers frequently become resistant to the therapy. For all these reasons it is evident that current classification and stratification methods are insufficient and that further research is necessary to unveil novel therapeutic vulnerabilities specifically directed to metastatic cells.

In this thesis, we focused our attention on DEPDC1A, an oncogene that is almost undetectable in normal adult tissues, while it is overexpressed in many aggressive tumours and strongly associated with poor patients' prognosis. In cancer cells, DEPDC1A has been mainly associated to proliferation and cell cycle (Huang et al., 2017), as well as migration and invasion (Girardini et al., 2011), and our unpublished work demonstrated a role for DEPDC1A in promoting metastasis formation. Here, thanks to a high-throughput transcriptomic approach, we have unveiled a novel oncogenic function of DEPDC1A in a highly metastatic triple negative breast cancer model. In particular, DEPDC1A is responsible for the transcriptional induction of genes involved in the fatty acids biosynthesis pathway (*ACLY*, *SCD1* and *ELOVL6*). Although a broader bioinformatic analysis in a transcriptomic dataset of metastatic breast cancers did not reveal a strong positive association between *DEPDC1A* and *ACLY, SCD1* and *ELOVL6* expression, an immunohistochemical staining in a set of 17 human breast cancer biopsies revealed a positive correlation between DEPDC1A-V1 and SCD1 protein levels. This discrepancy could be explained by the fact that in the bioinformatic analysis we were not able to discriminate between the V1 and V2 isoform expression; since we have the evidence that the V1 isoform is the responsible for the induction of the lipid metabolic traits, the presence of the V2 could mask the transcriptional effect.
The activation of this particular signature causes increased fatty acids accumulation, as demonstrated by increased lipid droplets content, and desaturation when DEPDC1A is

present. Lipid accumulation is a well-established phenotype in cancer cells which display a deranged lipid metabolism, and there is evidence that lipid droplets are required for cancer cell proliferation and aggressiveness (Koyzume and Miyagi, 2016; Tirinato et al., 2017), thus DEPDC1A, by activating lipogenesis, can influence cancer cell behaviour.

Owing to the fact that these genes are regulated by the transcription factor SREBP1, a master regulator of fatty acid metabolism, and that we found a great overlap between DEPDC1A and SREBP1 regulated genes (data not shown), we have demonstrated that these two proteins cooperate in the expression of SCD1. Mechanistically, we have shown that DEPDC1A physically interacts with SREBP1a and SREBP2 but not with SREBP1c, the binding specificity towards SREBP1a could be explained by the fact that it has a longer N-terminal transactivation domain than SREBP1c that could endow the protein with more affinity for DEPDC1A. Moreover, we have demonstrated that DEPDC1A is recruited by SREBP1 on the *SCD1* promoter, even if with a small fold enrichment with respect to IgG. This low enrichment might be explained by the fact that DEPDC1A does not bind directly the DNA, since it does not have a DNA binding domain. Nevertheless, the negative effect of DEPDC1A knockdown on SREBP1 transcriptional activity is evident by the luciferase experiments. This result indicates that DEPDC1A can act as transcriptional cofactor of SREBP1 and regulating SCD1 transcription.

Fatty acid biosynthesis and, in particular, desaturation is instrumental for the induction of aggressive phenotypes, such as migration and invasion (Baenke et al., 2013); in fact, when we interfered with SCD1 activity (either by siRNA or enzymatic inhibition) in DEPDC1A overexpressing cells, we were able to abolish the DEPDC1A dependent migratory ability, demonstrating that, in this context, desaturation of fatty acids is a crucial step for this oncogenic activity and that SCD1 inhibition can be an interesting therapeutic option.

Moreover, non-transformed breast cells (MCF-10A), which ectopically overexpress DEPDC1A and therefore have an increased SCD1 expression, become more sensitive in terms of viability to SCD1 enzymatic inhibition than empty-vector expressing control cells, indicating that lipid metabolic reprogramming induced by DEPDC1A might induce a potentially druggable dependency in cancer cells.

Our results open up to further implications on the consequences of this aberrantly activated metabolic axis on cancer cells. For instance, SCD1 and ELOVL6 are relevant

for the maintenance of specific fatty acids compositions that is known to impact on stemness and membrane fluidity, both crucial properties of aggressive cancer cells (Zhang et al., 2007; Marien et al., 2016; Li et al., 2017). Moreover, fatty acid synthesis, desaturation and lipid droplets formation are key processes for Reactive Oxygen Species (ROS) scavenging and endoplasmic reticulum stress attenuation, particularly relevant in highly proliferating cancer cells (Matsui et al., 2012; Fu et al., 2012). Furthermore, SCD1 has been shown to be required for lipid modifications of signalling molecules, such as WNT, in order to sustain lung cancer stem cells (Rios-Esteves et al., 2013; Noto et al., 2017).

Another important implication involves ACLY, the rate limiting enzyme for the production of the cytosolic acetyl-CoA, which is not only required for fatty acid biosynthesis but is crucial also for acetylation of histones, thus potentially impacting on the cancer cells' epigenetic landscape, which in turn underlies their plasticity and gives rise to adaptive clones that boost malignant progression (Kinnaird et al., 2016).

In recent years, a growing body of evidence indicates that metabolic rewiring might represent a double edged sword, since cancer cells have been shown to depend on it, and, furthermore, it represents a possible way to discriminate the cancerous tissue from the normal counterpart (Hay et al., 2016), and many clinical trials based on the inhibition of metabolic targets are ongoing.

Indeed, it is possible to target the transcriptional program activated by DEPDC1A. In particular here we have shown that SCD1 inhibition is able to dampen the oncogenic activity of DEPDC1A, but it does not affect normal cells. Pharmacological inhibition of SCD1 has been linked to toxicity to sebocytes in the skin and other tissues, but new inhibitors demonstrated to have no side effects towards non-transformed tissues (Theodoropoulos et al., 2016); with the rationale that cancer cells are strongly dependent on large amounts of lipids for biomass production and the synthesis of lipid-derived signalling molecules and that fatty acid desaturation is a crucial step for red-ox balance and membrane fluidity (Koizume and Miyagi, 2016; Peck and Schulze, 2016), targeting SCD1 can be a valuable option, possibly in concomitance with other drugs that inhibit lipid metabolism, such as statins, in order to simultaneously abrogate two major processes of lipid metabolism that are crucial for metastatic cells.

Interestingly, also ACLY and ELOVL6 are potential therapeutic targets, whose inhibitions has demonstrated to be promising both *in vitro* and *in vivo.* (Marien et al., 2016; Kinnaird et al., 2016).

In sum, understanding the alterations of metabolic pathways that could endow cancer cells with metastatic proclivity is of crucial importance. With this thesis we provided a novel transcriptional mechanism that is able to induce a metabolic reprogramming in breast cancer, which sustains pro-metastatic phenotypes. More importantly, we showed that this altered fatty acid metabolism represents a possible vulnerability that could be potentially employed, in combination with other inhibitors and conventional therapy, to selectively dampen breast cancer invasiveness and dissemination.

Data from our laboratory and other works indicate DEPDC1A as a cancer associated protein, and in our model we showed that it is induced by mutant p53. Previous work demonstrated that mutant p53 is able to induce lipid metabolism by direct interaction with SREBP2 and eliciting mevalonate pathway upregulation in triple negative breast cancer (Freed-Pastor et al., 2012). In this thesis we showed that another parallel mechanism is active to simultaneously upregulate fatty acid biosynthesis and enhance cancer aggressiveness, thus confirming that lipid metabolism upregulation is a crucial event in mutant p53 harbouring tumours. Interestingly, we evidenced that DEPDC1A is also a transcriptional target of SREBP1/2 making us hypothesize that SREBPs could be the transcription factors through which mutant p53 can induce DEPDC1A expression. Preliminary ChIP experiments indicate that SREBPs knockdown impairs mutant p53 binding on *DEPDC1A* promoter (data not shown), thus confirming our hypothesis that mutant p53 and SREBPs cooperation is crucial for DEPDC1A overexpression. Moreover, in this context, since both mutant p53 and DEPDC1A are able to bind and regulate SREBPs activity, it is possible to hypothesize that these protein can constitute a ternary complex able to regulate lipid metabolism and experiments will be performed in this direction.

Furthermore, we have evidence that DEPDC1A can be induced also in a mutant p53 independent manner and that other oncogenes could mediate its activation (such as MYC and E2F; data not shown), indicating that it is a common target of different oncogenic programs. For these reasons, the study of DEPDC1A roles and regulation acquires a broader importance, not only for its overexpression in many cancers with different genetic background, but also because its inhibition is expected to be detrimental selectively for cancer cells.

Interfering with DEPDC1A functions could be exerted in multiple ways, by using DEPDC1A as onco-antigen or by inhibiting its interaction with transcription factors.

For the first strategy, many efforts have been made to develop onco-vaccines against DEPDC1A. Some of them have already been used in combination with other cancer vaccines that have already been introduced within different clinical trials. The activation of the immune response towards cells overexpressing specific epitopes of DEPDC1A yielded interesting and promising results, indicating that this could be a real therapeutic strategy (Obara et al., 2012).
Regarding the interference with the aberrant interaction with the transcription factor, a cell-permeable dominant negative peptide (11R-DEP: 611-628) that interferes with the DEPDC1A-ZNF224 complex has been produced, and its usage induces growth arrest and apoptosis in bladder cancer cells in a *in vivo* mouse xenograft model (Harada et al., 2010).

For future directions, taking advantage of high-throughput metabolomics analysis, our goal is to confirm and unravel the role of DEPDC1A in regulating lipid metabolism in a panel of different cancers. Secondly, it will be important to understand if, *in vivo*, DEPDC1A bearing tumours can be effectively targeted with SCD1 inhibitors, alone or in combination with other drugs, and if this could avoid metastasis formation.
In parallel, we want to elucidate the mechanism through which DEPDC1A could influence SREBP1 activity in regulating *SCD1, ACLY* and *ELOVL6* transcription. Furthermore, taking advantage of the RNA-seq analysis, other DEPDC1A regulated processes will be explored that could impinge on tumour progression and dissemination. Finally, it will be important to investigate the physiological functions of DEPDC1A and the biochemical activities of its domains in both normal and cancerous tissues. Of particular interest is the fact that its expression is kept at very low levels in normal adult cells and that it is instead aberrantly expressed in cancer; it will be intriguing to dissect the oncogenic signalling and the mechanisms that underlie DEPDC1A activation. All these data will give novel and interesting insights on the role of DEPDC1A in cancer cell biology.

MATERIALS AND METHODS

Cell culture

MDA-MB-231 and MDA-MB-468 triple negative breast cancer cells, human embryonic kidney epithelial cells HEK 293T and 293GP packaging cells have been maintained in culture in Dulbecco's modified Eagle's Medium (DMEM) (Lonza) supplemented with 10% foetal bovine serum (FBS) (Euroclone) or lipoprotein depleted serum (LPDS) 1% (Biowest), penicillin (100 IU/ml) and streptomycin (100 IU/ml). MCF-7 breast cancer cells were cultured in EMEM medium (Lonza) supplemented with 10% FBS, penicillin (100 IU/ml) and streptomycin (100 IU/ml). MCF10-A, a normal mammary cell line, was maintained in culture in DMEM/F12 (Lonza) medium supplemented with 5% HS (horse serum), penicillin (100 IU/ml) and streptomycin (100 IU/ml) plus add of growth factor 10 µg/ml Insulin, 0.5 µg/ml Hydrocortisone and 20 µg/ml Epidermal Growth Factor (EGF). All cells were maintained at 37°C in 5% CO2.

Transfections

Cells were plated one-day before the transfection experiment. The appropriate plating density will depend on the growth rate and the condition of the cells. Cells that are 50–80% confluency were used on the day of the experiment.

For siRNA transfections, double-stranded RNA oligos (10 pmol/cm2) were transfected with Lipofectamine RNAi-MAX (Life Technologies) in antibiotic-free medium according to the manufacturer's instructions. Negative control siRNA was: AllStars negative control siRNA (Qiagen 1027281).

DNA transfections were done with Lipofectamine LTX and Plus Reagent (Invitrogen) or Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions.

Lentiviral particles were prepared by transient transfection of HEK-293T cells with the packaging plasmids and with pLenti expression vector as indicated, using standard calcium-phosphate method. After 48 h incubation at 32°C the virus-containing medium was filtered (0,45 µm filter), supplemented with 10% FCS and 8ug/ml polybrene and added to cells for 24h and then washed. Infected cells were enriched by drug selection (Puromycin, Blasticidin or Zeocyn, 2ug/ml each) for at least one week.

Retroviral packaging was made by calcium phosphate transfection of 293-GP packaging cells with the appropriate plasmids in combination with pMD2ENV coding for envelope proteins. After 8 hours, medium was changed with 10 ml of medium and cells incubated at 32°C. After 48-72h the virus-containing medium was filtered (0,45 μm filter) and supplemented with 10% FBS and 8ug/ml polybrene. The culture medium of target cells growing at low confluence (~30-40%) was replaced by the appropriate viral supernatant and incubated at 32°C for 24h. Infected cells were selected with puromycin or Zeocyn.

siRNAs sequences are listed in the table below:

siRNAs	SEQUENCE (Sense, 5'-3') (dTdT)
siDEPDC1A (I)	CCGUAGUCUAAGAUAACUA
siDEPDC1A (II)	CUAUCCAGUAAGGCUAUCA
siSCD1	CUACGGCUCUUUCUGAUCAUU
siSREBP1 (I)	CCACUCCAUUGAAGAUGUA
siSREBP1 (II)	AUCUCUGAAGGAUCUGGUG
siSREBP2 (I)	UGAGUUUCUCUCUCCUGAA
siSREBP2 (II)	UCAGAAUGUCCUUCUGAUG
siCTRL (QUIAGEN)	UNKNOWN

Plasmids

pLenti-DEPDC1A-V1 was generated by subcloning DEPDC1A-V1 coding sequence from pCAGGSn- DEPDC1A-V1 kindly provided by T. Katagiri in pLenti vector. pSR-shDEPDC1A plasmid was generated by cloning DEPDC1A (I) double stranded siRNA in pSuper Retro vector. The SREBP1a, 1c and 2 overexpressing vectors were obtained from addgene (cod: #26801, #26802, #26807).

RNA isolation and RT-PCR gene expression analysis.

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Spectrophotometric method was used to determine the Concentration and purity of the RNA. For quantitative RT-PCR mRNA was reversed transcribed into cDNA with Quantitect Reverse Transcriptase Kit (Quiagen). Real-time

PCR was carried out in triplicate with SsoAdvanced TM SYBER GREEN Supermix (BioRad) and the CFX96 Real-Time System (Bio-Rad). Relative expression levels were normalized to controls by using the comparative Ct (ΔΔCt).
The house-keeping gene Histone H3 was used to normalize the level of expression.

PRIMERS	SEQUENCE
ACLY FW	GACAGCACCATGGAGACCATGAAC
ACLY REV	GCCAATCTTAAAGCACCCAGGC
DEPDC1A FW	GATCTCCCTGAACCTCTACTTAC
DEPDC1A REV	CACTGGATCTATCTGAAACTGTG
H3 FW	GTGAAGAAACCTCATCGTTACAGGCCTGGT
H3 REV	CTGCAAAGCACCAATAGCTGCACTCTGGAA
ELOVL6 FW	CTCGAAATCAAGCGCTTTACAGA
ELOVL6 REV	AGGCAGCATACAGAGCAGAAA
SCD1 FW	CACTTGGGAGCCCTGTATGG
SCD1 REV	TGAGCTCCTGCTGTTATGCC

Protein extraction and western blot analysis.

For western blot analysis, cells were harvested and lysed in in 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM MgCl2, 1 mM EDTA, 1% Triton X-100 lysis buffer, supplemented with phosphatase and protease inhibitors (Sigma), and lysates were cleared by centrifugation. The proteins were separated using SDS-PAGE and blotted on nitrocellulose films; blocking was performed with 5% non-fat dry milk solution and the antibodies were incubated at 4°C over-night. Immunoreactivity was detected with anti-mouse and anti-rabbit secondary Antibodies HRP labeled (Sigma) using ECL Plus Western Blotting Substrate (Pierce).

For Co-immunoprecipitation (Co-IP) experiments the cells were harvested and lysed in 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM MgCl2, 1 mM EDTA, 1 % Triton X-100, 10 % glycerol supplemented with phosphatase and protease inhibitors, and lysates were cleared by centrifugation and pre-cleared with protein G-sepharose for 30 min before O/N incubation with indicated antibodies. Protein G-sepharose, incubated overnight with 0.5 mg/ml BSA, was then added to the lysates and incubated for 1h. Beads were washed three times in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40.

Antibodies

Antibodies used for western blot: monoclonal anti-SCD1 (Ab-19862 Abcam), monoclonal anti-SREBP1 (Sc-13551 Santacruz), polyclonal anti-SREBP2 (Ab-30682 Abcam), monoclonal anti-PLIN2 (ab181463 Abcam), monoclonal anti-HA (11867423001 Roche) and monoclonal anti-HA Y11 (Sc-805 Santacruz), monoclonal anti-Flag M2 (F3165 Sigma), Actin (A-9718 Sigma) and HSP90 (Sc-13119 Santacruz).

DEPDC1A antibody

For DEPDC1A detection we have generated a functional antibody against DEPDC1A. To this end we have developed a polyclonal anti-DEPDC1 antisera immunizing rabbits against a C-terminal region of 190 amino acids common to the two DEPDC1A variants (DEPDC1A-V1 and DEPDC1A-V2).

To generate the recombinant protein as immunogene, the plasmid pGEX 4T1 expressing a common region of two variants of DEPDC1 (621–811 amino acids of DEPDC1-V1) and contained S-transferase (GST) gene from Schistosoma japonicum upstream of the MCS, was expressed in Escherichia coli DH5α cells. Overnight culture obtained were diluted 1:50 in Luria Broth (LB) medium containing ampicillin and grown at 37°C with shaking to obtain a 0.3-0.6 OD 600. Then, 1 mM IPTG was added to the culture for overnight induction at 30°C. Induced E. coli. cells were harvested by centrifugation then the cells were suspended in 10 mL of cold lysis buffer (10 mL PBS, 1% Triton-X, 1.5 % DTT (w/v), and protease inhibitors (Sigma), and then applied the suspension to sonication. Sonication was carriedmout using intensity input at 8 on a Misonix Sonicator for 10 sec for “pulse on” and 30 sec for “pause” for each cycle, 6 cycles totally.

The sonicated cell lysates were centrifuged at 10,000x rpm for 15 min at 4 °C and the supernatant were removed to a new tube and incubated with glutathione-sepharose 4b beads (GE Healthcare) overnight at 4°C according to the supplier’s protocol. The quality of the GST fusion proteins was verified by Comassie blue staining (0.2 % Comassie blue R-250, 40 % Methanol, 10% Acetic acid). To remove E. coli’s proteins as contaminations, each DEPDC1A fragment protein was cut and extracted from sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel.

The extracted proteins were inoculated into rabbits, and subsequently the immune sera were purified on antigen affinity columns produced using the same antigenic peptide

cloned in pMal vector which encodes maltose-binding protein (MBP), according to the supplier's instructions.

Immunohistochemistry

Tissues were either fixed in 10% neutral-buffered formalin, processed, and embedded in paraffin. Prior to immunostaining, tissue sections were deparaffinised, rehydrated, and heated in citrate buffer pH 6 for antigen retrieval. Sections were incubated in methanol/ 30% H2O2 for 15 minutes at room temperature and blocked for 1-2 hours in normal horse serum/PBS/0.1 Tween solution (Vectastain). After the blocking, slides were incubated at 4°C with the indicated primary antibody, and then with the universal secondary antibody (Vectastain) for 45 minutes. To visualize the stain DAB solution (Vector Laboratories) was used followed by hematoxylin counterstaining.

Chromatin Immunoprecipitation

After specific treatments, MDA-MB-231 cells were cross-linked for 15 min with 1% formaldehyde, neutralized with 125 mM glycine pH2.5 and washed in PBS. Cells were scraped in PBS containing proteases inhibitors (protease inhibitor cocktail [Sigma], 1 mM PMSF), and centrifuged at 4000 rpm for 10 min at 4°C.

Cellular pellets were resuspended in hypotonic SDS lysis buffer (1% SDS, 10 mM EDTA, 50mM Tris-HCl pH 8.1, protease inhibitor cocktail [Sigma], 1 mM PMSF, 5 mM NaF) and incubated for 10 min at 4°C.

Chromatin was sonicated (power setting 5) with a Misonix Microson in 10" bursts followed by 50" of cooling on ice for a total sonication time of 2 min per sample.

Samples were then centrifuged for 10 minutes at 13000 rpm at 4°C and the supernatants were transferred to new micro-centrifuge tubes and diluted 2 fold in ChIP dilution Buffer (0.01% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl).

Chromatin was pre-cleared for 1 h at 4°C with protein A/G PLUS-Agarose (Santa Cruz Biotechnologies) and at this step 10% of the samples was taken as Input. The immunoprecipitation was performed O/N at 4°C with 1 ug of the indicated antibodies on the remaining 90%. The day after the DNA/protein complexes were recovered with protein A/G PLUS-Agarose and washed sequentially with 1ml of Low Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), 1ml of High Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM

Tris-HCl pH 8.1, 500 mM NaCl) and 1ml of LiCl Wash Buffer (250 mM LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1) and TE. RNase treatment was performed in TE for 30 min at 37°C.

The proteinA-G agarose/ DNA/Histones complexes now were eluted in 1% SDS 0.1 M $NaHCO_3$ solution vortexed and incubated for 15 minutes at room temperature with rotation, the samples were pelleted and the eluates were transferred into new micro-centrifuge tubes. 20 ul of NaCl 5M were added to the eluate and the de-crosslinking was obtained by putting the samples at 65°C O/N. In parallel, inputs were treated in the same way. After phenol/chloroform extraction and ethanol precipitation samples were resuspended in H_2O. Real-time qPCR was performed by using iTaq Universal SYBR Green Supermix (BIORAD). Promoter occupancy was calculated as the fold increase of normalized immunoprecipitated chromatin over the control IgG with the $2^{-\Delta\Delta Ct}$ method.

The primers used are listed below

PRIMERS	SEQUENCE
SCD1 PROMOTER REG A FW	GCTAGGCAGTTCATGGGCA
SCD1 PROMOTER REG A REV	GGGAGGGAGAGTACAGGGTAA
SCD1 PROMOTER REG B FW	TTTCAACTTGGGGAGCTGGG
SCD1 PROMOTER REG B REV	CCTGGCTTTATGATACATTGTCCC
SCD1 PROMOTER REG C FW	CAGAGAGAAAGCTCCCGACG
SCD1 PROMOTER REG C REV	CTGTAAACTCCGGCTCGTCA
SCD1 PROMOTER INTR 1 FW	GCAGTTTTACCTCGGTCGGA
SCD1 PROMOTER INTR 1 REV	CGCTCCTCATAGCGAGTCTG
DEPDC1A PROMOTER FW	GTAGACCGCAGGAGGGAGAA
DEPDC1A PROMOTER REV	TGAATAGCATAGAGGGCTGCG

Luciferase assays

Luciferase assays were performed in MDA-MB-231 cells with the PGL3 vector as reporter with SCD1 promoter sequence. Cell lysates were analyzed using the Dual-Luciferase Reporter Assay System (Promega). Luciferase reporters (300 ng cm−2) were transfected together with CMV–Renilla (30 ng cm−2) to normalize for transfection efficiency.

For luciferase assays in siRNA-transfected cells, siRNA transfection was achieved first and, after 48 h, transfection of plasmid DNA was performed and the cells were cultured in FBS 10% or LPDS 2% supplemented medium.

Migration assays.

For migration assay, transfected cells (1,5x105) were plated on 24 well PET inserts (8.0 μm pore size, Falcon), according to the manufacturer's instructions. After 18h, cells on the upper part of the membrane were removed with a cotton swabs and cells that passed through the filter were fixed in 4% PFA, stained with 0.05% crystal violet and counted. The data were represented as the means of at least three independent experiments with standard deviations indicated. Difference compared with the vector control.

Viability assay.

Cells (5*10^3 per well) were plated in 96-well plates and treated with SCD1 inhibitor (MF-438, Calbiochem) 2uM, Non treated cells and DMSO treated cells were used as controls. Cell viability was assayed with ATPlite (Perkin Elmer) according to the manufacturer's instructions using the EnSpire Multilabel Reader (Perkin Elmer).

Lipid droplets staining

Lipid droplets staining was performed as following. Briefly, cells were fixed in 4% paraformaldehyde for 15 min, and then washed in PBS. Cells were incubated with LD540 fluorescent dyes (50ng/ml) for 20 minutes at room temperature. Nuclei were counterstained with DAPI. LD540 was kindly provided by Cristoph Thiele Group.

The images were acquired through confocal microscopy, using a Nikon C1si confocal microscope, containing,457,477, 488, 514 argon lasers lines and 407, 561 or 640 nm diode laser. Light was delivered to the sample with an 80/20 reflector. The system was operated with a pinhole size of one Airy disk (30 nm). Electronic zoom was kept at minimum values for measurements to reduce potential bleaching. A 60X Oil Apo objective (with corresponding NA of 1.4) was used. Images were then processed for z-projection by using ImageJ 1.50 (NIH, Bethesda, USA).

For flow cytometry analysis of lipid droplets, the cells were harvested, pelleted and washed twice with PBS; then cells were re-suspended in 4% PFA and fixed for 20 minutes. After that, the cells were washed with PBS and stained in a 1ug/ml solution of BODIPY 493/503, (Invitrogen) for 30 minutes.

Analysis of FAMEs and desaturation.

Fatty acid composition of the different test cells was analysed through a standard method based on saponification of lipids, methylation of fatty acids and gas chromatography/mass spectrometry analysis of the obtained FAMEs. Fatty acids were identified on the basis of their retention times and MS spectra and comparison with the gas chromatography/mass spectrometry library. The identification of FAMEs was obtained by comparison of sample mass spectra with those available in the Wiley 275 libraries. Relative percentage for each component was calculated based on GC peak areas.

Gene expression data analysis and Functional Analysis.

Three biological mRNA replicates for each group (siControl or siDEPDC1A (I)) were performed on MDA-MB-231 cells. Total RNAs were extracted using Trizol reagent (Invitrogen), subjected to DNase-I (Ambion) treatment and RNAs were depleted of ribosomal RNA. Sequencing libraries for whole transcriptome analysis were prepared using ScriptSeq™ mRNA-Seq Library Preparation Kits (Epicentre® Biotechnologies, Madison, WI).

RNA-seq was performed on an Illimina HiSeq 2000 station using standard conditions. Demultiplexed raw reads (fastq) generated from the Illumina HiSeq were checked using FASTQC tool and all samples passed the quality standards. We aligned them to the reference genome (UCSC-hg38) using STAR (Dobin et al., 2013), using recommended options and thresholds. FeatureCounts (Liao et al., 2014) was used to generate gene counts based on GENCODE 25. Differential gene expression analysis was performed using Edge R (Robinson et al., 2010).

In order to identify the relationship between samples, the Euclidean distance between each pair of samples was calculated using the log-transformed values of cpm. Average linkage clustering was then used to generate a sample-to-sample distance heatmap, via the cluster3 software.

Genes with FDR<0.01 and fold change > 0.5 or <-0.5 were selected as differentially expressed and used as input for the functional analysis.

Starting from the list of differentially expressed genes we performed functional enrichment analysis (Huang et al., 2009) and gene set enrichment analysis (Subramanian et al., 2005).

Differentially expressed genes were further analysed using Ingenuity Pathway Analysis (IPA, Ingenuity® Systems, www.ingenuity.com). The prediction of the transcription factors and regulative molecules was obtained using the Upstream regulators function (IPA suite). For every upstream regulator an overlap p-value and a z-score are calculated: the p-value indicates the significance based on the overlap between dataset genes and known targets regulated by the molecule, while the z-score is used to infer the possible activation (z-score > 0) or inhibition (z-score < 0) of the molecule based on prior knowledge stored in the proprietary Ingenuity Knowledge Base.

All statistical analysis and calculations have been performed in Rstatistical environment.

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REFERENCES

Adorno, M., Cordenonsi, M., Montagner, M., Dupont, S., Wong, C., Hann, B., Solari, A., Bobisse, S., Rondina, M.B., Guzzardo, V., Parenti, A.R., Rosato, A., Bicciato, S., Balmain, A., Piccolo, S. (2009). A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. Cell. 137(1):87-98.

Amin, E., Jaiswal, M., Derewenda, U, Reis, K., Nouri, K., Koessmeier, K.T., Aspenström, P., Somlyo, A.V., Dvorsky, R., Ahmadian, M.R. (2016). Deciphering the Molecular and Functional Basis of RHOGAP Family Proteins: A SYSTEMATIC APPROACH TOWARD SELECTIVE INACTIVATION OF RHO FAMILY PROTEINS. J Biol Chem. 291(39):20353-71.

Baenke, F., Peck, B., Miess, H., Schulze, A. (2013). Hooked on fat: the role of lipid synthesis in cancer metabolism and tumour development. Dis Model Mech. 6(6):1353-63.

Ballon, D.R., Flanary, P.L., Gladue, D.P., Konopka, J.B., Dohlman, H.G., and Thorner, J. (2006). DEP-domain-mediated regulation of GPCR signaling responses. Cell 126, 1079-1093.

Barata, J.T., Silva, A., Brandao, J.G., Nadler, L.M., Cardoso, A.A., Boussiotis, V.A. (2004). Activation of PI3K is indispensable for interleukin 7-mediated viability, proliferation, glucose use, and growth of T cell acute lymphoblastic leukemia cells. J Exp Med. 200(5):659-69.

Ben-David, U., Gan, Q.F., Golan-Lev, T., Arora, P., Yanuka, O., Oren, Y.S., Leikin-Frenkel, A., Graf. M., Garippa, R., Boehringer, M., Gromo, G., Benvenisty, N. (2013). Selective elimination of human pluripotent stem cells by an oleate synthesis inhibitor discovered in a high-throughput screen. Cell Stem Cell. 12(2):167-79.

Bené, H., Lasky, D., Ntambi, J.M. (2001). Cloning and characterization of the human stearoyl-CoA desaturase gene promoter: transcriptional activation by sterol regulatory element binding protein and repression by polyunsaturated fatty acids and cholesterol. Biochem Biophys Res Commun.284(5):1194-8.

Bensaad, K., Tsuruta, A., Selak, M.A., Vidal, M.N., Nakano, K., Bartrons, R., Gottlieb, E., Vousden, K.H. (2006). TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell. 126(1):107-20.

Ben-Sahra, I., Howell, J.J., Asara, J.M., Manning, B.D. (2013). Stimulation of de novo pyrimidine synthesis by growth signaling through mTOR and S6K1. Science. 339(6125):1323-8

Ben-Sahra, I., Hoxhaj, G., Ricoult, S.J.H., Asara, J.M., Manning, B.D. (2016). mTORC1 induces purine synthesis through control of the mitochondrial tetrahydrofolate cycle. Science. 351(6274):728-733.

Bieging, K.T., Mello, S.S., Attardi, L.D. (2014). Unravelling mechanisms of p53-mediated tumour suppression. Nat Rev Cancer. 14(5):359-70.

Boutros, M., Paricio, N., Strutt, D.I., and Mlodzik, M. (1998). Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. Cell 94, 109-118.

Bozza, P.T., Viola, J.P. (2010). Lipid droplets in inflammation and cancer. Prostaglandins Leukot Essent Fatty Acids. 82(4-6):243-50.

Brosh, R., and Rotter, V. (2009). When mutants gain new powers: news from the mutant p53 field. Nat Rev Cancer 9, 701-713.

Brown, M.S., Goldstein, J.L. (1997). The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell. 89(3):331-40.

Brunn, G.J., Hudson, C.C., Sekulić, A., Williams, J.M., Hosoi, H., Houghton, P.J., Lawrence, J.C. Jr, Abraham, R.T. (1997). Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. Science.277(5322):99-101.

Budhu, A., Roessler, S., Zhao, X., Yu, Z., Forgues, M., Ji, J., Karoly, E., Qin, L.X., Ye, Q.H., Jia, H.L., Fan, J., Sun, H.C., Tang, Z.Y., Wang, X.W. (2013). Integrated metabolite and gene expression profiles identify lipid biomarkers associated with progression of hepatocellular carcinoma and patient outcomes. Gastroenterology. 144(5):1066-1075.

Bullock, A.N., Henckel, J., Fersht, A.R. (2000). Quantitative analysis of residual folding and DNA binding in mutant p53 core domain: definition of mutant states for rescue in cancer therapy. Oncogene. 19(10):1245-56.

Cairns, R.A., Harris, I.S., Mak, T.W. (2011). Regulation of cancer cell metabolism. Nat Rev Cancer. 11(2):85-95.

Chen, D., Ito, S., Hyodo, T., Asano-Inami, E., Yuan, H., Senga, T. (2017). Phosphorylation of DEPDC1 at Ser110 is required to maintain centrosome organization during mitosis. Exp Cell Res. 358(2):101-110.

Chen, S., and Hamm, H.E. (2006). DEP domains: More than just membrane anchors. Dev Cell 11, 436-438.
Cheng, C., Ru, P., Geng, F., Liu, J., Yoo, J., Wu, X., Cheng, X., Euthine, V., Hu, P., Guo, J., Lefai, E., Kaur, B., Nohturfft, A., Ma, J., Chakravarti, A., Guo, D. (2015). Glucose-Mediated N-glycosylation of SCAP Is Essential for SREBP-1 Activation and Tumor Growth. Cancer Cell. 28(5):569-581.

Cho, Y., Gorina, S., Jeffrey, P.D., Pavletich, N.P. (1994). Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. Science. 265(5170):346-55.

Civera, C., Simon, B., Stier, G., Sattler, M., and Macias, M.J. (2005). Structure and dynamics of the human pleckstrin DEP domain: distinct molecular features of a novel DEP domain subfamily. Proteins 58, 354-366.

Consonni, S.V., Maurice, M.M., Bos, J.L. (2014). DEP domains: structurally similar but functionally different. Nat Rev Mol Cell Biol. 15(5):357-62.

Curtis, C., Shah, S.P., Chin, S.F., Turashvili, G., Rueda, O.M., Dunning, M.J., Speed, D., Lynch, A.G., Samarajiwa, S., Yuan, Y., Gräf, S., Ha, G., Haffari, G., Bashashati, A., Russell, R., McKinney, S., et al., (2012). The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature18;486(7403):346-52.

Dang, C.V., O'Donnell, K.A., Zeller, K.I., Nguyen, T., Osthus, R.C., Li, F.. (2006). The c-Myc target gene network. Semin Cancer Biol. 2006 Aug;16(4):253-64.

Deberardinis, R.J., Lum, J.J., Thompson, C.B. (2006). Phosphatidylinositol 3-kinase-dependent modulation of carnitine palmitoyltransferase 1A expression regulates lipid metabolism during hematopoietic cell growth. J. Biol. Chem., 281. 37372-37380

DeBerardinis, R.J., Mancuso, A., Daikhin, E., Nissim, I., Yudkoff, M., Wehrli, S., Thompson, C.B. (2007). Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. Proc Natl Acad Sci U S A. 104(49):19345-50.

DeBerardinis, R.J., Chandel, N.S. (2016). Fundamentals of cancer metabolism. Sci Adv;2(5).

de Rooij, J., Rehmann, H., van Triest, M., Cool, R.H., Wittinghofer, A., and Bos, J.L. (2000). Mechanism of regulation of the Epac family of cAMP-dependent RapGEFs. J Biol Chem 275, 20829-20836.

Di Agostino, S., Strano, S., Emiliozzi, V., Zerbini, V., Mottolese, M., Sacchi, A., Blandino, G., Piaggio, G. (2006). Gain of function of mutant p53: the mutant p53/NF-Y protein complex reveals an aberrant transcriptional mechanism of cell cycle regulation. Cancer Cell. 10(3):191-202.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 29(1):15-21.

Dorrello NV, Peschiaroli A, Guardavaccaro D, Colburn NH, Sherman NE, Pagano M. (2006). S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. Science.314(5798):467-71.

Edinger, A.L., Thompson. C.B. (2002). Akt maintains cell size and survival by increasing mTOR-dependent nutrient uptake. Mol Biol Cell.13(7):2276-88.

Eriksson, M., Ambroise, G., Ouchida, A.T., Lima-Queiroz, A., Smith, D., Gimenez-Cassina, A., Iwanicki, M.P., Muller, P.A., Norberg, E., Vakifahmetoglu-Norberg, H. (2017). Effect of Mutant p53 Proteins on Glycolysis and Mitochondrial Metabolism. Mol Cell Biol. 37(24). pii: e00328-17.

Fallah, Y., Brundage, J., Allegakoen, P., Shajahan-Haq, A.N. (2017). MYC-Driven Pathways in Breast Cancer Subtypes. Biomolecules. 7(3).

Feng, Z., Levine, A.J. (2010). The regulation of energy metabolism and the IGF-1/mTOR pathways by the p53 protein. Trends Cell Biol. 20(7):427-34.

Feng, X., Zhang, C., Zhu, L., Zhang, L., Li, H., He, L., Mi, Y., Wang, Y., Zhu, J., Bu, Y. (2017). DEPDC1 is required for cell cycle progression and motility in nasopharyngeal carcinoma. Oncotarget. 8(38):63605-63619.

Fontemaggi, G., Dell'Orso, S., Trisciuoglio, D., Shay, T., Melucci, E., Fazi, F., Terrenato, I., Mottolese, M., Muti, P., Domany, E., Del Bufalo, D., Strano, S., Blandino, G. (2009). The execution of the transcriptional axis mutant p53, E2F1 and ID4 promotes tumor neo-angiogenesis. Nat Struct Mol Biol16(10):1086-93.

Freed-Pastor, W.A., Mizuno, H., Zhao, X., Langerod, A., Moon, S.H., Rodriguez-Barrueco, R., Barsotti, A., Chicas, A., Li, W., Polotskaia, A., et al. (2012). Mutant p53 disrupts mammary tissue architecture via the mevalonate pathway. Cell 148, 244-258.

Frezza, C., Gottlieb, E. (2009). Mitochondria in cancer: not just innocent bystanders. Semin Cancer Biol.19(1):4-11.

Fritz, V., Benfodda, Z., Rodier, G., Henriquet, C., Iborra, F., Avancès, C., Allory, Y., de la Taille, A., Culine, S., Blancou, H., Cristol, J.P., Michel, F., Sardet, C., Fajas, L. (2010). Abrogation of de novo lipogenesis by stearoyl-CoA desaturase 1 inhibition interferes with oncogenic signaling and blocks prostate cancer progression in mice. Mol Cancer Ther. 9(6):1740-54.

Fu, S., Watkins, S.M., Hotamisligil, G.S. (2012). The role of endoplasmic reticulum in hepatic lipid homeostasis and stress signaling. Cell Metab. 15(5):623-34.

Garrido, P., Morán, J., Alonso, A., González, S., González, C. (2013). 17β-Estradiol Activates Glucose Uptake via GLUT4 Translocation and PI3K/Akt Signaling Pathway in MCF-7 Cells. Endocrinology. 154(6):1979-89.

Gilkes, D.M., Semenza, G.L. (2013). Role of hypoxia-inducible factors in breast cancer metastasis. Future Oncol. 9(11):1623-36.

Girardini, J.E., Napoli, M., Piazza, S., Rustighi, A., Marotta, C., Radaelli, E., Capaci, V., Jordan, L., Quinlan, P., Thompson, A., et al. (2011). A Pin1/mutant p53 axis promotes aggressiveness in breast cancer. Cancer Cell 20, 79-91.

Godoy, A., Ulloa, V., Rodríguez, F., Reinicke, K., Yañez, A.J., García Mde, L., Medina, R.A., Carrasco, M., Barberis, S., Castro, T., Martínez, F., Koch, X., Vera, J.C., Poblete, M.T., Figueroa, C.D., Peruzzo, B., Pérez, F., Nualart, F. (2006). Differential subcellular distribution of glucose transporters GLUT1-6 and GLUT9 in human cancer: ultrastructural localization of GLUT1 and GLUT5 in breast tumor tissues. J Cell Physiol. 207(3):614-27.

Goldstein, J.L., DeBose-Boyd, R.A., Brown, M.S. (2006). Protein sensors for membrane sterols. Cell. 124(1):35-46.

Gottlieb, E., Vousden, K.H. (2010). p53 regulation of metabolic pathways. Cold Spring Harb Perspect Biol. 2(4):a001040.

Griffiths, B., Lewis, C.A., Bensaad, K., Ros, S., Zhang, Q., Ferber, E.C., Konisti, S., Peck, B., Miess, H., East, P., Wakelam, M., Harris, A.L., Schulze, A. (2013). Sterol regulatory element binding protein-dependent regulation of lipid synthesis supports cell survival and tumor growth. Cancer Metab.1(1):3.

Gusterson, B., (2009). Do "basal-like" breast cancers really exist? Nature reviews Cancer, 9(2):128–34.

Harada, Y., Kanehira, M., Fujisawa, Y., Takata, R., Shuin, T., Miki, T., Fujioka, T., Nakamura, Y., and Katagiri, T. (2010). Cell-permeable peptide DEPDC1-ZNF224 interferes with transcriptional repression and oncogenicity in bladder cancer cells. Cancer Res 70, 5829-5839.

Havas, K.M., Milchevskaya, V., Radic, K., Alladin, A., Kafkia, E., Garcia, M., Stolte, J., Klaus, B., Rotmensz, N., Gibson, T.J., Burwinkel, B., Schneeweiss, A., Pruneri, G., Patil, K.R., Sotillo, R., Jechlinger, M. (2017). Metabolic shifts in residual breast cancer drive tumor recurrence. J Clin Invest. 127(6):2091-2105.

Hay, N. (2016). Reprogramming glucose metabolism in cancer: can it be exploited for cancer therapy? Nat Rev Cancer. 16(10):635-49.

Holz MK, Ballif BA, Gygi SP, Blenis J. (2005). mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. Cell.123(4):569-80.

Hu, G., Zhang, Z., and Wensel, T.G. (2003). Activation of RGS9-1GTPase acceleration by its membrane anchor, R9AP. J Biol Chem 278, 14550-14554.

Hua, X., Nohturfft, A., Goldstein, J.L., Brown, M.S. (1996). Sterol resistance in CHO cells traced to point mutation in SREBP cleavage-activating protein. Cell. 87(3):415-26.

Huang, L., Chen, K., Cai, Z.P., Chen, F.C., Shen, H.Y., Zhao, W.H., Yang, S.J., Chen, X.B., Tang, G.X., Lin, X. (2017). DEPDC1 promotes cell proliferation and tumor growth via activation of E2F signaling in prostate cancer. Biochem Biophys Res Commun. 490(3):707-712.

Huang, D.W., Sherman, B.T., Lempicki, R.A. (2009). Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. Nature Protoc. 4(1):44-57.

Ikonen, E. (2008). Cellular cholesterol trafficking and compartmentalization. Nat Rev Mol Cell Biol. 9(2):125-38.

Im, S.S., Yousef, L., Blaschitz, C., Liu, J.Z., Edwards, R.A., Young, S.G., Raffatellu, M., Osborne, T.F. (2011). Linking lipid metabolism to the innate immune response in macrophages through sterol regulatory element binding protein-1a. Cell Metab. 13(5):540-9.

Jiang, P., Du, W., Wang, X., Mancuso, A., Gao, X., Wu, M., Yang, X. (2011). p53 regulates biosynthesis through direct inactivation of glucose-6-phosphate dehydrogenase. Nat Cell Biol. 13(3):310-6.

Joerger, A.C., Fersht, A.R. (2008). Structural biology of the tumor suppressor p53. Annu Rev Biochem. 77:557-82.

Jong, Y.J., Li, L.H., Tsou, M.H., Chen, Y.J., Cheng, S.H., Wang-Wuu, S., Tsai, S.F., Chen, C.M., Huang, A.T., Hsu, M.T., Lin, C.H. (2004). Chromosomal comparative genomic hybridization abnormalities in early- and late-onset human breast cancers: correlation with disease progression and TP53 mutations. Cancer Genet Cytogenet. 148(1):55-65.

Kaiser, A.M., Attardi, L.D. (2018). Deconstructing networks of p53-mediated tumor suppression in vivo. Cell Death Differ. 25(1):93-103.

Kandoth, C., McLellan, M.D., Vandin, F., Ye, K., Niu, B., Lu, C., Xie, M., Zhang, Q., McMichael, J.F., Wyczalkowski, M.A., Leiserson, M.D.M., Miller, C.A., Welch, J.S., Walter. M.J., Wendl, M.C., Ley, T.J., Wilson, R.K., Raphael, B.J., Ding, L., et al. (2013). Mutational landscape and significance across 12 major cancer types. Nature. 502(7471):333-339

Kanehira, M., Harada, Y., Takata, R., Shuin, T., Miki, T., Fujioka, T., Nakamura, Y., and Katagiri, T. (2007). Involvement of upregulation of DEPDC1 (DEP domain containing 1) in bladder carcinogenesis. Oncogene 26, 6448-6455.

Kharrat A, Millevoi S, Baraldi E, Ponting CP, Bork P, Pastore A. (1998). Conformational stability studies of the pleckstrin DEP domain: definition of the domain boundaries. Biochim Biophys Acta. 1385(1):157-64.

Kassambara, A., Schoenhals, M., Moreaux, J., Veyrune, J.L., Rème, T., Goldschmidt, H., Hose, D., Klein, B. (2013). Inhibition of DEPDC1A, a bad prognostic marker in multiple myeloma, delays growth and induces mature plasma cell markers in malignant plasma cells. PLoS One. 8(4):e62752.

Kim, H.M., Jung, W.H., Koo, J.S. (2015). Expression of Yes-associated protein (YAP) in metastatic breast cancer. Int J Clin Exp Pathol. 8(9):11248-57.

Kim, M.P., Lozano, G. (2018). Mutant p53 partners in crime. Cell Death Differ. 25(1):161-168.

Kinnaird, A., Zhao, S., Wellen, K.E., Michelakis, E.D. (2016). Metabolic control of epigenetics in cancer. Nat Rev Cancer. 16(11):694-707.

Koizume, S., Miyagi, Y. (2016). Lipid Droplets: A Key Cellular Organelle Associated with Cancer Cell Survival under Normoxia and Hypoxia. Int J Mol Sci. 17(9).

Kozma, R., Ahmed, S., Best, A., and Lim, L. (1996). The GTPase-activating protein n-chimaerin cooperates with Rac1 and Cdc42Hs to induce the formation of lamellipodia and filopodia. Mol Cell Biol 16, 5069-5080.

Kretschmer, C., Sterner-Kock, A., Siedentopf, F., Schoenegg, W., Schlag, P.M., and Kemmner, W. (2011). Identification of early molecular markers for breast cancer. Mol Cancer 10, 15.

Kroemer, G., Pouyssegur, J. (2008). Tumor cell metabolism: cancer's Achilles' heel. Cancer Cell.13(6):472-82.

Li, J., Condello, S., Thomes-Pepin, J., Ma, X., Xia, Y., Hurley, T.D., Matei, D., Cheng, J.X. (2017). Lipid Desaturation Is a Metabolic Marker and Therapeutic Target of Ovarian Cancer Stem Cells. Cell Stem Cell. 20(3):303-314.

Liao, Y., Smyth, G.K., Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics. 30(7):923-30.

Long, J.P., Li, X.N., Zhang, F. (2016). Targeting metabolism in breast cancer: How far we can go? World J Clin Oncol. 7(1):122-30.

Luo, X., Cheng, C., Tan, Z., Li, N., Tang, M., Yang, L., Cao, Y. (2017). Emerging roles of lipid metabolism in cancer metastasis. Mol Cancer.16(1):76.

Macheda, M.L., Rogers, S., Best, J.D. (2005). Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. J Cell Physiol.202(3):654-62.

Manning BD, Cantley LC. (2007). AKT/PKB signaling: navigating downstream. Cell.129(7):1261-74.

Mantovani, F., Walerych, D., Del Sal, G. (2017). Targeting mutant p53 in cancer: a long road to precision therapy. FEBS J;284(6):837-850.

Marchesi S, Montani F, Deflorian G, D'Antuono R, Cuomo A, Bologna S, Mazzoccoli C, Bonaldi T, Di Fiore PP, Nicassio F. (2014). DEPDC1B coordinates de-adhesion events and cell-cycle progression at mitosis. Dev Cell. 31(4):420-33.

Marien, E., Meister, M., Muley, T., Gomez Del Pulgar, T., Derua, R., Spraggins, J.M., Van de Plas, R., Vanderhoydonc, F., Machiels, J., Binda, M.M., Dehairs, J., Willette-Brown, J., Hu, Y., Dienemann, H., Thomas, M., Schnabel, P.A., Caprioli, R.M., Lacal, J.C., Waelkens, E., Swinnen, J.V. (2016). Phospholipid profiling identifies acyl chain elongation as a ubiquitous trait and potential target for the treatment of lung squamous cell carcinoma. Oncotarget. 7(11):12582-97.

Martemyanov, K.A., Lishko, P.V., Calero, N., Keresztes, G., Sokolov, M., Strissel, K.J., Leskov, I.B., Hopp, J.A., Kolesnikov, A.V., Chen, C.K., et al. (2003). The DEP domain determines subcellular targeting of the GTPase activating protein RGS9 in vivo. J Neurosci 23, 10175-10181.

Marín-Hernández, A., Rodríguez-Enríquez, S., Vital-González, P.A., Flores-Rodríguez, F.L., Macías-Silva, M., Sosa-Garrocho, M., Moreno-Sánchez, R. (2006). Determining and understanding the control of glycolysis in fast-growth tumor cells. Flux control by an over-expressed but strongly product-inhibited hexokinase. FEBS J. 273(9):1975-88.

Matsui, H., Yokoyama, T., Sekiguchi, K., Iijima, D., Sunaga, H., Maniwa, M., Ueno, M., Iso, T., Arai, M., Kurabayashi, M. (2012). Stearoyl-CoA Desaturase-1 (SCD1) Augments Saturated Fatty Acid-Induced Lipid Accumulation and Inhibits Apoptosis in Cardiac Myocytes. PLoS One. 2012;7(3):e33283.

Mego, M., Mani, S.A., Cristofanilli, M. (2010). Molecular mechanisms of metastasis in breast cancer--clinical applications. Nat Rev Clin Oncol. 7(12):693-701.

Mi, Y., Zhang, C., Bu, Y., Zhang, Y., He, L., Li, H., Zhu, H., Li, Y., Lei, Y., Zhu, J. (2015). DEPDC1 is a novel cell cycle related gene that regulates mitotic progression. BMB Rep. 48(7):413-8.

Muller, P.A., Caswell, P.T., Doyle, B., Iwanicki, M.P., Tan, E.H., Karim, S., Lukashchuk, N., Gillespie, D.A., Ludwig, R.L., Gosselin, P., Cromer, A., Brugge, J.S., Sansom, O.J., Norman, J.C., Vousden, K.H. (2009). Mutant p53 drives invasion by promoting integrin recycling. Cell. 139(7):1327-41.

Muller, P.A., Vousden, K.H. (2013). p53 mutations in cancer. Nat Cell Biol. 15(1):2-8.

Murphy, K.L., Dennis, A.P., Rosen, J.M. (2000). A gain of function p53 mutant promotes both genomic instability and cell survival in a novel p53-null mammary epithelial cell model. FASEB J. 14(14):2291-302.

Nakakuki, M., Shimano, H., Inoue, N., Tamura, M., Matsuzaka, T., Nakagawa, Y., Yahagi, N., Toyoshima, H., Sato, R., Yamada, N. (2007). A transcription factor of lipid synthesis, sterol regulatory element-binding protein (SREBP)-1a causes G(1) cell-cycle arrest after accumulation of cyclin-dependent kinase (cdk) inhibitors. FEBS J. 274(17):4440-52.

Nik-Zainal S, Davies H, Staaf J, Ramakrishna M, Glodzik D, Zou X, Martincorena I, Alexandrov LB, Martin S, Wedge DC, Van Loo P, et al. (2016). Landscape of somatic mutations in 560 breast cancer whole-genome sequences. Nature. 534(7605):47-54.

Norum J.H., Andersen, K., Sørlie, T. (2014). Lessons learned from the intrinsic subtypes of breast cancer in the quest for precision therapy. Br J Surg.101(8):925-38.

Noto, A., De Vitis, C, Pisanu ME, Roscilli G, Ricci G, Catizone A, Sorrentino G, Chianese G, Taglialatela-Scafati O, Trisciuoglio D, Del Bufalo D, Di Martile M, Di Napoli A, Ruco L, Costantini S, Jakopin Z, Budillon A, Melino G, Del Sal G, Ciliberto G, Mancini R. (2017). Stearoyl-CoA-desaturase 1 regulates lung cancer stemness via stabilization and nuclear localization of YAP/TAZ. Oncogene. 36(32):4573-4584.

Obara W, Ohsawa R, Kanehira M, Takata R, Tsunoda T, Yoshida K, Takeda K, Katagiri T, Nakamura Y, Fujioka T. (2012). Cancer peptide vaccine therapy developed from oncoantigens identified through genome-wide expression profile analysis for bladder cancer. Jpn J Clin Oncol. 42(7):591-600.

Okayama, H., Kohno, T., Ishii, Y., Shimada, Y., Shiraishi, K., Iwakawa, R., Furuta, K., Tsuta, K., Shibata, T., Yamamoto, S., et al. (2012). Identification of genes upregulated in ALK-positive and EGFR/KRAS/ALK-negative lung adenocarcinomas. Cancer Res 72, 100-111.

Parrales, A., Iwakuma, T. (2015). Targeting Oncogenic Mutant p53 for Cancer Therapy. Front Oncol.;5:288.

Pascual, G., Avgustinova, A., Mejetta, S., Martín, M., Castellanos, A., Attolini, C.S., Berenguer, A., Prats, N., Toll, A., Hueto, J.A., Bescós, C., Di Croce, L., Benitah, S.A. (2017). Targeting metastasis-initiating cells through the fatty acid receptor CD36. Nature. 541(7635):41-45.

Peck, B., Schug, Z.T. Zhang, Q., Dankworth, B., Jones, D.T., Smethurst, E., Patel, R., Mason, S., Jiang, M., Saunders, R., Howell, M., Mitter, R., Spencer-Dene, B., Stamp, G., McGarry, L., James, D., Shanks, E., Aboagye, E.O., Critchlow, S.E., Leung, H.Y., Harris, A.L., Wakelam, M.J.O., Gottlieb. E., Schulze, A. (2016). Inhibition of fatty acid desaturation is detrimental to cancer cell survival in metabolically compromised environments. Cancer Metab.4:6.

Peck, B., Schulze, A. (2016). Lipid desaturation - the next step in targeting lipogenesis in cancer? FEBS J. 283(15):2767-78.

Perou, C.M., Sørlie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Rees, C.A., Pollack, J.R., Ross, D.T., Johnsen, H., Akslen, L.A., Fluge, O., Pergamenschikov, A., Williams, C., Zhu, S.X., Lønning, P.E., Børresen-Dale, A.L., Brown, P.O., Botstein, D., et al. (2000). Molecular portraits of human breast tumours. Nature, 406(6797), pp.747–752.

Peterson, T.R., Sengupta, S.S., Harris, T.E., Carmack, A.E., Kang, S.A., Balderas, E., Guertin, D.A., Madden, K.L., Carpenter, A.E., Finck, B.N., Sabatini, D.M.. (2011). mTOR complex 1 regulates lipin 1 localization to control the SREBP pathway. Cell. 2011 Aug 5;146(3):408-20.

Piccolo, S., Dupont, S., Cordenonsi, M. (2014). The biology of YAP/TAZ: hippo signaling and beyond. Physiol Rev. 94(4):1287-312,

Plas, D.R., Talapatram S., Edingerm A.L., Rathmell, J.C., Thompson, C.B. (2001). Akt and Bcl-xL promote growth factor-independent survival through distinct effects on mitochondrial physiology. J Biol Chem;276(15):12041-8

Polotskaia, A., Xiao, G., Reynoso, K., Martin, C., Qiu, W.G., Hendrickson, R.C., Bargonetti, J. (2015). Proteome-wide analysis of mutant p53 targets in breast cancer identifies new levels of gain-of-function that influence PARP, PCNA, and MCM4. Proc Natl Acad Sci U S A. 112(11):E1220-9.

Ponting C.P., Bork, P. (1996). Pleckstrin's repeat performance: a novel domain in G-protein signaling? Trends Biochem Sci. 21(7):245-6.

Pouysségur J, Dayan F, Mazure NM. (2006). Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature.441(7092):437-43.

Prat, A., Parker, J.S., Karginova, O., Fan, C., Livasy, C., Herschkowitz, J.I., He, X., and Perou, C.M. (2010). Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res 12, R68.

Qiao, J., Mei, F.C., Popov, V.L., Vergara, L.A., and Cheng, X. (2002). Cell cycle-dependent subcellular localization of exchange factor directly activated by cAMP. J Biol Chem 277, 26581-26586.

Radhakrishnan, A., Sun, L.P., Kwon, H.J., Brown, M.S., Goldstein, J.L. (2004). Direct binding of cholesterol to the purified membrane region of SCAP: mechanism for a sterol-sensing domain. Mol Cell. 15(2):259-68.

Ridley, A.J. (2006). Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. Trends Cell Biol. 16(10):522-9.

Rios-Esteves, J., Resh, M.D. (2013). Stearoyl CoA desaturase is required to produce active, lipid-modified Wnt proteins. Cell Rep.4(6):1072-81.

Rivlin, N., Brosh, R., Oren, M., Rotter, V. (2011). Mutations in the p53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis. Genes Cancer. 2(4):466-74.

Robinson, D.R., Wu, Y.M., Lonigro, R.J., Vats, P., Cobain, E., Everett, J., Cao, X., Rabban, E., Kumar-Sinha, C., Raymond, V., Schuetze, S., Alva, A., Siddiqui, J., Chugh, R., Worden, F., Zalupski, M.M., Innis, J., Mody, R.J., Tomlins, S.A., Lucas, D., Baker, L.H., Ramnath, N., Schott, A.F., Hayes, D.F., Vijai, J., Offit, K., Stoffel, E.M., Roberts, J.S., Smith, D.C., Kunju, L.P., Talpaz, M., Cieślik, M., Chinnaiyan, A.M. (2017). Integrative clinical genomics of metastatic cancer. Nature. 548(7667):297-303.

Robinson, M.D., McCarthy, D.J., Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 26(1):139-40.

Röhrig, F., Schulze, A. (2016). The multifaceted roles of fatty acid synthesis in cancer. Nat Rev Cancer. 16(11):732-749.

Romero-Garcia, S., Moreno-Altamirano, M.M., Prado-Garcia, H., Sánchez-García, F.J. (2016). Lactate Contribution to the Tumor Microenvironment: Mechanisms, Effects on Immune Cells and Therapeutic Relevance. Front Immunol. 7:52.

Santa-Maria, C.A., Gradishar, W.J. (2015). Changing Treatment Paradigms in Metastatic Breast Cancer: Lessons Learned. JAMA Oncol. 1(4):528-34;

Santos, C.R., Schulze, A. (2012). Lipid metabolism in cancer. FEBS J. 279(15):2610-23.

Scaglia, N., Igal, R.A. (2008). Inhibition of Stearoyl-CoA Desaturase 1 expression in human lung adenocarcinoma cells impairs tumorigenesis. Int J Oncol. 33(4):839-50.

Schwartzenberg-Bar-Yoseph, F., Armoni, M., Karnieli, E. (2004). The tumor suppressor p53 down-regulates glucose transporters GLUT1 and GLUT4 gene expression. Cancer Res. 64(7):2627-33.

Sendoel A, Maida S, Zheng X, Teo Y, Stergiou L, Rossi CA, Subasic D, Pinto SM, Kinchen JM, Shi M, Boettcher S, Meyer JN, Manz MG, Bano D, Hengartner MO. (2014). DEPDC1/LET-99 participates in an evolutionarily conserved pathway for anti-tubulin drug-induced apoptosis. Nat Cell Biol. 16(8):812-20

Semenza, G.L. (2007). Hypoxia-inducible factor 1 (HIF-1) pathway. Sci STKE. 2007(407).

Shah, S.P., Roth, A., Goya, R., Oloumi, A., Ha, G., Zhao, Y., Turashvili, G., Ding, J., Tse, K., Haffari, G., et al. (2012). The clonal and mutational evolution spectrum of primary triple-negative breast cancers. Nature, 486(7403), pp.395–399.

Shajahan-Haq, A.N., Cook, K.L., Schwartz-Roberts, J.L., Eltayeb, A.E., Demas, D.M., Warri, A.M., Facey, C.O., Hilakivi-Clarke, L.A., Clarke, R. (2014). MYC regulates the unfolded protein response and glucose and glutamine uptake in endocrine resistant breast cancer. Mol Cancer. 13:239.

Shembade, N., Ma, A., Harhaj, E.W. (2010). Inhibition of NF-kappaB signaling by A20 through disruption of ubiquitin enzyme complexes. Science. 327(5969):1135-9.

Shen, L., O'Shea, J.M., Kaadige, M.R., Cunha, S., Wilde, B.R., Cohen, A.L., Welm, A.L., Ayer, D.E. Metabolic reprogramming in triple-negative breast cancer through Myc suppression of TXNIP. Proc Natl Acad Sci U S A. 112(17):5425-30.

Shimomura, I., Hammer, R.E., Richardson, J.A., Ikemoto, S., Bashmakov, Y., Goldstein, J.L., Brown, M.S. (1998). Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. Genes Dev. 12(20):3182-94.

Sorrentino G, Ruggeri N, Specchia V, Cordenonsi M, Mano M, Dupont S, Manfrin A, Ingallina E, Sommaggio R, Piazza S, Rosato A, Piccolo S, Del Sal G. (2014). Metabolic control of YAP and TAZ by the mevalonate pathway. Nat Cell Biol;16(4):357-66.

Sounni, N.E., Cimino, J., Blacher, S., Primac, I., Truong, A., Mazzucchelli, G., Paye, A., Calligaris, D., Debois, D., De Tullio, P., Mari, B., De Pauw, E., Noel, A. (2014). Blocking lipid synthesis overcomes tumor regrowth and metastasis after antiangiogenic therapy withdrawal. Cell Metab. 20(2):280-94.

Soussi, T., Wiman, K.G. (2015). TP53: an oncogene in disguise. Cell Death Differ. 22(8):1239-49.

Spandl, J., White, D.J., Peychl, J., Thiele, C. (2009). Live cell multicolor imaging of lipid droplets with a new dye, LD540. Traffic. 10(11):1579-84.

Strano, S., Munarriz, E., Rossi, M., Cristofanelli, B., Shaul, Y., Castagnoli, L., Levine, A.J., Sacchi, A., Cesareni, G., Oren, M., Blandino, G. (2000). Physical and functional interaction between p53 mutants and different isoforms of p73. J Biol Chem. 275(38):29503-12.

Strano, S., Fontemaggi, G., Costanzo, A., Rizzo, M.G., Monti, O., Baccarini, A., Del Sal, G., Levrero, M., Sacchi, A., Oren, M., Blandino, G. (2002). Physical interaction with human tumor-derived p53 mutants inhibits p63 activities. J Biol Chem. 277(21):18817-26.

Subramanian, A., Tamayo, P., Mootha,. V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., Mesirov, J.P. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A. 102(43):15545-50

Székely, B., Silber, A., Pusztai, L. (2017). New Therapeutic Strategies for Triple-Negative Breast Cancer. Oncology (Williston Park). 31(2):130-7.

Sztalryd, C., Kimmel, A.R. (2014). Perilipins: lipid droplet coat proteins adapted for tissue-specific energy storage and utilization, and lipid cytoprotection. Biochimie. 96:96-101.

Teicher, B.A., Linehan, W.M., Helman, L.J. (2012). Targeting Cancer Metabolism. Clin Cancer Res. 18(20):5537-45.

Theodoropoulos P.C., Gonzales, S.S., Winterton, S.E., Rodriguez-Navas, C., McKnight, J.S., Morlock, L.K., Hanson, J.M., Cross, B., Owen, A.E., Duan, Y., Moreno, J.R., Lemoff, A., Mirzaei, H., Posner, B.A., Williams, N.S., Ready, J.M., Nijhawan. D. (2016). Discovery of tumor-specific irreversible inhibitors of stearoyl CoA desaturase. Nat Chem Biol. 12(4):218-25.

Tirinato, L., Pa.gliari. F., Limongi, T., Marini, M., Falqui, A., Seco, J., Candeloro, P., Liberale, C., Di Fabrizio, E. (2017). An Overview of Lipid Droplets in Cancer and Cancer Stem Cells. Stem Cells Int. 1656053.

Torre, L.A., Bray, F,, Siegel, R.L., Ferlay, J., Lortet-Tieulent, J., Jemal, A. (2015). Global cancer statistics, 2012. CA Cancer J Clin.65(2):87-108.

Tosi, A., Dalla Santa, S., Cappuzzello, E., Marotta, C., Walerich, D., Del Sal, G., Zanovello, P., Sommaggio, R., Rosato, A. (2017). Identification of a HLA-A*0201-restricted immunogenic epitope from the universal tumor antigen DEPDC1. Oncoimmunology. 6(8):e1313371

Valentino, E., Bellazzo, A., Di Minin, G., Sicari, D., Apollonio, M., Scognamiglio, G., Di Bonito, M., Botti, G., Del Sal, G., Collavin, L. (2017). Mutant p53 potentiates the oncogenic effects of insulin by inhibiting the tumor suppressor DAB2IP. Proc Natl Acad Sci U S A. 114(29):7623-7628.

van Riggelen J, Yetil A, Felsher DW. (2010). MYC as a regulator of ribosome biogenesis and protein synthesis. Nat Rev Cancer.10(4):301-9.

Vander Heiden, M.G., Cantley, L.C., Thompson, C.B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation.324(5930):1029-33.

Végran, F., Boidot, R., Oudin, C., Defrain, C., Rebucci, M., Lizard-Nacol, S. (2007). Association of p53 gene alterations with the expression of antiapoptotic survivin splice variants in breast cancer. Oncogene. 26(2):290-7.

Vigil, D., Cherfils, J., Rossman, K.L., Der, C.J. (2010). Ras superfamily GEFs and GAPs: validated and tractable targets for cancer therapy? Nat Rev Cancer. 10(12):842-57.

Walerych, D., Napoli, M., Collavin, L., Del Sal, G. (2012). The rebel angel: mutant p53 as the driving oncogene in breast cancer. Carcinogenesis;33(11):2007-17.

Wennerberg, K., Rossman, K.L., Der, C.J. (2005). The Ras superfamily at a glance. J Cell Sci.118(Pt 5):843-6.

Walerych, D., Lisek, K., Sommaggio, R., Piazza, S., Ciani, Y., Dalla, E., Rajkowska, K., Gaweda-Walerych, K., Ingallina, E., Tonelli, C., Morelli, M.J., Amato, A., Eterno, V., Zambelli, A., Rosato, A., Amati, B., Wiśniewski, J.R., Del Sal, G. (2016). Proteasome machinery is instrumental in a common gain-of-function program of the p53 missense mutants in cancer. Nat Cell Biol;18(8):897-909.

Walther T.C., Farese, R.V. Jr. (2012). Lipid droplets and cellular lipid metabolism. Annu Rev Biochem. 81:687-714.

Warburg O. (1925) The metabolism of carcinoma cells. The Journal of Cancer Research;9(1):148–163.

Warburg O. (1956) On the origin of cancer cells. Science. 123(3191):309–314.

Ward, P.S., Thompson, C.B. (2012). Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. Cancer Cell.21(3):297-308.

Wharton, K.A., Jr. (2003). Runnin' with the Dvl: proteins that associate with Dsh/Dvl and their significance to Wnt signal transduction. Dev Biol 253, 1-17.

Wieman, H.L., Wofford, J.A., Rathmell, J.C. (2007). Cytokine stimulation promotes glucose uptake via phosphatidylinositol-3 kinase/Akt regulation of Glut1 activity and trafficking. Mol Biol Cell;18(4):1437-46.

Wong, H.C., Mao, J., Nguyen, J.T., Srinivas, S., Zhang, W., Liu, B., Li, L., Wu, D., and Zheng, J. (2000). Structural basis of the recognition of the dishevelled DEP domain in the Wnt signaling pathway. Nat Struct Biol 7, 1178-1184.

Xiao, D., Ren, P., Su, H., Yue, M., Xiu, R., Hu, Y., Liu, H., Qing, G. (2015). Myc promotes glutaminolysis in human neuroblastoma through direct activation of glutaminase 2. Oncotarget. 2015 Dec 1;6(38):40655-66.

Yahagi N, Shimano H, Matsuzaka T, Najima Y, Sekiya M, Nakagawa Y, Ide T, Tomita S, Okazaki H, Tamura Y, Iizuka Y, Ohashi K, Gotoda T, Nagai R, Kimura S, Ishibashi S, Osuga J, Yamada N. (2003). p53 Activation in adipocytes of obese mice. J Biol Chem.278(28):25395-400.

Yeudall, W.A., Vaughan, C.A., Miyazaki, H., Ramamoorthy, M., Choi, M.Y., Chapman, C.G., Wang, H., Black, E., Bulysheva, A.A., Deb, S.P., Windle, B., Deb, S. (2012). Gain-of-function mutant p53 upregulates CXC chemokines and enhances cell migration. Carcinogenesis. 33(2):442-51.

Zhang, X.H., Zhao, C., Ma, Z.A. (2007). The increase of cell-membranous phosphatidylcholines containing polyunsaturated fatty acid residues induces phosphorylation of p53 through activation of ATR. J Cell Sci. 120(Pt 23):4134-43.

Zhang, C., Liu, J., Liang, Y., Wu, R.., Zhao, Y., Hong, X., Lin, M., Yu, H., Liu, L., Levine, A.J., Hu, W., Feng, Z. (2013). Tumour-associated mutant p53 drives the Warburg effect. Nat Commun. 4:2935.

Zhou, G., Wang, J., Zhao, M., Xie, T.X., Tanaka, N., Sano, D., Patel, A.A., Ward, A.M., Sandulache, V.C., Jasser, S.A., Skinner, H.D, Fitzgerald, A.L., Osman, A.A., Wei, Y., Xia, X., Songyang, Z., Mills, G.B., Hung, M.C., Caulin, C., Liang, J., Myers, J.N. (2014). Gain-of-function mutant p53 promotes cell growth and cancer cell metabolism via inhibition of AMPK activation. Mol Cell. 54(6):960-7.