Universidade de Aveiro Departamento de Ciências Médicas

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Inês de Albuquerque Almeida Batista

Contribuição da metiltransferase SETD7 para a proliferação e diferenciação celular

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Doutora Luisa Alejandra Helguero, Professora Auxiliar Convidada do Departamento de Ciências Médicas da Universidade de Aveiro.

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Palavras-chave SETD7; metilação; transcrição; proliferação; diferenciação.

Resumo

A SETD7 foi originalmente identificada como uma metiltransferase da histona 3, catalisando a monometilação da sua lisina 4, o que, por sua vez, provoca a abertura da cromatina e activação da transcrição genética. Recentemente, tem sido demonstrado que várias outras proteínas, tais como ERα, HIF-α, STAT3 e DNMT, são alvo de metilação pela SETD7. Devido à grande variedade de funções desempenhadas pelos alvos moleculares da SETD7, esta enzima é um potencial regulador de vários processos vitais para as células, isto é, do ciclo celular, resposta a danos no ADN, diferenciação e proliferação. De facto, SETD7 já começou a ser o foco de muitos estudos. Ainda assim, pouco se sabe sobre como SETD7 influencia esses processos e sua contribuição para o desenvolvimento de cancro, facto que despertou a nossa curiosidade para o estudo do papel da SETD7 na proliferação e diferenciação celular. Aqui mostramos que SETD7 é regulada negativamente pelo EGF e pela consequente activação da via de MAPK em células epiteliais mamárias. Além disso, SETD7 também diminui o número de células epiteliais mamárias e a sua expressão parece ser induzida durante a diferenciação celular em resposta a hormonas lactogénicas. Adicionalmente, dois potenciais alvos de SETD7 (STAT3 e HOXB2) exibem padrões de expressão semelhantes a SETD7 em resposta ao EGF e hormonas lactogénicas, o que sugere que a SETD7 pode desempenhar um papel na sua regulação em células mamárias.

Keywords SETD7; methylation; gene transcription; proliferation; differentiation.

Abstract

SETD7 was originally identified as a histone methyltransferase, catalyzing the monomethylation of histone 3 at its fourth lysine and, thereby, triggering the opening of chromatin and gene transcription activation. Recently, several other non-histone proteins, such as ERα, HIF-α, STAT3 and DNMT, have been also shown to be methylated by SETD7. Due to the great variety of functions played by SETD7 molecular targets, this enzyme has the potential to arise as an important regulator of vital cellular processes, namely cell cycle, DNA damage response, differentiation and proliferation. In fact, SETD7 has now begun to be the focus of many studies. Still, little is known about how SETD7 influences these processes and its contribution to cancer development, a fact which has ignited our curiosity to study SETD7 role in cell proliferation and differentiation. Here we show that SETD7 is negatively regulated in proliferative mammary epithelial cells by EGF and the activation of the MAPK pathway. Furthermore, SETD7 also decreases mammary epithelial cell number and its expression seems to be induced during differentiation in response to lactogenic hormones. Additionally, two potential targets of SETD7 (STAT3 and HOXB2) exhibit similar expression patterns to SETD7 in response to EGF and lactogenic hormones which suggests that SETD7 plays a role in their regulation in mammary cells.

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List of Abbreviations

AKAP6	A kinase anchor protein 6
ASH1L	Absent, Small or Homeotic 1-like protein
AML	Acute myeloid leukemia
ARTD1	ADP-ribosyltransferase diphtheriatoxin-like 1
AREG	Amphiregulin
AR	Androgen Receptor
BTC	Betacellulin
CREB	cAMP response element-binding protein
CSC	Cancer stem cells
CENPC1	Centromere protein C1
Co-REST	Co-repressor of element-1 silencing transcription factor
CBP	CREB-binding protein
DEAF1	Deformed epidermal autoregulatory factor 1
DEX	Dexamethasone
DMSO	Dimethyl sulfoxide
dH_2O	Distilled water
DNMT1	DNA (cytosine-5)-methyltransferase 1
DNMT	DNA methyltransferase
TTK	Dual specificity protein kinase
ELF5	E74-like factor 5
EGFR	EGF receptor
ESC	Embryonic stem cells
EZH2	Enhancer of zeste homolog 2
EGF	Epidermal growth factor
EPR	Epiregulin
MEK	ERK kinase
E2	Estradiol
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FXR	Farnesoid X receptor
FoxO3	Forkhead Box O3
CCND1	gene encoding cyclin D1;
CDKN1A	gene encoding p21
HB-EGF	Heparin-binding EGF-like growth factor
HDM	Histone demethylase
HMT	Histone methyltransferase
HOX	Homeobox
HIV-Tat	Human immunodeficiency virus transactivator
HIF-1α	Hypoxia-inducible factor 1 alpha
IGF2	Insulin-like growth factor-2
IRF1	Interferon Regulatory Factor 1
IL	Interleukin

JAK	Janus kinase
JARID1	Jumonji AT-rich interactive domain 1
LT-RC	Long-term repopulating stem cell
lum-SC	Luminal unipotent stem cells
KMT	Lysine methyltransferases
LSD1	Lysine-specific histone demethylase 1
MaSC	Mammary stem cell
MAPKK	MAPK kinase
MKP1	MAPK phosphatase 1
MED1	Mediator Complex 1
MeCP2	Methyl-CpG Binding Protein 2
MAPK	Mitogen-activated protein kinase
MLL	Mixed lineage leukemia protein
MCP	Monocyte chemoattractant protein
MPS1	Monopolar spindle 1
MEF	Mouse embryonic fibroblast
MMTV	Mouse mammary tumor virus
MINT	Msx2-interacting nuclear target protein
MYND	Myeloid-Nervy-DEAF1
myo-SC	Myoepithelial unipotent stem cells
MyoD	Myogenic differentiation protein
NAD^+	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor-kappa B
PCAF	p300/CBP-associated factor
PDX1	Pancreatic and duodenal homeobox 1
PRC2	PcG repressive complex 2
PPARBP	Peroxisome proliferator-activated receptor binding protein
PPAR-γ	Peroxisome proliferator-activated receptor-gamma
PTEN	Phosphatase and tensin homologue
PI3K	Phosphoinositide 3 kinase
PLCγ	Phospholipase C gamma
PARP1	Poly-ADP-ribose polymerase 1
PcG	Polycomb group
PRDM9	Positive Regulatory-domain zinc finger protein 9
PGC-1α	PPAR-γ co-activator alpha
PIC	Pre-initiation complex formation
PR	Progesterone receptor
PRL	Prolactin
PRLR	Prolactin receptor
AKT	Protein kinase B
PKC	Protein kinase C
RANKL	Receptor activator NF-κB ligand
pRb	Retinoblastoma tumor suppressor protein
RT	Room temperature

SAM	S-adenosylmethionine
SMYD	SET and MYND domain-containing protein
ST-RC	Short-term repopulating stem cell
STAT	Signal transducer and activator of transcription
SIRT1	Sirtuin 1
SDS	Sodium dodecyl sulfate
SAGA	Spt-Ada-Gcn5-acetyltransferase
SET	Su(var)3-9-enhancer of zeste-trithorax
TAF	TATA-box-binding protein associated factor
TDLU	Terminal ductal-lobular unit
TEB	Terminal end bud
TFIID	Transcription factor IID
TGFα	Transforming growth factor alpha
TNFα	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
Wnt4	Wingless-type MMTV integration site family member 4
YAP	Yes-associated protein
YY1	Yin yang 1
ZDHC8	Zinc finger DHHC (Asp-His-His-Cys) domain-containing protein 8

1. State of the Art

1.1. Introduction

Cellular differentiation is an essential biological process by which cells become competent and commit to a specific cell lineage, being important during both embryonic development and adult life (1). It is also now known as an important feature to have in consideration in the clinical evaluation of tumor aggressiveness and potential response to treatment. Generally, a high degree of differentiation is associated with a better prognosis. In fact, differentiated cancer cells have a higher degree of similarity with each other and with their tissue of origin, are more cohesive and less invasive (2).

Increasing evidence suggests that cell differentiation is deeply regulated by chromatin remodeling and histone post-translational modifications (1). Specifically, methylation of histone H3 at its fourth lysine (K4) is now established as an active mark for gene transcription (functioning as a distinctive signal for the recruitment of specific transcription factors) and cell differentiation (3-7). SETD7 is a H3K4 methyltransferase (8, 9) also known to methylate several other histone and non-histone substrates involved in distinct cellular processes, including cell cycle regulation, DNA damage response, apoptosis, chromatin modulation, proliferation and differentiation (10-12). Thus, the potential of SETD7 as a regulator of these processes and, thereby, of cell's homeostasis, is an interesting lead to follow. However, little is still known about SETD7 role in physiological and pathological processes. For this reason, we decided to study SETD7 expression in proliferation and differentiation of mammary epithelial cells. We believe this project to be of major importance as only by understanding how SETD7 influences these processes in normal cells, can one then establish its role in breast cancer development, progression and invasion.

In summary, this project main goal is to establish the correlation between SETD7 and cellular differentiation and proliferation, as well as its function and regulation, in mammary epithelial cells. In addition, the expression and potential role of some of SETD7 molecular targets during these processes will also be explored.

1.2. Mammary development

1.2.1. Mammary development during Embryogenesis, Puberty and Pregnancy/Lactation

The mammary gland is a specialized epidermal appendage, whose main function is to produce and provide milk to the newborn. This not only assures the nutrition of the baby during its first months but also the provision of immune factors, which helps to protect the baby from infections. On the other hand, it is also important for the development of unique bonds between mother and infant during nursing (13).

This process of production and delivery of milk is called lactation, which is possible due to the existence of a complex network of branched ducts that constitute the mammary epithelium (14).

The ductal network is embedded within the mammary fat pad constituted by adipocytes. This stroma also comprises fibroblasts, immune cells and blood vessels (13).

Mammary gland morphogenesis can be divided in five main stages: embryonic, pubertal, gestational, lactation and involution (or regression) (figure 1) (15). In the embryo, mammary development starts as a primary ectodermal thickening within the 4-6 months of gestation forming the mammary line, that then breaks up into individual placodes (a pair in humans) (16). At this stage, this primary bud contains two different cell populations: central and peripheral (or basal) (17). These placodes project into the underlying mesenchyme originating the epithelial buds (18). The epithelial buds then form branches and, at near term, the breast consists of a ductal tree of approximately five ducts embedded in the mammary fat pad and an external nipple that connects them to the body surface (13). Similarly to what occurs in the formation of other epithelial appendages, all of these changes rely on coordinated signaling between the epithelium and the underlying mesenchyme (19).

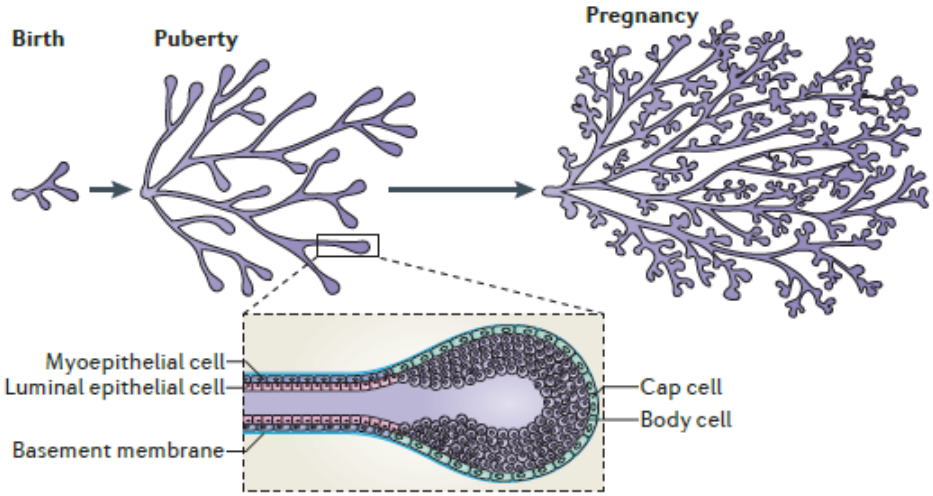

Figure 1 – Different stages of mammary development and TEBs structure. Adapted from (14).

After birth, the breasts involute and become quiescent until puberty (20), when, in females, the formation of secondary ductal branches takes place (17). In the pubertal stage, upon secretion of ovarian hormones like estrogen and progesterone, the mammary epithelium grows exponentially (21) and the immature ducts elongate, which leads to the formation of the terminal end buds (TEBs) (figure 1). Thereby, TEBs are structures that can be found at the end of the primary ducts and differ from them on three main features: they have an inner multilayer luminal epithelium (in contrast to the single layer present in the primary ducts) also delimited by an outer monolayer of myoepithelial cells, and own a large amount of mammary stem cells which, thus, allows them to have high levels of proliferation (22). It is important to notice that the primary TEBs, present in the beginning of the pubertal development, possess an outer layer of cap cells and an inner multilayer of body cells, which are highly proliferative and will give rise to the myoepithelium and luminal epithelium, respectively (23). Once deep in the fat pad, the TEBs bifurcate, forming new primary branches that then divide into secondary branches that will form a complex network of mammary branches through the entire fat pad. Simultaneously, stromal expansion can also be

observed in this phase (20). Moreover, lobular-alveolar structures, termed terminal ductal-lobular units (TDLUs) (figure 2), are formed at the end of these secondary ducts (19).

During pregnancy, an extensive and accelerated proliferation of the epithelium takes place, with further branching, lobuloalveolar development and terminal differentiation (24), which is triggered by progesterone and prolactin (23). These structures (alveoli) contain milk-producing alveolar cells that are responsible for the synthesis and delivery of milk during lactation (13). The myoepithelium that forms the outer layer of these structures is very important at this phase, consisting of specialized, contractile cells that permit the release of milk through the ducts (14). Upon lactation cessation, the mammary glands involute, i.e., they suffer a coordinated apoptosis and remodeling process, reversing the previous growth. The breast structure is now similar to the existing one before pregnancy (24).

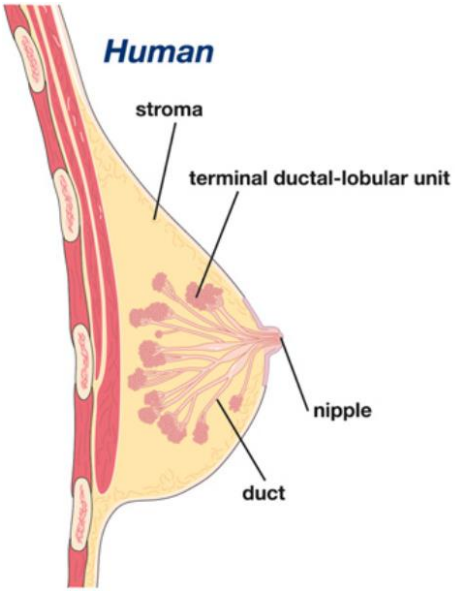

Figure 2 – Schematic representation of the human breast. Adapted from (15).

1.2.2. Mammary Stem Cells (MaSCs) and mammary epithelial hierarchy

All of the changes described above, especially the ones that occur during pregnancy, and the fact that the human mammary gland maintains its regenerative capacity for decades, are thought to rely on the presence of mammary stem cells (MaSCs). As represented in figure 3, there is a hierarchy of stem and progenitor cells that ultimately give rise to the mature lobular and ductal structures (23).

There are different cell-surface markers that can be used to discriminate between the different mammary cell subpopulations, like CD24 (heat-stable antigen), CD29 (β1-integrin) and CD49f (α6-integrin) (25, 26). MaSCs have been shown to have higher levels of CD24, CD29 and CD49f and low levels of Sca-1, however, neither of these are exclusive of MaSCs which suggests that further research and discovery of specific markers is required (25). CD44 is a transmembrane receptor

that can serve as a stem cell marker as well, since it has been found to be highly present in MaSCs and continues to be expressed in myoepithelial cells, being involved in ductal outgrowth, cell-cell adhesion between luminal and myoepithelial cells and bilayer organization (27). It was also found that MaSCs don't possess both estrogen (ER) and progesterone receptors (PR). However, as mentioned, it has been well established that these two steroid hormones are essential for mammary epithelial expansion and differentiation. Thus, these processes may be mediated by ER/PR positive (ER+/PR+) cells signaling to MaSCs via paracrine mechanisms. In other words, estrogen and progesterone stimulate ER-/PR-dependent gene transcription within the ER+/PR+ cells (that seem to be non-dividing cells from the luminal epithelium), promoting the release of certain factors. Then, these factors attach to specific receptors on the MaSCs surface and ultimately regulate MaSCs proliferation and differentiation (25).

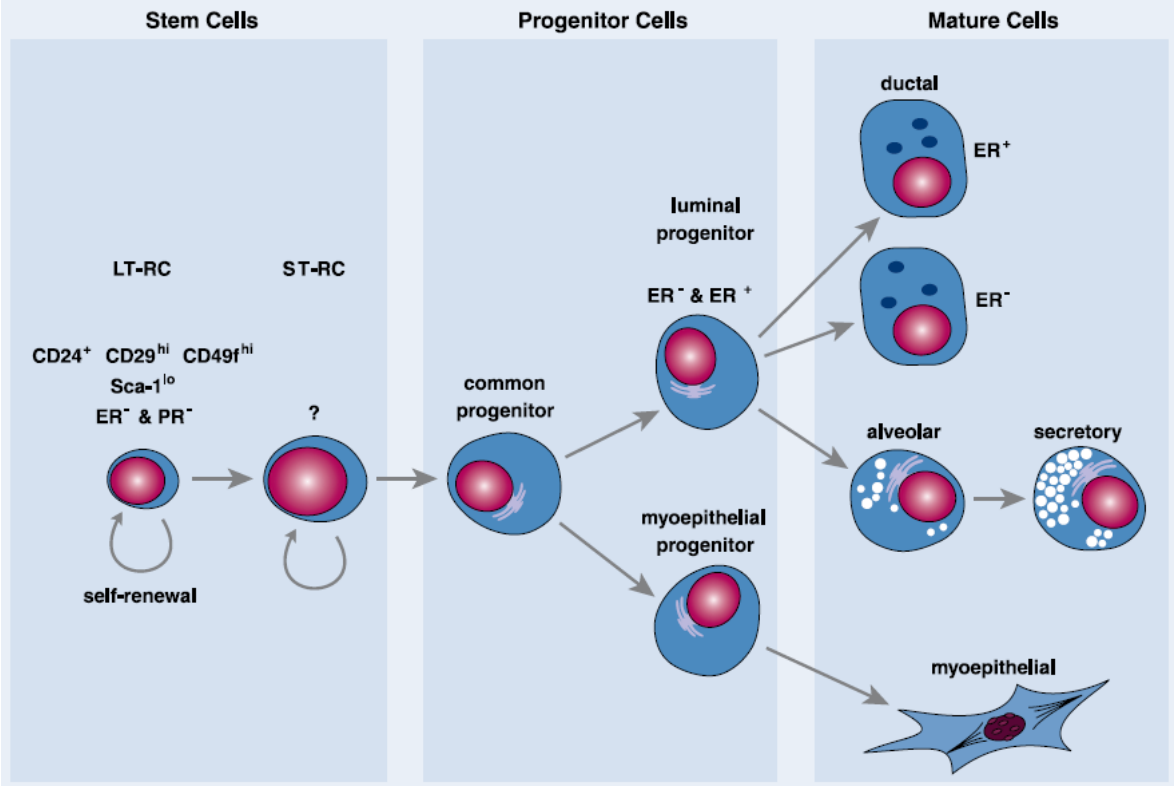

Figure 3 – Hypothetical model of the mammary epithelial hierarchy. The mammary cellular hierarchy is still poorly understood but it is hypothesized that there may exist a hierarchy of multiple stem and progenitor cells. MaSCs are multipotent, long-term, self-renewal adult stem cells that can differentiate into luminal and myoepithelial progenitor cells. Then, these progenitor cells commit to the luminal (ductal and alveolar) and myoepithelial lineages, respectively. ER, Estrogen receptor; LT-RC, Long-term repopulating stem cell; PR, Progesterone receptor; ST-RC, Short-term repopulating stem cell. Taken from (25).

1.2.3. Proliferation and differentiation of the mammary gland

All of the changes that occur during the different stages of mammary gland development are driven by different pathways, most of which are related to pituitary and ovarian hormones that

act in specific receptors located on the mammary gland. The following hormones are particularly relevant to guide and control the developmental changes occurring in puberty and adulthood (28).

1.2.3.1. Estrogen and EGFR signaling

Estrogen is a hormone released by the ovaries that has a major role in pubertal mammary growth during ductal morphogenesis through the activation of ERα. However, ERα is only expressed in a subpopulation of luminal cells (ERα+ cells) and therefore it is thought that these cells drive proliferation of ERα negative (ERα-) cells through paracrine signals. In addition to its role in ductal morphogenesis, estrogen also promotes alveolar cells growth and maintenance during pregnancy (28).

Epidermal growth factor receptor (EGFR), a transmembrane ERBB/HER receptor tyrosine kinase present in both epithelial and stromal cells (28), has long been shown to be implicated in mammary gland development and to mediate estrogen proliferative effects (29-31). In fact, one of EGFR ligands, Amphiregulin (AREG), is a strong candidate for the factor that is released by ERα+ cells (28, 32). There is also evidence supporting a role for EGF as a mediator of estrogen paracrine signaling (30, 31, 33). In fact, EGF was shown to promote mammary epithelial cell proliferation in synergy with estrogen (33). Other members of the EGF-like family of growth factors include transforming growth factor-α (TGFα), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), epiregulin (EPR), and epigen. All of these EGFR ligands are originally expressed as transmembrane precursors whose extracellular domain is proteolytically cleaved to release the mature growth factor that will activate EGFR (34). Activated EGFR can then activate multiple signaling pathways involved in cell proliferation and survival, such as the phosphoinositide 3 kinase/protein kinase B (PI3K/AKT), Janus kinase/signal transducers and activators of transcription (JAK/STAT) and mitogen-activated protein kinase (MAPK) pathways (figure 4) (35-37).

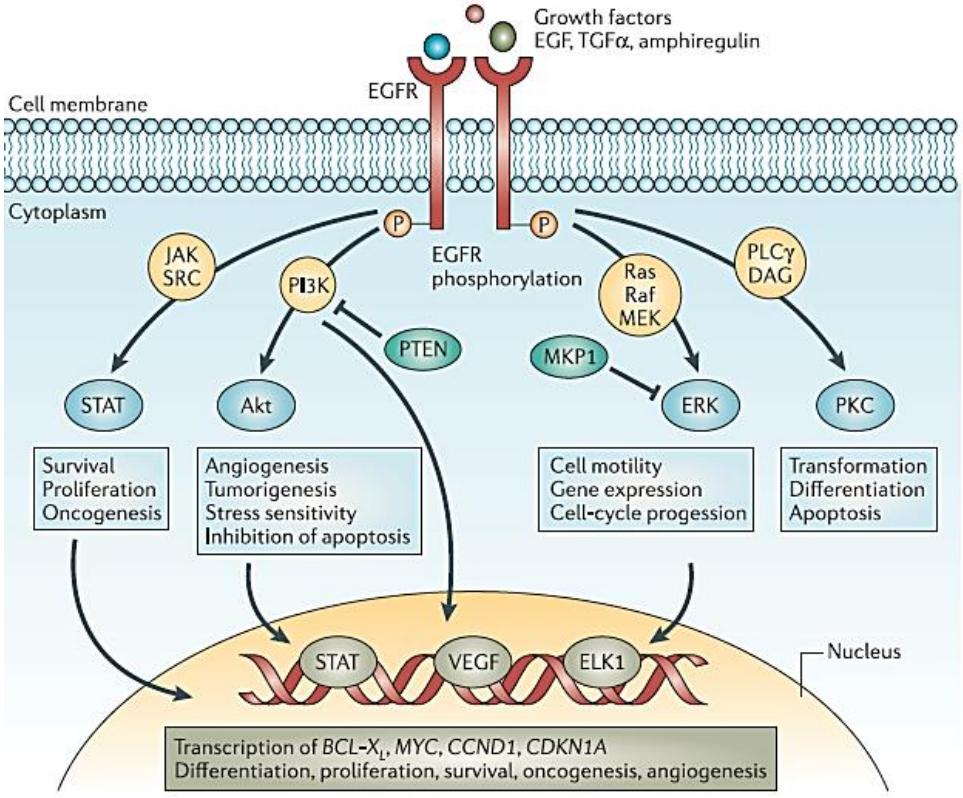

Figure 4 – Main pathways activated downstream of EGFR. AKT, Protein kinase B; CCND1, gene encoding cyclin D1; CDKN1A, gene encoding p21; EGF, epidermal growth factor; EGFR, EGF receptor; ERK/MAPK, extracellular signal-regulated kinase; JAK, Janus kinase; MEK/MAPKK, ERK kinase; MKP1, MAPK phosphatase 1; PI3K, phosphoinositide 3 kinase; PKC, protein kinase C; PLCγ, phospholipase Cγ; PTEN, phosphatase and tensin homologue; STAT, signal transducers and activators of transcription; TGFα, transforming growth factor-α; VEGF, vascular endothelial growth factor. Taken from (38).

1.2.3.2. Progesterone

Progesterone is an ovarian hormone that contributes to the development of side-branches and alveologenesis during pregnancy. For that reason it is crucial for the formation of mature structures capable of producing and secreting milk (that holds nutrients vital for the newborn). PR is only expressed in a subpopulation of luminal cells (about 30% of them) and, similarly to estrogen, this suggests that progesterone promotes cell proliferation through paracrine signals (28). Between the several possible paracrine mediators of the progesterone function, the receptor activator of nuclear factor kappa B1 (NF-κB1) ligand (RANKL) and the Wingless-type mouse mammary tumor virus (MMTV) integration site family member 4 (Wnt4) seem to be the more promising.

1.2.3.3. Prolactin

Prolactin (PRL) is a hormone secreted by the pituitary gland that collaborates with progesterone in lobuloalveolar development, in order to generate a lactation-competent gland during pregnancy. It can also act indirectly by stimulating progesterone production in the ovaries. Prolactin binds to a member of the class I cytokine receptor superfamily, PRLR, which enhances

several signaling pathways such as JAK/STAT, MAPK and PI3K/AKT pathways. The interaction PRL-PRLR triggers JAK2/STAT5 signaling pathway activation (28), enhancing transcription via the transcription factor STAT5 phosphorylation (by JAK2) and its nuclear translocation. This cascade of events lead to induction of RANKL (in synergy with progesterone), which ultimately triggers mammary epithelial growth and alveologenesis. PRL also induces insulin-like growth factor 2 (IGF2), which accelerates alveolar growth (39). Prolactin, in association with insulin and cortisol, are also key factors in lactogenesis (that occurs from mild pregnancy to a few days before parturition), where the mammary epithelium differentiates and begins to produce all of the milk components. Further epithelial expansion and an increase in the expression of genes involved in milk secretion can occur after birth of the new infant as suckling begins (40-42). This process is also driven by STAT5 that appears to regulate the transcription and secretion of milk proteins (41).

1.2.3.4. Insulin

Insulin is essential for lactation since it enhances milk protein production in synergy with PRL. Recent data suggests that insulin increases the transcription of E74-like factor 5 (ELF5) and, thus, of STAT5 (since ELF5 is a co-regulator and amplifier of STAT5 activity), which furthers its phosphorylation in result to PRL signaling and leads to an increase in the transcription of milk protein genes such as α-lactalbumin and β-casein (40, 43).

1.3. Tumorigenesis and cancer development

Tumorigenesis is a multistep process, which depends on accumulation of DNA mutations. However, only a small portion of these mutations will contribute for malignant transformation and, therefore, for cancer initiation and progression. Also, to drive this transformation, these mutations may intensify cells proliferation rate, make them resistant to apoptosis and activate specific pathways involved in cell growth and division, giving these cells a selective proliferative advantage (44). Moreover, nowadays it is acknowledged that cancer cells main features are: they are self-sufficient in growth signals, insensitive to anti-growth and pro-apoptotic signals, thereby having unlimited self-renewal potential. They are also capable of stimulating blood vessels growth (angiogenesis) and of invading other tissues and form metastases (45). Additionally, cancer development can be instigated not only by genetic alterations but also by dysregulation of the epigenetic machinery. Accordingly, epigenetic and genetic alteration can cause aberrant gene expression and genomic instability and, possibly, predispose to cancer (46).

1.3.1. Cancer and Stem cells

Considering the current knowledge about cancer pathology, it is thought that cancer originates from stem cells or cells that have acquired stem cell properties. One of stem cells' key characteristics is the fact that they have a long lifespan, which makes them major candidates for the accumulation of mutations and subsequent malignant transformation. On the other hand,

cancer stem cells are non-differentiated cells that can give rise to specific cell lineages, thus perpetuating mutations through uncontrolled mitotic divisions. The cancer stem cells capacity to differentiate is also a probable cause for cancer heterogeneity and therapeutic resistance (44). Currently it is not established if cancer stem cells originate from normal adult stem cells or result from de-differentiation of cancer cells (47-49).

1.4. Epigenetic modifications

Epigenetic modifications are hereditable changes in the genetic expression patterns mediated by epigenetic regulatory mechanisms, without a change in DNA sequence. These mechanisms comprise DNA methylation, histone modifications and RNA silencing by noncoding RNAs (50). Most of these changes are generated during cellular differentiation and can be passed through to descendant cells, being maintained even after multiple cycles of cell division (51).

DNA methylation, catalyzed by DNA methyltransferases (DNMTs), occurs on cytosine residues predominantly in the sequence CpG (52). This causes the silencing of the genetic material (genes and non-coding regions), which is particularly important during embryogenesis (50). On the other hand, histone modifications consist of enzyme-dependent histone residues posttranslational changes, including acetylation, phosphorylation, methylation, ubiquitylation and sumoylation (50, 53). This kind of epigenetic modification will be further explained ahead. Lastly, RNA silencing consists of posttranscriptional mRNA silencing by small non-coding RNAs, commonly called microRNAs (50). MicroRNAs are partially or completely complementary to their target mRNA, which allows them to recognize and bond with specific mRNAs. Then, this may cause mRNA degradation or inhibition, preventing mRNA translation into proteins. This is important for the maintenance and control of the global gene expression patterns (51).

Epigenetic changes play an important role in the regulation of gene expression and chromosomal stability. In other words, they ensure that the correct genes are transcribed, at the right time and amount within each specific tissue. For these reasons, it is plausible to assume that any perturbation of this process can lead to the dysregulation of gene transcription and of normal cellular processes and indirectly contribute to cancer initiation and progression. Moreover, alteration of the normal methylation patterns due to changes in the enzymatic activities of the histone methyltransferases (HMTs) and histone demethylases (HDMs) has been associated with breast, prostate, lung and brain cancers as well as leukemia (52).

1.5. Histone methyltransferases

In eukaryotic cells the genetic material is enclosed in the nucleus and packaged into chromatin, a complex structure composed of DNA and proteins. Moreover, in mammalian cells, chromatin is arranged into compacted and highly organized structures, the chromosomes (54). Chromatin's fundamental unit is the nucleosome formed by 145-147 base pairs of DNA, wrapped around an

octamer of four core histones: H2A, H2B, H3 and H4. Each nucleosome core is then stabilized by a linker histone (e.g., H1), that in cooperation with other non-histone proteins further direct nucleosome packaging and organization into chromosomes (55, 56).

Histones can suffer posttranslational modifications, including acetylation, phosphorylation and methylation. These modifications (that mostly occur on the N-terminal tail domains) are catalyzed by specific histone modifying enzymes and can alter the interactions between nucleosomes, DNA-nucleosomes and nucleosomes/DNA-regulatory factors, leading to the regulation of chromatin organization, mitosis and DNA transcription, replication, recombination and repair (53, 55, 57).

Histone methylation is catalyzed by HMTs that transfer a methyl group to a lysine or to an arginine. All the lysine methyltransferases (KMTs), except Dot1 that methylates H3 at K79, contain a Su(var)3-9-Enhancer of Zeste-Trithorax (SET) domain that is responsible for catalysis of cofactor S-adenosylmethionine (SAM), with subsequent transference of a methyl group to a lysine. Similarly, arginine methyltransferases (PRMTs) also catalyze the transference of a methyl group from SAM to an arginine residue within its substrates. Contrarily to other histone post-translational modifications, methylation does not alter histone charge, having little to no effect on DNA-histone interactions. Methylation can rather serve as a distinctive signal for the recruitment of methylation "reader" proteins that can trigger transcriptional activation or suppression depending on the modified histone and residue position in the histone primary structure. Lysines and arginines can be either mono- or di-methylated. Additionally, lysines can also be tri-methylated (6, 53, 58).

Due to its involvement on gene expression and chromatin architecture regulation, epigenetic modifications can be implicated on cell malignant transformation and subsequent aberrant proliferation. Thus, the dysregulation of histone modifying enzymes' activity may lead to aberrant histone modification and give rise to cancer through inhibition of tumor suppressors and/or oncogenes activation (51, 53). In fact, because of HTMs ability to control gene transcription, the correct regulation of HTMs expression and activity is crucial for the normal function and fate determination of cells. Therefore, dysregulation of HTMs is nowadays increasingly acknowledged as a hallmark for cancer development and progression, and therefore several HTMs are currently in the spotlight of cancer research because of their potential oncogenic or tumor suppressor roles (52, 59, 60). One example is SETD7 which has been found to be associated with but have divergent functions in various cancers (61-65).

1.5.1. Histone H3 methylation at K4

Epigenetic modifications, such as histone modifications, are responsible for the control of the genetic material expression or silencing, being crucial during the different development stages, apoptosis and aging. H3 methylation at K4 mark is enriched at actively transcribed regions, and has been shown to be important for an efficient control of normal development, nucleosomal function and homeostasis. Thereby, dysregulation of this modification can lead to impairment of normal cellular regulatory mechanisms and is related to cancer development. Furthermore,

H3K4me1/me2/me3 dysregulation may mediate anti-apoptotic, proliferative, tumor-induced angiogenesis and inflammation pathways activation (66). Currently, H3K4 methylation modifiers (methyltransferases and demethylases) are known to have a role in embryonic development and regulation of stem cells' fates, being required for stem cells self-renewal or differentiation and important for future cancer stem cells studies (6, 7, 67).

H3 methylation in its fourth lysine is often found near the promoters of actively transcribed genes (68). This is emphasized by the fact that H3K4me2 are often found in coding regions and about 75% of all human promoters are marked by H3K4me3 (6, 69). H3K4 methylation is present in the intergenic regions within Homeobox (HOX) gene clusters, which are some of the most important genes regulated by H3K4 methylation (68). HOX genes encode a family of transcriptional regulators that are involved in embryonic development. During the initial stages of development HOX genes are downregulated, whereas it can become increasingly activated during embryogenic development, coordinating tissue-specific cell proliferation and differentiation (with HOX loss-of-function resulting in embryonic lethality) (70-72). Similarly to embryogenesis, HOX expression during adulthood is tissue-dependent. In fact, the different HOX genes exhibit a tissue-specific expression pattern, regulating cell renewal (73), hematopoiesis (74), cell fate and differentiation and tissue homeostasis (75-77). Nonetheless, HOX genes dysregulation has been associated with cancer. Interestingly, HOX genes can either be upregulated or downregulated in cancer and either have an oncogenic or a tumor suppressor effect, depending on the tissue context and cancer type. For example, HOXB13 was found to be upregulated in ER+ breast cancers from patients that had been treated with tamoxifen, being responsible for tamoxifen resistance and an increase in cancer cells proliferation, migration and invasion (77-79). On the other hand, HOXB13 is downregulated in colorectal and prostate cancers, functioning as a tumor suppressor (78). HOXB2 was also shown to function as a tumor suppressor (inhibiting tumor growth and promoting apoptosis) in breast cancer (80) and acute myeloid leukemia (AML) (81). In fact, HOXB2 is correlated with better a prognosis in breast cancer patients (80). Conversely, HOXB2 overexpression has been associated with cervical (82, 83), lung (84), and pancreatic (85) cancers progression, invasiveness and recurrence. On the other hand, H3K4 methylation antagonizes Enhancer of zeste homolog 2 (EZH2) repression of Polycomb group (PcG)-regulated genes. EZH2 is the catalytic subunit of PcG repressive complex 2 (PRC2) (initially described as a repressor of the HOX genes by catalyzing H3K27 trimethylation) (86-88). PRC2 is essential for the regulation of transcriptional silencing during embryonic development, especially in the early stages, mediating lineage decisions (89, 90).

The forth lysine of H3 histones can be methylated by several SET-domain containing enzymes such as SET1A, SET1B, mixed lineage leukemia proteins 1 to 5 (MLL1 to MLL5), SET7/9, SET and Myeloid-Nervy-DEAF1 (MYND) domain-containing protein 1 to 3 (SMYD1 to SMYD3), Absent, Small or Homeotic 1-like protein (ASH1L), SET domain and Mariner transposase fusion protein (SETMAR) and Positive Regulatory-domain zinc finger protein 9 (PRDM9) (6, 91). These can methylate H3 at K4 into all three states, being involved in transcriptional activation (6, 69).

H3K4 demethylation is controlled by lysine demethylase 5/Jumonji adenine-thymine (AT)-rich interactive domain 1 (KDM5/JARID1) and lysine-specific histone demethylase 1 (LSD1), that act as transcriptional repressors (67).

1.5.1.1. SETD7 and its histone and non-histone substrates

SETD7 (also known as SET7 or SET9) is a lysine methyltransferase that comprises a SET domain and it catalyzes the transference of a methyl group to a lysine residue of various histones and non-histone subtracts. These potential SETD7 substrates are involved in distinct cellular processes, for instance, in cell cycle regulation, DNA damage response, RNA polymerase II dependent gene transcription, chromatin modulation and differentiation. Hence, SETD7 can play a critical role in several physiological and pathological processes (10-12). Moreover, SETD7 knockdown has been shown to cause embryonic lethality, which indicated that it plays an important role in development. On the other hand, SETD7 was also shown to be important for β-cells' function and skeletal muscle differentiation, as described below (92).

Histone H3 can be monomethylated by SETD7 at its lysine 4, which can lead to transcriptional activation by preventing chromatin condensation and by facilitating transcriptional factors binding to the promoter regions of specific genes (figure 5) (93). For example, SETD7-driven methylation of H3 at K4 takes part in the expression of the tumor necrosis factor alpha (TNF-α) gene by facilitating NFκB-p65 subunit binding to gene promoters of NFκB-dependent inflammatory genes, such as TNFα, monocyte chemoattractant protein-1 (MCP-1) and interleukin 8 (IL-8) (whose dysregulation can give rise to several inflammatory diseases, including atherosclerosis, insulin resistance, diabetes and metabolic syndrome, as well as cancer). H3K4 methylation is also associated with RELA expression, which encodes for NFκB-p65. Additionally, SETD7 also directly methylates p65 at K37, restricting p65 to the nucleus and promoting its association with NFκB-dependent promoters, or at K315 and K316, resulting in negative regulation of p65 translocation to the nucleus (92, 94).

A recent study suggests that SETD7 is required for skeletal muscle gene expression and myogenic differentiation by directly interacting with Myogenic differentiation (MyoD) protein, a transcriptional factor that binds to the regulatory region of muscle differentiation genes to activate gene expression. The interaction between MyoD and SETD7 facilitates SETD7 access to the silencing nucleosome and monomethylation of H3K4, leading to increased affinity of MyoD to the myogenic regulatory regions. SETD7 also prevents Suv39h1 (a methyltransferase that transfers three methyl group to H3K9) association with MyoD (by competing with Suv39h1 for MyoD association), preventing its inhibitory effect over differentiation genes through H3K9 methylation (12).

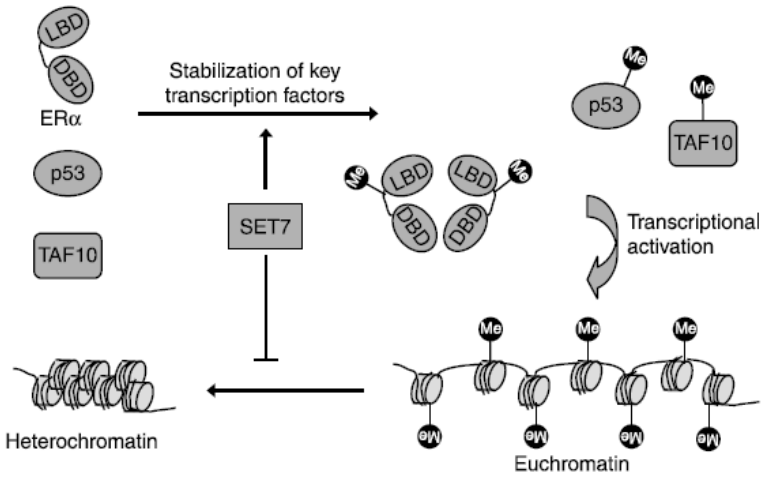

Figure 5 – SETD7 substrates and gene expression. H3K4 methylation by SETD7 prevents chromatin condensation (i.e., chromatin adopts an "open" conformation – euchromatin) and enhances gene transcription. SETD7 also methylates non-histone proteins, including transcription factor. Methylation of ERα, p53 and TAF10 results in their stabilization, thereby improving their activity as transcriptional activators. ERα, Estrogen receptor α; SETD7, SET domain containing lysine methyltransferase 7; TAF10, TATA-box-binding protein associated factor 10. Taken from (93).

SETD7 is also required for normal pancreatic and duodenal homeobox 1 (Pdx1)-mediated insulin synthesis by pancreatic β-cells, since the interaction between SETD7 and this transcriptional factor, Pdx1, leads to the recruitment of SETD7 and consequent H3K4 methylation in the promoter region of the Ins1/2 gene (with its subsequent activation and preproinsulin expression) (92, 95-97). Having this in mind, SETD7 may play an important role in development through cellular differentiation and tissue-specific gene activation (92).

As described in Table 1, methylation of specific lysine residues on non-histone proteins (such as ERα, p53 and TAF10) by SETD7 has also been described, improving their ability to serve as transcriptional activators (figure 5) (93). Recent studies have demonstrated that SETD7 also methylates several lysines of H2A and H1.4, as well as, the lysine K15 of H2B (92).

These findings highlight the importance of SETD7 and H3K4 methylation in several physiological processes and the basis of its mechanism of action. Furthermore, one can question if this enzyme is also linked to pathological processes, like cancer, diabetes or inflammatory diseases. Therefore, it would be interesting to conduct additional research to further explore SETD7 importance and expression patterns in these diseases.

Table 1 - SETD7 targets and respective effects upon SETD7-mediated methylation.

SETD7 targets		Effects	Ref.s
Histones	**H3**	H3 methylation at K4, enhances transcriptional activation by preventing chromatin condensation.	(93)
	H2A **H2B**	Methylates histones H2A and H2B when in a free state. Little is known about the functional effect of this modification.	(92, 98)

	H1.4	H1.4 is methylated by SETD7 in six different lysine residues (K121, K129, K159, K171, K177 and K192) which can influence H1 binding to DNA and its function in chromatin compaction.	(92, 99)
Chromatin associated	**MeCP2** (Methyl-CpG Binding Protein 2)	MeCP2 is a nuclear protein that recognizes and binds methylated DNA, functioning as a promoter repressor. SETD7 was reported to methylate MeCP2 at K347, although the effects of this methylation are still a mystery.	(92, 100)
	PPARBP (Peroxisome Proliferator-Activated Receptor Binding Protein) or **MED1** (Mediator Complex 1)	PPARBP has been demonstrated to be methylation by SETD7 at K1006, however, the functional purpose of this modification is still to be defined. PPARBP is a coactivator complex of the transcription machinery that is recruited in order to enhance the expression of RNA polymerase II transcribed genes, regulating the cell cycle, DNA repair, peroxisome proliferation, differentiation, proliferation and apoptosis. Med1 gene was shown to be overexpressed in breast, prostate and hepatic cancer.	(98, 101, 102)
	CENPC1 (Centromere protein C1)	CENPC1-K414 monomethylation is catalyzed by SETD7. Little is known about the biologic effects of this modification, with the need to further study how SETD7 can influence CENPC1 function. CENPC1 associate with centromeric DNA and assists the assembly of the kinetochore to the centromere.	(92, 98, 103)
Transcriptional factors and Co-activators / repressors	**p53**	SETD7 methylation at K372 results in the stabilization of p53 and inhibition of p53 nuclear export, which leads to transcriptional activation of the p53 target genes.	(92, 104, 105)
	TAF10 (TATA-box-binding protein associated factor 10)	TAF10 is methylated at K189, which increases TAF10-RNA polymerase II affinity and, as a result, stimulates TAF10 mediated transcription. TAFs are transcription factors, part of the Transcription Factor IID (TFIID) complex, that bind gene promoters and trigger pre-initiation complex formation (PIC), which regulates gene transcription initiation by RNA polymerase II. TAFs enhance transcription by interacting with transcriptional activators and as readers of epigenetic marks.	(92, 93, 106-108)
	TAF7	Similarly to TAF10, TAF7 is also monomethylated by SETD7 at K5.	(92, 105, 109)
	ERα	Enhances ERα-driven transcription by altering ERα recruitment and binding to target gene regulatory sequences through K302 methylation.	(92, 93, 110)
	E2F1	E2F1 is destabilized by methylation at K185 by SETD7, which prevents E2F1 accumulation during DNA damage and the activation of its pro-apoptotic target gene p73. This highlights SETD7 potential role in cell cycle regulation (and, by association, in cancer proliferation). However, other reports suggest that E2F1 methylation at K185 by SETD7 also enhances E2F1-dependent transcriptional activation (although it enhances E2F1 proteosomal degradation).	(92, 111-113)

MINT (Msx2-interacting nuclear target protein)	SETD7 methylates MINT at K2076. MINT functions as transcription repressor involved in the regulation of several processes such as cell cycle, craniofacial development, neural cell fate and apoptosis. Yet, MINT methylation function is still unknown.	(98, 114)
IRF1 (Interferon Regulatory Factor 1)	Lysine K126 of IRF1 seems to be methylated by SETD7. IRF1 is a transcription regulator of immune responses and hematopoietic development. Further studies should focus on the discovery of SETD7 modification effects over IRF1 function.	(98, 115)
HIV-Tat (Human immunodeficiency virus transactivator)	By methylating Tat at K51, SETD7 enhances Tat-dependent transactivation of several viral and cellular genes, contributing to viral replication and HIV-1 pathogenesis.	(92, 116, 117)
FoxO3 (Forkhead Box O3)	FoxO3 is an activator of genes involved in several cellular regulatory pathways, such as stress resistance, cell cycle arrest, differentiation, apoptosis and metabolism. It also is described in the literature as a tumor suppressor. FoxO3 is methylated at K271, which leads to an increase in its transcriptional activity.	(92, 118)
NFκB-p65	SETD7-driven methylation of H3 at K4 or of p65 at K37 facilitates NFκB-p65 subunit binding to gene promoters of NFκB-dependent inflammatory genes, such as TNF-α, MCP-1 and IL-8.	(92, 94, 119)
AR (Androgen Receptor)	SETD7 methylates AR (nuclear and cytoplasmic) at K630, which leads to enhanced transcriptional activity of AR and subsequent expression of PSA and NKX3-1.	(92, 120)
FXR (Farnesoid X receptor)	FXR is a nuclear receptor that regulates bile acid homeostasis (by inhibiting its synthesis) and lipid, cholesterol and glucose metabolism in the liver and intestines. FXR also promotes liver regeneration and inhibits transcription of pro-inflammatory genes which may explain why FXR-knockout in mice significantly increases liver tumor incidence. SETD7-dependent methylation of FXR at K206 enhances transcription of two FXR target genes, SHP and BSEP. FXR lysines K210 and K460 are also putatively methylated by SETD7, although this still needs to be proven.	(121-128)
pRb (Retinoblastoma tumor suppressor protein)	pRb is monomethylated at K873 by SETD7, which is necessary for Rb-dependent cell cycle arrest, transcriptional repression, and Rb-dependent differentiation.	(92, 105, 129)
STAT3	STAT3, a transcription factor normally activated in response to several cytokines and growth factors involved in cell growth and survival, is dimethylated at K140 by SETD7 in response to IL-6 signaling, which inhibit STAT3 binding to DNA promoters.	(92, 105, 130)
HIF-1α (Hypoxia-inducible factor 1α)	Under hypoxic conditions HIF is activated, inducing the transcription of genes involved in cell's adaptation to cellular hypoxia. More specifically, it can alter cellular energy metabolism and promote angiogenesis, maintaining tissue integrity and homeostasis. Recent studies (65, 131) show that SETD7 stabilizes HIF-1α and induces HIF-1α-dependent gene transcription by	(131-133)

		methylating HIF-1α at K32 and inhibiting its ubiquitination and degradation. SETD7 also methylates H3K4 at the promoters of HIF-1α-activated genes. This may suggest that SETD7 is involved in metabolic adaptation in hypoxic cancer cells. By contrast, a different study (132) states that SETD7 may negatively regulate HIF-1α transcriptional activity. Therefore, additional research must be conducted in other to clarify SETD7 effects over HIF-1α function.	
	PGC-1α (Peroxisome proliferator-activated receptor-gamma (PPAR-γ) co-activator α)	PGC-1α is a transcription co-activator that interacts with nuclear receptors, including PPAR-γ, and transcription factors to facilitate gene transcription. It is required for the regulation of mitochondrial biogenesis, energy metabolism and adaptive thermogenesis. PGC-1α has also an anti-inflammatory role in muscle tissue. PGC-1α is methylated at K779, which is essential for the recruitment of Mediator and Spt-Ada-Gcn5-acetyltransferase (SAGA) complexes and, thus, for the transcription of PGC-1α target genes. In fact, SETD7 knockdown decreased PGC-1α capacity to bind MED1, MED17 and the SAGA complex component CCDC101/SGF29, which consequently impaired PGC-1α capacity to stimulate transcription.	(134-137)
	YAP (Yes-associated protein)	YAP is a transcriptional coactivator of proliferation and anti-apoptotic genes, whose activity is inhibited upon activation of the Hippo signaling pathway by cell-cell contact. YAP is methylated by SETD7 at K494, which prevents YAP translocation to the nucleus, decreasing YAP target genes (Ctgf and Cdc20) expression. Moreover, $SETD7^{+/+}$ mouse embryonic fibroblasts (MEFs) were more sensitive to contact inhibition of proliferation than $SETD7^{-/-}$ MEFs, which may be related to YAP methylation and consequent cytoplasmic sequestration.	(138, 139)
	PDX1 (Pancreatic And Duodenal Homeobox protein 1)	PDX1 is a transcription factor essential for pancreatic development, promoting cells differentiation. PDX1 also promotes pancreatic regeneration and regulates mature β-cells function, proliferation and survival as well as β-cell-related genes transcription (such as insulin). PDX1 has been associated with diabetes and cancer development, being overexpressed in several cancers, such as pancreatic and gastric cancer. Maganti *et al.* (2015) found that PDX1 is methylated at K123 and K131, with K131 been necessary for PDX1 transcriptional activity. They then proposed that the methylation of these two lysines may be catalyzed by SETD7 as PDX1 transcriptional activity is significantly increased (by about 40%) by SETD7. However, there is not enough evidence to prove this hypothesis.	(140-147)
	YY1 (Yin Yang 1)	YY1 is a transcription factor that is methylated by SETD7 at two lysine residues, K173 and K411. YY1 can either activate or repress gene expression, regulating	(148-150)

		cell proliferation and differentiation, DNA repair and apoptosis. Evidence suggests that YY1 methylation by SETD7 promotes YY1-dependent transactivation of specific genes involved in cell cycle regulation and cell proliferation. Moreover, alike other SETD7 substrates YY1 plays a role in cancer development and progression. Interestingly, YY1 can function either as an oncogene (promoting cancer proliferation) or a tumor-suppressor, depending on the type of cancer. Therefore, SETD7 is a putative regulator of YY1 oncogenic or tumor-suppressor functions.	
Enzymes	**ZDHC8** (Zinc finger DHHC (Asp-His-His-Cys) domain-containing protein 8)	ZDHC8 is an S-acyltransferase that mediates the attachment of fatty acids on to cysteine residues (S-acylation), which regulates their solubility, attachment to membranes, distribution over specific organelles, folding and stability. ZDHC8 methylation by SETD7 occurs at K300. Up to now, no functional effect has been described.	(98, 151, 152)
	TTK (Dual specificity protein kinase) or **MPS1** (Monopolar spindle 1)	It was verified that TTK is methylated by SETD7 at K708, however, the functional effects of this modification still need to be cleared. TTK is a dual-specificity protein kinase that phosphorylates several proteins involved in DNA damage-induced cell cycle arrest, spindle pole duplication and chromosome alignment and segregation during mitosis. Dysregulation of this mitotic kinase may be involved in cancer development since it would result in uncontrolled proliferation.	(98, 153, 154)
	DNMT1 (DNA cytosine-5-methyltransferase 1)	Methylation of DNMT1 (a maintenance DNA methyltransferase that specifically methylates DNA at CpG residues, normally involved in transcriptional repression) at K142 by SETD7 results in a decrease in DNMT1 by facilitating its polyubiquitination and subsequent proteasome-mediated degradation.	(92)
	SIRT1 (Sirtuin 1)	In response to DNA damage, SIRT1 methylation at K233, K235, K236 and K238 is catalyzed by SETD7, which inhibits SIRT1-p53 association and, consequently, enhances p53 acetylation and transactivation. This ultimately leads to apoptotic cell death, which indicates that SIRT1 is an oncogenic protein and in the absence of SETD7 protects cells from apoptosis.	(92, 155)
	PCAF (p300/CBP-associated factor)	STED7 methylates six different lysine residues of PCAF (K78, K89, K638, K671, K672 and K692) – an acetyltransferase implicated in several cellular processes –, regulating PCAF localization (methylated PCAF was found to localize to the nucleus) and possibly its function.	(92, 156)
	PARP1 (Poly-ADP-ribose polymerase 1) or **ARTD1** (ADP-ribosyltransferase diphtheria	PARP1is a nuclear enzyme that transfers ADP-ribose from nicotinamide adenine dinucleotide (NAD^+) to nuclear proteins (e.g., histones, transcription factors or PARP1 itself – auto-inhibition). PARP1 is best known as a DNA damage sensor, promoting DNA repair and	(157-163)

	toxin-like 1)	maintenance of genomic integrity. PARP1 also regulates chromatin replication, transcriptional activation, differentiation, cell death and cell cycle arrest. PARP1 methylation at K508 by SETD7 was shown to enhance PPAR1 enzymatic activity in basal conditions and upon oxidative and DNA stress.	
Others	**LIN28A**	LIN28A is RNA-binding protein that inhibits RNA translation. LIN28A is expressed in embryonic stem cells (ESCs) and cancer stem cells (CSC), conferring self-renewal properties and pluripotency to these cells. LIN28A also inhibits cell differentiation by preventing pri-/pre-let-7 maturation (a microRNA whose mature form promotes cell differentiation). LIN28A is methylated by SETD7 at its lysine K135, which promotes its localization to the nucleus. In fact, although LIN28A is predominantly located in the cytoplasm (blocking pre-let-7 maturation), methylated LIN28A (by SETD7) localizes to the nucleus. In addition, SETD7-mediated methylation stabilizes LIN28A and enhances its affinity to pri-let-7, maximizing the inhibition of let-7 maturation by LIN28A in the nucleus.	(164-166)
	AKAP6 (A kinase anchor protein 6)	AKAP6 methylation at lysine K604 is catalyzed by SETD7. AKAP6 functions as an anchor protein that localizes signaling enzymes with their substrates, facilitating their interaction. In other words, AKAP6 is responsible for the spatial and temporal coordination of cellular signaling. Currently, little is still known about AKAP6 methylation effects.	(98, 167, 168)
	Cullin 1	Cullin 1, a protein required for ubiquitin-dependent protein degradation, plays an important role in cell cycle progression and early embryogenesis through ubiquitination of several proteins, such as p27 and p21 (repressing their inhibitory effect over cyclin-dependent kinases). Cullin 1 methylation by SETD7 occurs at K73 but further studies need to be conducted in other to better understand its functional effects.	(98, 169)

1.5.1.1.1. SETD7 substrates and its potential association with cancer

Numerous SETD7 substrates, such as ERα (110), p53 (170) and FoxO3 (118), have been shown to play a pivotal role in carcinogenesis and cancer progression. In addition, studies using embryonic fibroblasts obtained from SETD7 heterozygous and null mice demonstrated that these are more susceptible to malignant transformation in comparison to cells from healthy mice (118). Furthermore, SETD7 knockdown in p53-negative cells increased colony formation, which can be an indicative of the potential SETD7 role in cells growth retardation and death, even in the absence of this pro-apoptotic factor (113). This may suggest that SETD7 acts as a tumor suppressor through the regulation of its substrates (118). However, conflicting results are described in the literature regarding STED7 function in physiological and pathological scenarios (61-65, 92, 139, 171, 172).

On the other hand, LSD1, a demethylase that usually counteracts the SETD7 effects over SETD7-methylated substrates, was found to be present in high levels in prostate carcinoma, neuroblastoma and breast and colon cancers. Moreover, LSD1 knockdown led to breast cancer cells growth retardation (113). This finds may suggest that SETD7 pro-apoptotic activity may be highly counteracted by LSD1 (113, 118, 170).

To conclude, the data presented above may suggest that SETD7 expression and its activity as a tumor suppressor or stimulator may be regulated on a tissue/cellular-context dependent basis, and that LSD1 possibly takes part in this regulation.

1.5.1.1.2. SETD7 substrates

1.5.1.1.2.1. Nuclear receptor ERα

ERα is a nuclear receptor that, once activated, binds to specific DNA sequences, recruits co-activators and functions as a transcriptional activator of several genes. ERα is essential for mammary epithelial cells proliferation and ductal morphogenesis during puberty (28, 173, 174).

Currently, ERα plays a significant role in breast cancer prognosis and management. In fact, about two-thirds of newly diagnosed breast cancers are ERα+ and will potentially respond well to endocrine therapy with anti-estrogens (93, 110). However, after a while some patients develop resistance and recur (175), which can be caused by posttranslational modifications that can affect ERα-mediated gene transcription efficiency (176) as, for example, methylation by SETD7. A study by Subramanian *et al.* (2008) revealed that SETD7 methylates ERα at K302 and is necessary for ERα function. Moreover, they also proposed that SETD7 is involved in ERα-dependent transcription regulation in breast cancer since the knockdown of SETD7 can lead to impaired recruitment of ERα to its target genes and to a decreased estrogen-driven transcriptional activation in human breast cancer cells (110). This was supported by a recent study in which ERα protein levels diminished after SETD7 inhibition or knockdown due to ERα destabilization and consequent degradation (177). Thus, one can foresee a potential role for SETD7 as a key factor in ERα regulation in cancer. For that reason, future research must focus on achieving a better understanding of ERα regulation by SETD7 in cancer cells. SETD7 potential use as a cancer treatment's target should also be explored.

1.5.1.1.2.2. STAT3

STAT3 is also a SETD7 substrate, whose function as a transcriptional factor is inhibited by SETD7-dependent dimethylation at K140 (105, 130, 178). The STAT family of transcriptional factors is typically involved in biological processes like development, differentiation, immune response to pathogens and metabolism and is overexpressed in several diseases, including cancer. STAT3 is activated in the cytoplasm by JAK, becoming phosphorylated and prompted to translocate into the nucleus to transactivate anti-apoptotic (such as Mcl-1 and Bcl-xL), proliferative (like cyclin D1 and c-Myc) and inflammatory genes, like it is illustrated in figure 6 (179, 180). STAT3 can also induce apoptosis in functionally differentiated mammary epithelial cells upon weaning (181), but it was also found to be constitutively activated and to have a critical role in breast cancer

initiation (179, 182), as well as in other cancer cell lineages (including colon, gastric, lung, head and neck, skin and prostate)(179). Therefore, one possible approach for cancer treatment is to inhibit STAT3 (179, 180), which could be accomplished through SETD7 methylation (105, 130).

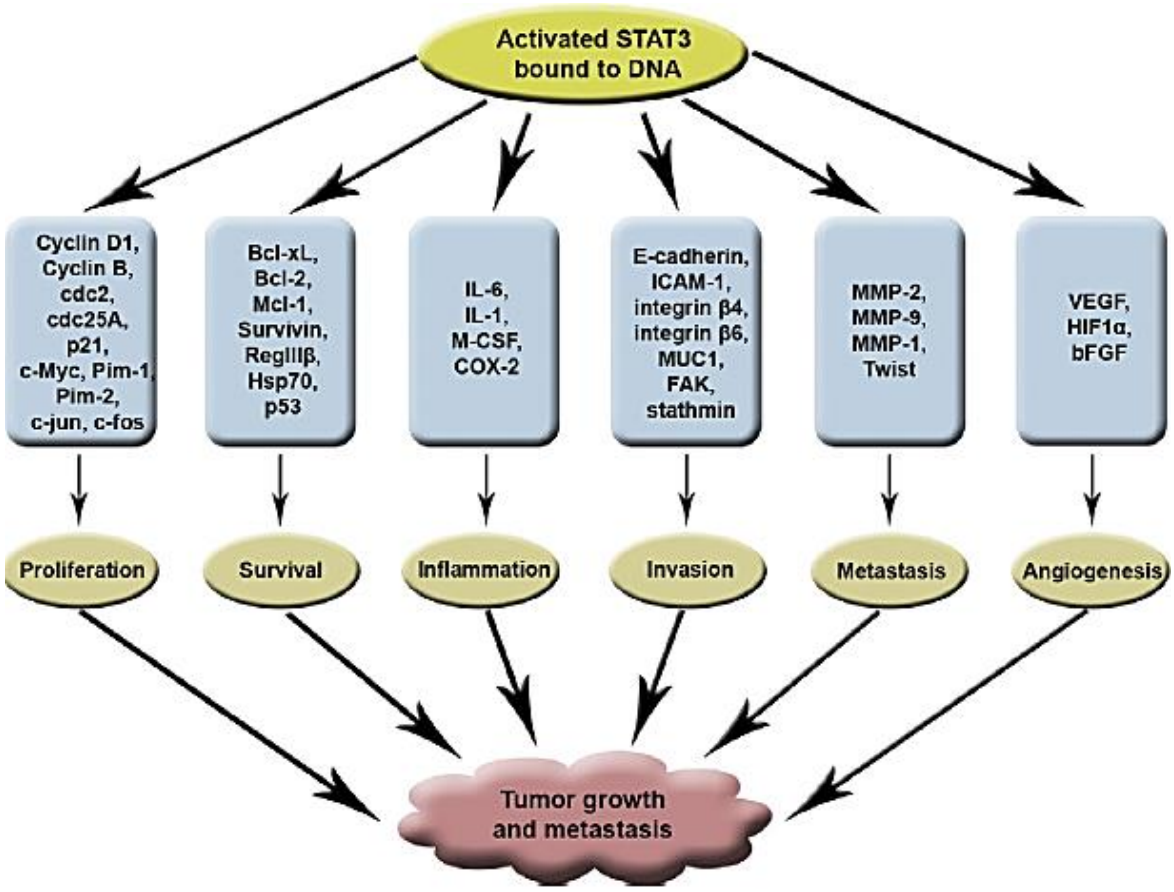

Figure 6 – STAT3 transcriptional targets and their effects during cancer development. Taken from (179).

1.6. Future perspectives

Nowadays, SETD7 is acknowledged as important for the regulation of different cellular processes. However, SETD7-driven methylation produces a great variety of results depending on the substrate methylated and possibly on the cellular context. We should also consider that most of the data presented here regarding SETD7 targets is qualitative and not quantitative. Therefore it would be interesting to explore if the methylation levels of each substrate by SETD7 is significant or not. Note that many of SETD7 substrates are inter-related, which means that though methylation of one of them, SETD7 may control several of its substrates. Moreover, while several SETD7 targets are master regulators of proliferation and differentiation, the physiological and pathological relevance of SETD7 is still unclear and should be investigated.

Regarding H3 histone, some studies (92, 98) show that H3K4 methylation by SETD7 may be more efficient at a peptide level than the methylation of some of the SETD7 non-histone subtracts (e.g.,

PCAF). The pointed reason for this variance is the SETD7 preference to methylate substrates that possess one of the following amino sequences: KSK, RSK and KAK (with K representing the target lysine). On the other hand, H3K4 methylation is a mark for transcriptional activation of genes involved in cellular differentiation and proliferation, thereby instigating our curiosity about SETD7's relevance in these processes.

In this work we intend to disclose SETD7's relevance in regulating proliferation of undifferentiated and differentiated mammary epithelial cells; underlining a possible role for SETD7 in breast cancer growth regulation.

2. Aims of the Study

SETD7 has been found to methylate numerous substrates, including histones and non-histone proteins, which are involved in various cellular processes, such as apoptosis, cell cycle, DNA repair, gene expression and cell differentiation and proliferation. This highlights SETD7 role as a putative regulator of these processes. In fact, recent studies have started to focus not only on SETD7-mediated regulation of its various substrates but also on SETD7 importance to the different cellular mechanisms in which these substrates are involved. However, SETD7 role in mammary cell proliferation and differentiation is not established.

Thus, the central aim of this work is to analyze if SETD7 affects proliferation of mammary epithelial cells in different stages of differentiation.

The specific aims are:

- To evaluate SETD7 expression through cell differentiation
- To establish a correlation between SETD7 protein levels and expression of proteins known to regulate proliferation (ERalpha and STAT3), apoptosis (STAT3) and differentiation (HOXB2).
- To characterize SETD7 subcellular localization and expression upon MAPKK/MEK inhibition.
- To study if SETD7 has a role in mammary epithelial cell proliferation.
- To study the effect of the SETD7 inhibitor (R)-PFI-2 over SETD7 expression.

3. Materials and Methods

In order to fulfil the objectives proposed above, the experimental design represented in Figure 7 was achieved.

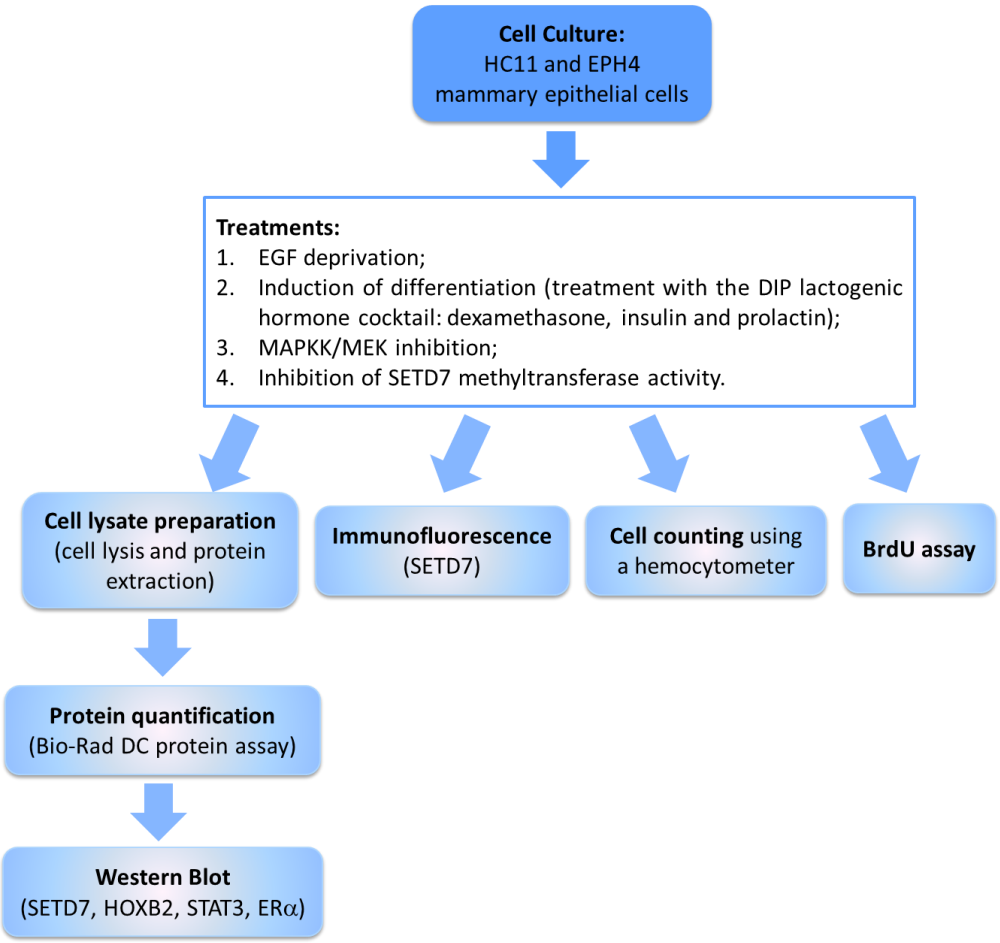

Figure 7 – Schematic representation of the methodology used throughout this project. EGF, Epidermal growth factor; ERα, Estrogen receptor α; HOXB2, Homeobox b2; MAPKK, Mitogen-activated protein kinase kinase; STAT3, Signal transducer and activator of transcription 3; SETD7, SET domain containing lysine methyltransferase 7.

3.1. Cell Culture

3.1.1. Cell lines

Throughout this project the HC11 cell line (ATCC CRL-3062, American Type Culture Collection, Manassas, VA, USA) was used. HC11 cells are non-tumorigenic murine mammary epithelial adherent cells widely used to study mammary epithelial cell differentiation as well as proliferation and signal transduction. HC11 cells routinely maintained in an undifferentiated stem cell-like state by culturing with high FBS concentration and 10 ng/mL EGF. HC11 are made competent for further differentiation by lowering FBS concentration and withdrawal of EGF. HC11 are functionally differentiated by incubation with lactogenic hormones, such as dexamethasone,

insulin and prolactin. These three hormones together form the DIP lactogenic differentiation cocktail (or DIP medium), normally used to induce differentiation of mammary epithelial cells (183, 184).

To confirm our findings (in the HC11 cell line), we also used another mammary epithelial cell line known as EPH4 (ATCC CRL-3063), exposing them to the same experimental conditions as the HC11 cells. EPH4 cells are mammary epithelial adherent cells derived from the IM-2 cell line, which was originally isolated from the mammary gland of a pregnant BALB/c mouse (185, 186), alike the HC11 cell line, that is a clonal derivative of COMMA-1D cells. Furthermore, also alike HC11 cells, EPH4 cells are responsive to lactogenic hormones, undergoing differentiation when exposed to DIP medium (184).

3.1.2. Cell culture

HC11 and EPH4 cells were grown at 37ºC (humidified atmosphere, 5% carbon dioxide) in T75 cell culture flasks in complete growth medium containing RPMI 1640 medium (PAA Laboratories, Pasching, Austria) supplemented with 10% fetal bovine serum (FBS) (Gibco by Life Technologies, CA, USA), 100 μg/mL gentamicin (Gibco), 10 ng/mL EGF and 5 μg/mL insulin medium (Sigma-Aldrich, St. Louis, MO, USA). The culture medium was renewed every 2-3 days. Cells were trypsinised once the culture reached about 80% confluence. Trypsinization was performed using 2-3mL trypsin-EDTA (1x, 0.05%/0.02% in PBS; PAA Laboratories) for approximately 4 minutes at 37ºC.

3.1.3. Treatments

3.1.3.1. Initial considerations

(R)-PFI-2 (kind gift from Dr. Peter J. Brown, SGC University of Toronto) is a selective and potent inhibitor of the methyltransferase activity of SETD7, inhibiting SETD7 *in vitro* with an IC50 value of about 2.0 nM. (R)-PFI-2 competes with the substrate and prevents its binding to SETD7 by occupying the substrate lysine-binding groove of SETD7, thereby, inhibits SETD7 catalytic activity. However, (R)-PFI-2 has a cofactor-dependent inhibitory mechanism of action, i.e., it only binds to SETD7 and inhibits SETD7 methyltransferase activity after the binding of its cofactor (SAM) to the enzyme (187).

U0126 (Tocris, Bristol, UK) is a selective, non-competitive inhibitor of MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) 1 and MEK2 with an IC50 of about 70 nM and 60 nM, respectively (188). U0126 inhibits phosphorylation and subsequent activation of extracellular signal-regulated kinase ERK1 and ERK2.

Both (R)-PFI-2 and U0126 were diluted in dimethyl sulfoxide (DMSO) and the same volume of DMSO was used as a vehicle control.

3.1.3.2. For Western Blot

In order to analyze protein effect of EGF, DIP, U0126 or (R)-PFI-2 on protein expression, cells were plated in 60- or 100-mm plastic culture dishes and allowed to grow to 60% confluence in complete growth medium. Next, this medium was removed and the cells attached to the culture dish were washed twice with sterile PBS (PBS tablets by Gibco). Next, the following treatments were added to the cells:

1. EGF deprivation: cells were incubated for 2h, 4h, 8h, 16h and 24h in RPMI 1640 medium supplemented with 2% FBS, 100 μg/mL gentamicin and 5 μg/mL insulin (from now on designated EGF-free medium), with (controls) or without 10 ng/mL EGF.
2. DIP medium (induction of differentiation): cells were incubated for 3h, 6h, 18h, 48h and 72h in EGF-free medium and 1 μg/mL prolactin and 100 nM dexamethasone. Simultaneously, untreated controls (i.e., cells that were incubated in the same conditions and medium, without adding prolactin or dexamethasone) were prepared, to which ethanol was added (at the same volume as dexamethasone), since dexamethasone is dissolved in ethanol (vehicle).
3. SETD7 methyltransferase activity inhibition: cells were incubated for 16h and 24h in EGF-free medium with or without adding 10 ng/mL EGF, and 8 nM of (R)-PFI-2 (inhibitor of SETD7 methyltransferase activity) or equal volume of DMSO were added to the cells.
4. MAPKK/MEK inhibition: cells were incubated for 16h in EGF-free medium with or without adding 10 ng/mL EGF, and 1 μM of U0126 (inhibitor of MAPKK/MEK) or equal volume of DMSO were added to the cells.

After the treatments, cell lysates were obtained according to the procedure described below.

3.1.3.3. For immunofluorescence

For immunofluorescence assay, cells were plated in 24-well plates and grown on sterile 18x18 mm glass microscope coverslides until they reached 70% confluence.

1. MAPKK/MEK inhibition: The cells were initially grown in 0.5 mL/well of complete growth medium. After cells reached 70% confluence, the plate was washed twice with sterile PBS and incubated in 0.5 mL/well of EGF-free medium for 24h. Thereafter, cells were treated with EGF-free medium with or without 10 ng/mL EGF and with or without 1 μM of U0126. The cells were incubated in these treatments for 16h. Then, they were washed twice with sterile PBS and fixed with 4% buffered formalin for 10 minutes at room temperature (RT). Following washes with PBS, cells were stored at 4ºC and stained (see the protocol for immunofluorescence staining described below) at the nearest suitable opportunity.

3.1.3.4. For cell counting

For the cell counting experimental procedure, about 2000 cells/well were plated in 24-well plates and treated according to the protocol described below:

1. EGF-deprivation and SETD7 methyltransferase activity inhibition: The cells were initially seeded for 24h in 0.5 mL/well of complete growth medium. After 24h, this medium was replaced with 0.5 mL/well of EGF-free medium, with or without 10 ng/mL EGF and with or without 1 nM, 8 nM and 10 nM of (R)-PFI-2 and the remaining 4 wells were treated with DMSO.
2. DIP medium and SETD7 methyltransferase activity inhibition: cells were initially seeded for 24h in 0.5 mL/well of EGF-free growth medium. After 24h, this medium was replaced with 0.5 mL/well of experimental medium: EGF-free medium or DIP medium with or without with or without 1 nM, 8 nM and 10 nM of (R)-PFI-2 (4 wells for each treatment).

The cells were incubated in the treatments described for 3 days and then counted (see cell counting assay protocol below).

3.1.3.5. For BrdU assay

For the BrdU assay, about 700 cells/well were plated in a 96-well plate and seeded in 100 μL /well of complete growth medium for 24h. Then, 200 μL/well of EGF-free medium and the following treatments were added (4 well for each treatment):

1. DMSO;
2. 8 nM of (R)-PFI-2;
3. 10 ng/mL EGF + DMSO;
4. 10 ng/mL EGF + 8 nM of (R)-PFI-2.

The cells were incubated in these treatments for 2 days and then renovated, adding 10 μM BrdU (Cell Proliferation ELISA, BrdU (colorimetric) kit, REF. 11 647 229 001, version 14, Roche, Mannheim, Germany) to each well.

3.2. Western Blot analysis protocol

3.2.1. Preparation of cell lysates

After removal of the culture medium, the cell monolayer was washed twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 1.8 mM $KHPO_4$ and 10 mM Na_2HPO_4, pH 7.4). Using a cell scrapper, cells were scraped off the culture dish and the resulting cell suspension was pipeted into a 1.5 mL microtube and placed on ice and later centrifuged at 14000 rpm for 1 minute. The supernatant was discarded and the resulting pellet stored at -70ºC. Thereafter, the pellets were placed on ice and then suspended in 100 μL per $1x10^6$ cells of RIPA lysis buffer (150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, 25 mM Tris–HCl pH 7.6) plus proteinase inhibitor cocktail (1:100), phosphatase inhibitor 2 cocktail (1:100), phosphatase inhibitor 3 cocktail (1:100) and 1M DTT (1:1000) (to lyse the cells and preserve the integrity of the samples). The resulting suspension was vortexed and incubated on ice for 10 minutes and then vortexed again and placed on ice for other 10 minutes. Next, the samples were centrifuged at 14000 rpm for 15 minutes at

4°C to pellet the cell debris. The supernatant was transferred to a new microtube and then stored at -70ºC.

3.2.2. Protein quantification

In a 96-well plate, the samples were diluted 1:4 with distilled water (dH_2O). In parallel, dilutions of bovine serum albumin (BSA) (used as a protein standard to obtain a standard curve) in dH_2O were prepared to obtain final concentrations of 0.3, 0.6, 1.25 and 2.5 mg/ml of BSA, using of a stock solution of 10 mg/mL of BSA (Sigma). From the resulting solutions (samples and BSA diluted in dH_2O), 5 µL were pipetted to two clean wells (duplicates). Also, 2 wells were prepared with 5 µL of dH_2O (blank standard). Then, 25 µL of Bio-Rad DC protein assay reagent A + reagent S (20 µL of reagent S for each 1mL of reagent A) and 20 µL of reagent B were added to each well. After incubating the plate for 15 minutes at RT, absorbance was read at 750 nm. Next, a standard curve was obtained using the absorbance values of the standards and the protein concentration of each sample was determined.

3.2.3. Preparation of samples for Western blot analysis

Using the protein concentration values obtained, the volume of each sample required to load an equal amount of protein into the wells of the gel was calculated and then mixed with Laemmli buffer 5x (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue and 16% β-mercaptoethanol) at a ratio of 1:4 (or if 2x at a ratio of 1:1). The protein samples were always kept on ice throughout this process. After short spin, the protein samples were denaturated at 100°C for 5 minutes and then placed on ice.

3.2.4. Protein separation by SDS-PAGE, blotting and detection

The proteins were separated according to their size by Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresis was performed at a continuous voltage of 200V using the Bio-Rad Mini-Protean 3 Electrophoresis System, in running buffer (192 mM glycine, 25 mM Tris, 3.5 mM SDS). Then, proteins were transferred from the gel to the Immobilon-P polyvinylidene fluoride (PVDF) transfer membrane (previously activated in methanol) with the Trans-Blot Turbo System (Biorad; 30 minutes transfer at 25 V and 1.0 A) in transfer buffer (192 mM glycine, 25 mM Tris, 20% methanol). After the transfer, membranes were stained with Ponceau S to verify if the transfer was performed correctly. The membranes were washed several times with dH_2O until the bands were no longer visible and then 5 minutes with Tween-TBS (20mM Tris, 150 mM NaCl, pH 7.6 and 0.05% Tween-20) before incubating the membranes with blocking solution (5% dry milk in Tween-TBS) for at least 1 hour. The membranes were then incubated with the primary antibody [anti-SETD7 (ab14820, Abcam; dilution: 1/1500), anti-HOXB2 (ab86386, Abcam; dilution: 1/1000), anti-STAT3 (sc-482, Santa Cruz Biotechnology; dilution:

1/300), anti-ERα (sc-542, Santa Cruz Biotechnology; dilution: 1/300)] in Tween-TBS for at least 2 hours. After incubation with the primary antibody and washing the membranes three times with Tween-TBS for 10 minutes, the membranes were incubated for 1 hour with the secondary antibody [anti-mouse and anti-rabbit IgG (Sigma, dilution: 1/5000)] diluted in Tween-TBS. Afterwards, the membranes were washed twice with Tween-TBS and once with TBS for 10 minutes. Detection was performed using WesternBright ECL HRP substrate and LucentBlue X-Ray films (Advansta, CA, USA).

To reassure that equal amounts of protein were loaded into the wells we used alpha-tubulin as a housekeeping protein reference that would later serve to normalize the results obtained for the target protein. Membranes were stripped in mild stripping buffer (200 mM glycine, 3.5 mM SDS and 1% Tween-20, pH 2.2) for 10 minutes (twice) and washed twice with PBS for 10 minutes and then twice with Tween-TBS for 5 minutes. Afterwards the same protocol as the described above was used (starting from the blocking step), using anti-alpha tubulin (NB600-506, Novus Biologicals, CO, USA; dilution: 1/5000) as the primary antibody and anti-rat IgG (Sigma, dilution: 1/5000) as the secondary antibody.

Lastly, the X-ray films were digitalized and the relative intensity of the bands was determined by densitometric analysis performed using Quantity One Basic Software version 4.6.6 (Bio-Rad).

3.3. Immunofluorescence analysis protocol

After fixation and storage at 4ºC in PBS (as described), the cells were washed twice in PBS (0.5 mL/well) for 5 minutes and permeabilized with 0.5% Triton-X-100 in PBS (250 μL/well) for 10 minutes. Then, the cells were washed three times with PBS (1 mL/well) for 5 minutes and incubated for 30 minutes in blocking solution (0.01% tween and 15% FBS in PBS, 250 μL/well). Afterwards, cells were incubated overnight with the primary antibody in PBS [anti-SETD7 (ab14820, Abcam; dilution: 1/300), 150 μL/well]. The next day, the cells were washed three times for 5 minutes in PBS (1 mL/well), incubated for 30 minutes to 1 hour with the secondary antibody [anti-mouse IgG, Alexa fluor 568, Life Technologies, CA, USA (dilution: 1/300)] diluted in 150 μL/well of blocking solution and then washed again with PBS (1 mL/well), three times for 5 minutes. To counterstain the nuclei, 0.1 μg/mL DAPI (Sigma) was added. After an incubation of 10 minutes with DAPI, the cells were washed in PBS for 10 minutes and the coverslides were mounted onto microscope slides with Prolong Gold anti-fade reagent (Life Technologies). The slides were then analyzed under an inverted microscope (Nikon, Eclipse Ti-U) and the images acquired using the same settings with the NIS-Elements D imaging software. The fluorescence intensity was also measured with NIS-Elements D software. Subsequently, the images where equally edited with Adobe Photoshop CS6, applying the same adjustments to all images.

3.4. Cell counting protocol

The experimental medium was removed and the cells washed with 250 μL/well of sterile PBS. The cells were incubated in 250 μL/well of trypsin-EDTA for 4 minutes (or until completely detached from the plate) at 37ºC. Then, 10 μL of FBS was added to each well and the cells counted using a Neubauer improved counting chamber under a phase contrast microscope. Each treatment was carried out in quadruplicates. Experiments were repeated at least twice.

3.5. BrdU labelling and detection protocol

For this assay the Cell Proliferation ELISA, BrdU (colorimetric) kit (REF. 11 647 229 001, Roche) was used. As mentioned, after 2 days, the treatments were renewed and the cells simulated with 10 μM BrdU and incubated overnight in this labelling medium. Then, 100 μL of FixDenat (fixation solution) were added to each well. After an incubation of 30 minutes at RT, this solution was removed and the cells incubated for 90 minutes with 70 μL/well anti-BrdU-POD working solution (dilution: 1:100) at RT. Afterwards, the wells were washed three times for 5 minutes with 200 μL/well PBS 1X and then 70 μL/well of substrate solution were added. The cells were incubated in this solution from 5 to 20 minutes and the absorbance was measured at 370 nm (and 492 nm – reference wavelength). Values used were in the linear range. Each treatment was carried out in quadruplicates.

3.6. Statistical analysis

Data was analyzed using GraphPad software. Differences between control and treatments was analyzed using One-Way ANOVA and Dunnet´s post-test. Differences between two groups were analyzed with Student´s t test. Differences were considered significant if $p < 0.05$.

4. Results

Previously, we found that when HC11 cells enter the differentiation program (48h of EGF withdrawal) SETD7 mRNA is upregulated and continues to be high in functionally differentiated cells (72h treatment with DIP) (189). Therefore, in this section, we aimed to establish at which time point after EGF depletion SETD7 protein is increased.

4.1. SETD7 protein levels increase in response to EGF deprivation

In order to study SETD7 expression in undifferentiated mammary epithelial cells, we used HC11 cells grown with EGF and depleted of EGF for the indicated time points.

At the early time points (2, 4 and 8 hours) no significant changes could be observed in SETD7 levels (figure 8). However, SETD7 expression increased after 16h of EGF removal and this effect was maintained for the 24h time point, with an increase of 21% and 13% in SETD7 expression, respectively.

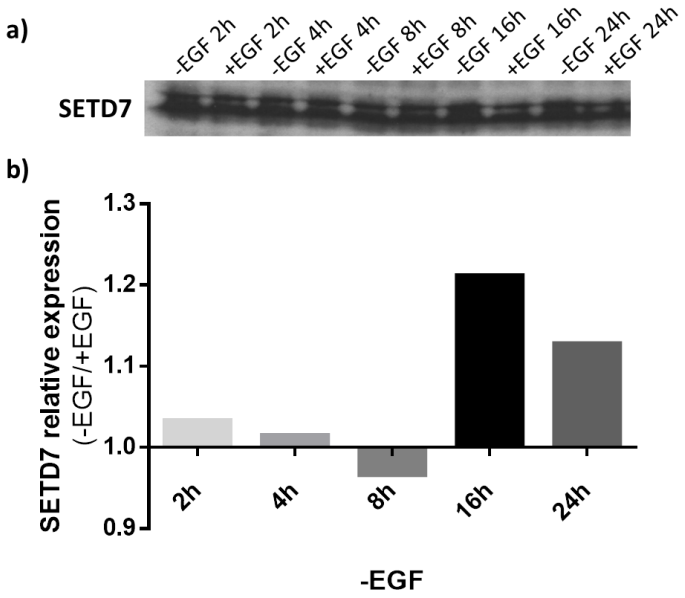

Figure 8 – Effect of EGF on SETD7 expression. HC11 cells were depleted of EGF for the indicated time intervals. Afterwards, SETD7 protein levels were analyzed by Western Blot. (**a**) SETD7 expression throughout the different treatment periods with and without EGF. (**b**) Relative quantification of the intensity of the bands in the blot. For each treatment period, -EGF intensity values were related to those obtained for proliferating cells (+EGF controls), which were set to one.

4.2. SETD7 protein levels increase upon MAPK inhibition

The objective of this experiment was to study if the MAPK pathway (that can be activated by EGF and linked to cell growth and proliferation (190)) is responsible for SETD7 downregulation observed previously in undifferentiated mammary epithelial cells. Therefore, we compared SETD7

protein levels in HC11 cells co-treated with EGF-containing medium (EGF-free medium + 10 ng/mL EGF) and 1 μM U0126 for 16h.

As it is shown in the blot (and respective relative quantification) on figure 9a and 9b, we found that SETD7 slightly increases (by about 17%) upon MAPK inhibition in undifferentiated HC11 cells (+EGF). This was corroborated by immunofluorescence (figure 9c,d). In fact, upon treatment with U0126, we can observe a significant increase in SETD7 expression in HC11 cells treated either with +EGF or -EGF medium, in comparison to controls (not treated with U0126). Furthermore, U0126 reverses EGF negative effect on SETD7 protein expression.

SETD7 localization is also evident on figure 9d. Indeed, independently of the treatment that the cells were subjected to, SETD7 localizes predominantly in the nucleus but can also be found in the cytosol (figure 9d). Furthermore, the intensity of SETD7 cytoplasmic fluorescence seems to be higher in the undifferentiated cells (+EGF) treated with U0126 than controls.

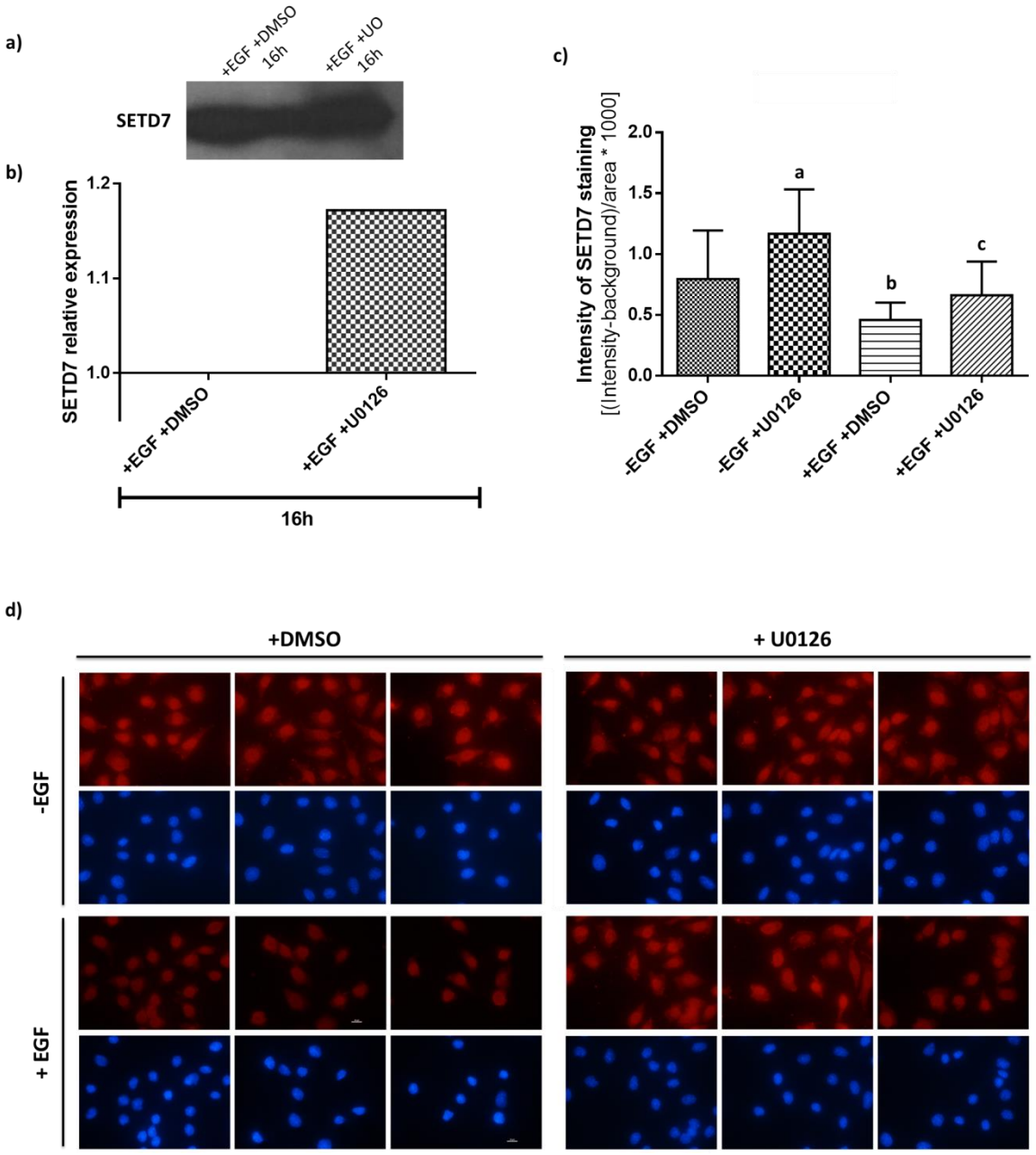

Figure 9 – U0126 effect on SETD7 expression. (**a**) and (**b**) HC11 cells were co-treated with EGF-free medium + 10 ng/mL EGF (+EGF) and 1 μM U0126 (or DMSO – control) for 16h. Afterwards, SETD7 protein levels were analyzed by Western Blot. (**a**) SETD7 expression in undifferentiated cells treated with U0126. (**b**) Relative quantification of the intensity of the bands in the blot (the +DMSO/-U0126 control was set to one). For the immunofluorescence assay [(**c**) and (**d**)], HC11 cells were co-treated with EGF-free medium or +EGF medium and 1 μM of U0126 and incubated in this medium for 16h before fixation. DMSO was used as a vehicle control. SETD7 staining was then analyzed. (**c**) Fluorescence intensity of SETD7 staining. Mean ± SD of the values obtained is shown. a and b, $p < 0.0001$ *vs.* -EGF +DMSO; c, $p < 0.05$ *vs.* +EGF +DMSO. (**d**) Analysis of SETD7 expression in HC11 cells by immunofluorescence. In blue are the cell nuclei stained with DAPI and in red is SETD7 staining. Representative of one experiment.

4.3. SETD7 activity inhibition by (R)-PFI-2 increases cell number

As previously stated we found that SETD7 protein levels increase upon removal of EGF which is an important factor needed for the proliferation and survival of HC11 cells (191). This suggests that SETD7 may negatively influence cell proliferation. In order to further characterize how SETD7 influences cell proliferation, we analyzed if its inhibition by (R)-PFI-2 would be translated in an increase in cell number of undifferentiated HC11 and EPH4 cells. For this, cells were treated with EGF-free medium and different concentrations of (R)-PFI-2 (1 nM, 8 nM or 10 nM) or DMSO. Controls to assess the response of the cells included EGF-free medium plus 10 ng/mL EGF (figure 10).

In cells treated with either +EGF or –EGF medium, cell number increased following SETD7 inhibition (figure 10). Moreover, although the number of cells is significantly lower (by 30% and 27% in HC11 and EPH4, respectively) in untreated cells incubated in –EGF medium (-EGF+DMSO) in comparison to cells incubated in +EGF medium (+EGF+DMSO), this is reversed upon SETD7 inhibition by 8 nM and 10 nM (R)-PFI-2, resulting in cell number values similar to +EGF treated cells (40% and 25% increments in HC11 and EPH4 cells, respectively). Thus, SETD7 methyltransferase activity seems to be necessary for cell transition from a highly proliferative state to low proliferative state. It is important to notice that similar results were obtained in both HC11 (figure 10a) and EPH4 cells (figure 10b) which indicates that the effect that we are observing is reproducible in more than one experimental model, and therefore biologically relevant.

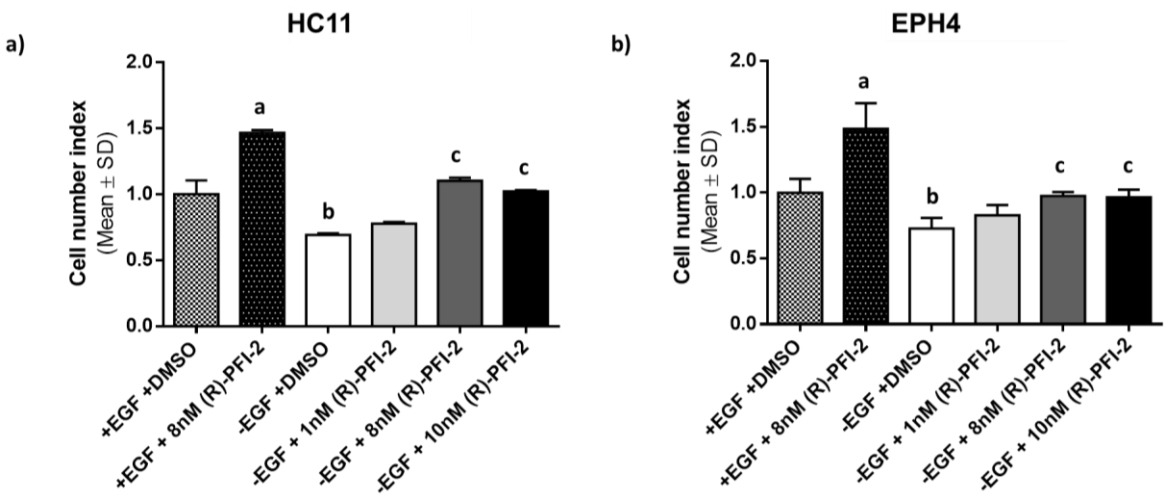

Figure 10 – HC11 and EPH4 cell counting assay. Cells were treated with EGF-free medium, with or without 10 ng/mL EGF, and different concentrations of (R)-PFI-2 (1 nM, 8 nM and 10 nM). After 3 days, cells were counted in a Neubauer improved counting chamber and the relative cell number variation was calculated by setting untreated +EGF control values to 1. Mean ± SD from one experiment carried out in quadruplicates is shown for (**a**) HC11 (a and b, $p < 0.0001$ *vs.* +EGF +DMSO; c, $p < 0.0001$ *vs.* –EGF +DMSO and no significant differences *vs.* +EGF +DMSO) and (**b**) EPH4 (a, $p < 0.0001$ *vs.* +EGF +DMSO; b, $p < 0.05$ *vs.* +EGF +DMSO; c, $p < 0.05$ *vs.* –EGF +DMSO and no significant differences *vs.* +EGF +DMSO) cells. Both graphs are representative of 2 independent experiments.

4.4. Cell proliferation is not significantly affected by (R)-PFI-2

In order to determine whether the later results concerning (R)-PFI-2 effect on cell number in undifferentiated and competent cells (figure 11) were caused by an increase in cell proliferation, a BrdU assay was then conducted. The assay was conducted using the same mammary epithelial cell lines (HC11 and EPH4) as the cell counting assay (figure 11). No significant differences in BrdU incorporation could be observed between cells treated with (R)-PFI-2 and controls (figure 11). This applies to both proliferating and non-proliferating cells from both cell lines. In other words, according to our results (R)-PFI-2 had no significant effect on cell proliferation in both HC11 (figure 11a) and EPH4 (figure 11b) cell lines. Furthermore, these results suggest that the augment in cell number previously reported for cells treated with (R)-PFI-2 did not result from an increase in cell proliferation.

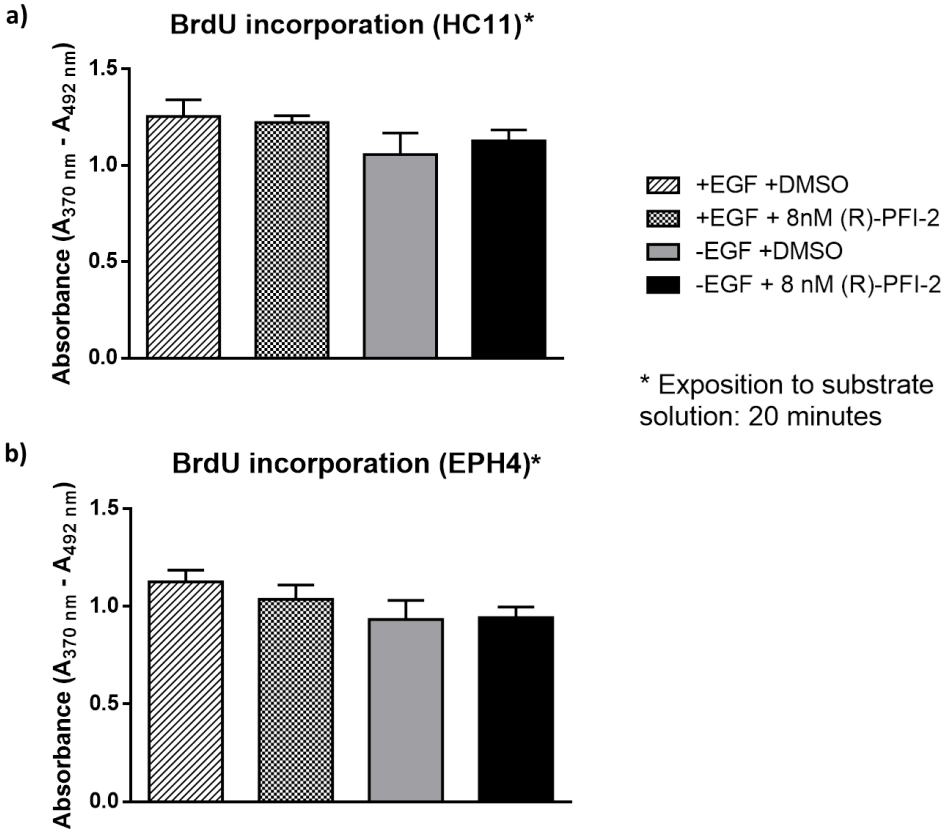

Figure 11 – BrdU labeling of HC11 and EPH4 cells. HC11 and EPH4 cells were cultured in EGF-free medium, with or without 10 ng/mL EGF, and 8 nM of (R)-PFI-2 or DMSO (vehicle control) for 2 days. Then, these experimental media were renewed and supplemented with 10 μM BrdU. Cells were incubated overnight in these media, proceeding then with the Brdu assay. Absorbance was measured at 370 nm and 492 nm (reference wavelength). Mean ± SD from one experiment carried out in quadruplicates is shown for (**a**) HC11 and (**b**) EPH4 cells. No significant differences were found between treatments for either experiment. Both graphs are representative of 2 independent experiments.

4.5. SETD7 levels increase upon inhibition of its methyltransferase activity by (R)-PFI-2

In this project we also tested the impact of (R)-PFI-2 on SETD7 expression. For this purpose, HC11 cells were co-treated with or without EGF and 8 nM of (R)-PFI-2. We found that, although (R)-PFI-2 is an inhibitor of SETD7 methyltransferase activity, when we add (R)-PFI-2 to the cells for either 16h or 24h the quantity of SETD7 increases (figure 12). In fact, SETD7 protein levels increase about 28% and 21% in cells co-treated with or without EGF and (R)-PFI-2 for 16h, respectively, and about 19% in –EGF+(R)-PFI-2 treated cells for 24h.

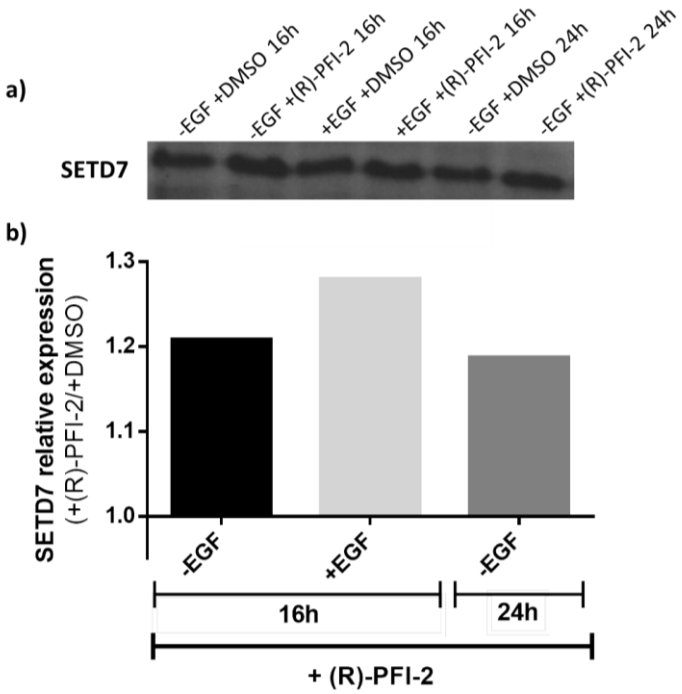

Figure 12 – (R)-PFI-2 effect on SETD7 expression. (**a**) and (**b**) HC11 cells were co-treated with or without EGF and 8 nM (R)-PFI-2 (or DMSO – vehicle control) for 16h and 24h. Afterwards, SETD7 protein levels were analyzed by Western Blot. (**a**) SETD7 expression in proliferative (+EGF) and less-proliferative (-EGF) cells treated with (R)-PFI-2 for 16h and 24h. (**b**) Relative quantification of the intensity of the bands in the blot. Intensity values obtained for cells treated with (R)-PFI-2 were related to those obtained for untreated cells, which were set to one. Representative of 2 experiments.

4.6. ERα, STAT3 and HOXB2 expression in response to EGF – correlation to SETD7 protein levels

Next, we investigated if expression of two of SETD7's non-histone substrates that are known to induce proliferation (ERα (110, 192, 193) and STAT3 (35, 36, 178)), apoptosis (STAT3) (181) and one involved in differentiation (HOXB2) (194) were associated to SETD7 protein induction following EGF removal. Accordingly, the cellular lysates obtained for the western blot analysis of SETD7 expression were used to study ERα, STAT3 and HOXB2 protein levels.

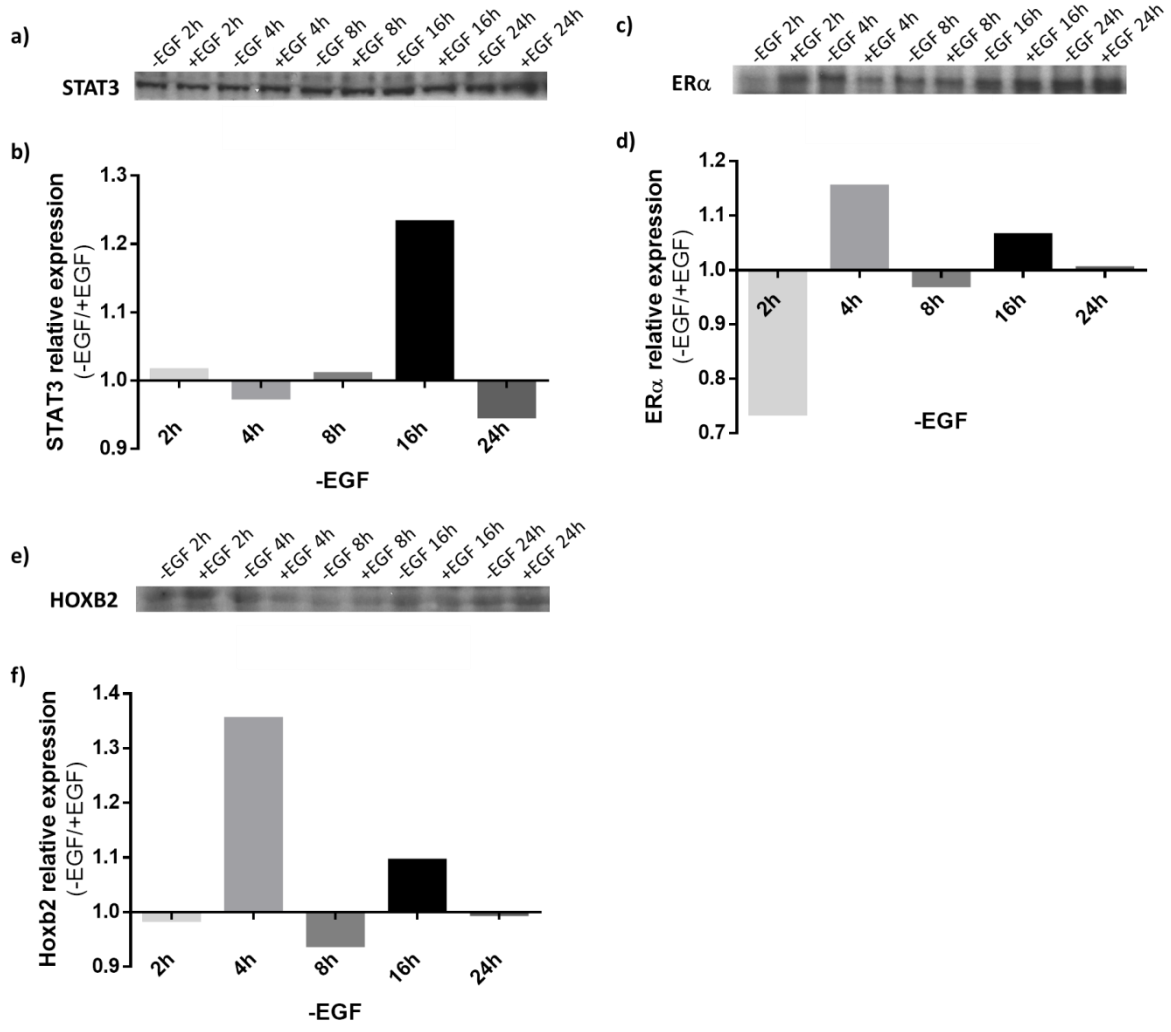

Figure 13 – Effect of EGF on STAT3, ERα and HOXB2 expression. HC11 cells were stimulated with or without EGF for the indicated time intervals. Afterwards, STAT3, ERα and HOXB2 protein levels were analyzed by Western Blot. (**a**), (**c**) and (**e**) show STAT3, ERα and HOXB2 expression (respectively) throughout the different treatment periods with EGF-free medium in comparison to +EGF controls. (**b**), (**d**) and (**f**) show the relative quantification of the intensity of the bands in the corresponding blot. For each treatment period, -EGF intensity values were related to those obtained for proliferating cells, which were set to one.

As it can be observed on figure 13a and 13b, STAT3 protein levels are fairly maintained when we remove EGF from the medium for 2h, 4h and 8h. However, as it happens with SETD7, STAT3

protein levels rise 23% after an incubation of 16h with EGF-free medium. Therefore, we found an association between SETD7 and STAT3 protein increase following EGF depletion. However, this effect is not maintained after 24h. On the other hand, EGF seems to have a strong short-term positive effect on ERα protein levels (as it can be seen on figure 13c and 13d at the 2h mark), and no significant differences can be detected in ERα expression in HC11 cells treated for 8h, 16h and 24h with or without EGF. Consequently, we did not find an association between ERα and SETD7 protein increase. Finally, HOXB2 levels vary through the different incubation periods (figure 13e and 13f), being higher in cells that were deprived from EGF for 4h and 16h (increases of 35% and 9,5%, respectively) but slightly lower (about 6%) at the 8h incubation period in comparison to the values obtained for cells treated with EGF. Therefore, although we observed an association between HOXB2 and SETD7 increase after 16h EGF depletion, there are clearly other factors modulating HOXB2 protein.

Thereafter, we also studied STAT3 protein levels upon inhibition of SETD7 methyltransferase activity by (R)-PFI-2. HC11 cells were treated with EGF-free medium and 8 nM of (R)-PFI-2 or DMSO. Controls consisted of cells also treated with EGF-free medium and 8 nM of (R)-PFI-2 or DMSO, adding 10 ng/mL EGF.

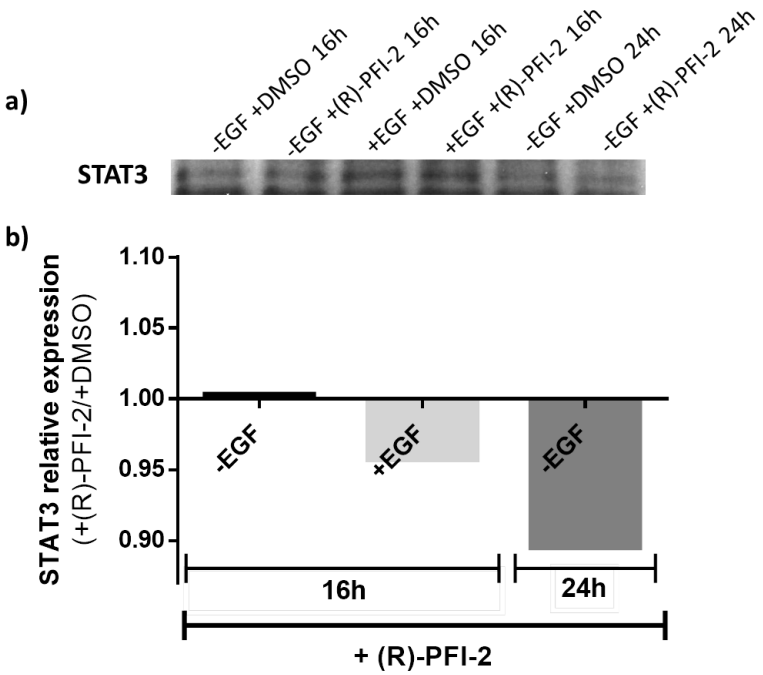

Figure 14 – (R)-PFI-2 effect on STAT3 expression. (**a**) and (**b**) HC11 cells were co-treated with or without EGF and 8 nM (R)-PFI-2 (or DMSO – vehicle control) for 16h and 24h. Afterwards, STAT3 protein levels were analyzed by Western Blot. (**a**) STAT3 expression in proliferative and competent (lower proliferation) cells treated with (R)-PFI-2 for 16h and 24h. (**b**) Relative quantification of the intensity of the bands in the blot. Intensity values obtained for cells treated with (R)-PFI-2 were related to those obtained for untreated cells, which were set to one.

As it is shown on figure 14, STAT3 protein levels did not suffer a significant change in HC11 cells treated either with or without EGF and (R)-PFI-2 for 16h; therefore, it seems that SETD7 methyltransferase activity is not necessary for STAT3 increase and stability at 16h.

4.7. SETD7 is regulated by lactogenic stimuli

The main objective of this project was to test if SETD7 protein levels are regulated in functionally differentiated cells. Therefore, we analyzed SETD7 expression in HC11 cells that were exposed to a lactogenic hormone cocktail of dexamethasone, insulin and prolactin (i.e., DIP medium) to induce differentiation. Controls were treated with EGF-free medium.

Following DIP-treatment for 6h, there was a marked increase (of 45%) in SETD7 protein levels (figure 15). However, before (i.e., in cells treated with DIP medium for 3h) and in the following hours, no significant differences can be observed in SETD7 protein levels in comparison to controls.

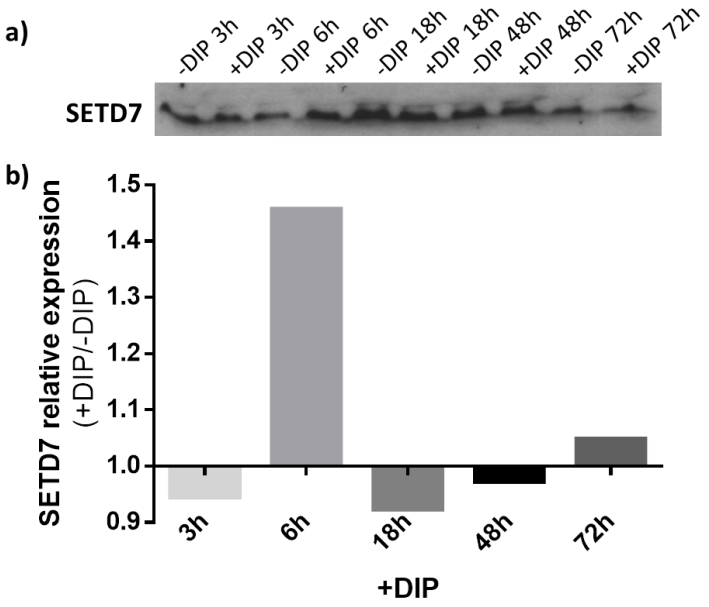

Figure 15 – Effect of lactogenic stimuli on SETD7 expression. HC11 cells were stimulated with DIP medium (+DIP) for the indicated time intervals. Controls were treated with EGF-free medium (-DIP). Afterwards, SETD7 protein levels were analyzed by Western Blot. (**a**) SETD7 expression throughout the different treatment periods with DIP medium in comparison to –DIP controls. (**b**) Relative quantification of the intensity of the bands in the blot. For each treatment period, +DIP intensity values were related to those obtained for cells treated with –DIP medium, which were set to one.

4.8. (R)-PFI-2 is capable of reversing the negative effect of lactogenic hormones on cell number

Subsequently, we studied how (R)-PFI-2 affects cell number in functionally differentiated HC11 cells, which do not normally proliferate. Here, we compared (R)-PFI-2 effect on cell number upon treatment with EGF-free medium and DIP medium. In this case, untreated cells that were incubated in EGF-free medium (-EGF+DMSO) were used as controls (figure 16). In this experiment, cell number was increased by about 70% when cells were treated with 8 nM of (R)-PFI-2 in DIP medium compared to cells only treated with DIP medium and DMSO, which indicates that (R)-PFI-2 is also capable of reversing DIP-mediated cell differentiation and that if SETD7 is inhibited in this cellular stage it can result in aberrant proliferation.

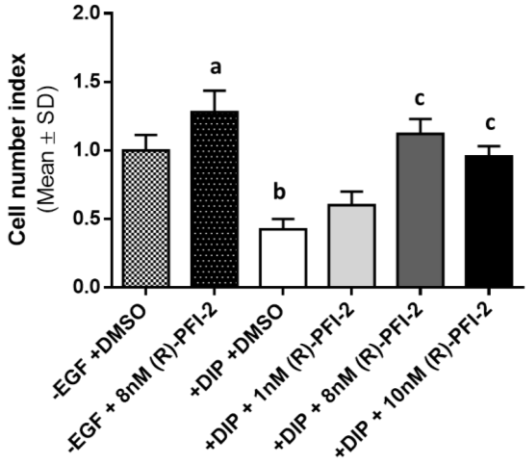

Figure 16 – HC11 cell counting assay. Cells were treated with EGF-free or DIP medium and different concentrations of (R)-PFI-2 (1 nM, 8 nM and 10 nM). After 3 days, cells were counted in a Neubauer improved counting chamber and the relative cell number variation was calculated by setting untreated -EGF control values to 1. Mean ± SD from one experiment carried out in quadruplicates is shown. a, $p < 0.05$ *vs.* –EGF +DMSO; b, $p < 0.0001$ *vs.* –EGF +DMSO; c, $p < 0.0001$ *vs.* +DIP +DMSO and no significant differences *vs.* -EGF +DMSO. Representative of 2 experiments.

4.9. STAT3 and HOXB2 regulation by lactogenic hormones

In order to further explore an association between STAT3 and HOXB2 protein levels and SETD7, STAT3 and HOXB2 were analyzed by western blot (figure 17) using the same cellular lysates that were obtained for the analysis of SETD7 expression upon exposition to lactogenic hormones.

Alike SETD7, STAT3 is increased (by 48%) in cells treated with DIP medium for 6h (figure 17a and 17b). The lactogenic cocktail also seems to positively regulate STAT3 after 72h (which then suffers an increase of 14.5%). During the remaining treatment periods, we did not observe a significant change in STAT3 protein levels. Consequently, we observe an association between STAT3 and SETD7 protein levels.

On the other hand, although HOXB2 protein levels are 32% lower in the extracts treated with DIP medium for 3h, but it increases after 18h and up to 72h exposure to lactogenic hormones (figure 17c and 17d). Therefore, we did not observe any association between HOXB2 and SETD7 protein levels.

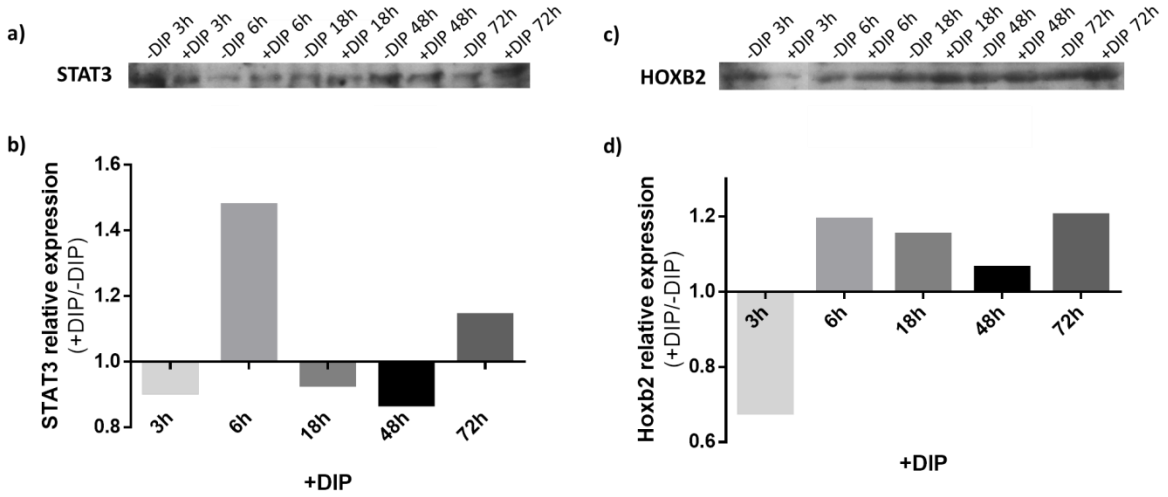

Figure 17 – Effect of lactogenic stimuli on STAT3 and HOXB2 protein levels. HC11 cells were stimulated with DIP medium (+DIP) for the indicated time intervals. Cells treated with EGF-free medium (-DIP) were used as controls. Afterwards, STAT3 and HOXB2 protein levels were analyzed by Western Blot. (**a**) and (**c**) show STAT3 and HOXB2 expression (respectively) throughout the different treatment periods with DIP medium in comparison to –DIP controls. (**b**) and (**d**) show the relative quantification of the intensity of the bands in the corresponding blot. For each treatment period, +DIP intensity values were related to those obtained for cells treated with –DIP medium, which were set to one.

5. Discussion

Cellular differentiation is an essential biologic process by which cells become competent and commit to a specific lineage, being important not only for embryonic development but also during adult life (1). For example, cellular differentiation is crucial during pregnancy for the terminal differentiation and the formation of alveolar structures necessary for the production and delivery of milk during lactation (13, 23, 24). Furthermore, de-regulation of differentiation is commonly found in cancer (2, 195, 196).

Recent studies have demonstrated that differentiation is deeply regulated by chromatin remodeling and histone post-translational modifications (1). Specifically, H3K4 methylation has been associated with gene transcription (functioning as a distinctive signal for the recruitment of specific transcription factors) and cell differentiation (3-7). Moreover, over the years increasing evidence suggests that SETD7 (a H3K4 methylase) may be involved in cell differentiation through the methylation of H3K4 or other of its substrates (12, 139). For that reason we decided to study SETD7 expression in proliferation and differentiation of mammary gland epithelial cells, with focus on protein targets that are transcription factors and regulate these biological processes. For this purpose, we used a well-established model of mammary epithelial cell differentiation: HC11 cell line (183, 184). These cells are routinely grown with EGF to keep them in an undifferentiated/highly proliferative state and are induced to differentiate by depletion of EGF followed by addition of a lactogenic stimulus.

Before we go on with the discussion of our findings, it is important to notice a small problem that we had in some of our western blot assays. During this project we used alpha-tubulin as a housekeeping protein reference to normalize the results obtained for the target protein. However, it was not possible to obtain good results for tubulin due to flaws during the stripping step (the mild stripping solution did not work). Nevertheless, before each western blot we quantified the total protein amount in each sample in order to assure that equal amounts of protein were loaded into the wells of the stacking gel. Moreover, increasing evidence shows that the housekeeping protein used nowadays are susceptible to suffer variations depending on the tissue, experimental conditions and treatment being tested and may even prejudice the accuracy of the analysis taking place (197, 198). Having that in mind, although it is not the ideal situation, we believe it is not indispensable to use these housekeeping proteins for our analysis. Therefore, when the data concerning the amount of tubulin present in the loaded samples was unavailable we assumed it to be equal for all wells.

EGF is a growth factor well-known for promoting cell growth, survival and proliferation and for its involvement in breast cancer development and metastasis (35, 190, 199). In fact, HC11 cells are dependent on EGF and insulin to proliferate and survive (191). Hence, in order to investigate if there is any biological association between SETD7 and mammary epithelial cells proliferation we examined how EGF influences SETD7 expression. SETD7 protein expression was not immediately induced after removal of EGF. Nevertheless, our findings show that SETD7 protein levels increase after 16h of EGF depletion and are maintained for 24h. Therefore, we can associate SETD7 expression to a diminished cell number. In fact, in a subsequent experiment, both EPH4 and HC11 cells exhibited a clear increase in the number of cells after the inhibition of SETD7 activity by (R)-PFI-2, whether they were co-treated with or without EGF. Cell number is the result of two

biological processes: proliferation and apoptosis. We did not observe any significant difference in proliferation in cells treated with (R)-PFI-2, therefore, the increase in cell number is possibly a result of reduced apoptosis. Thus, our study suggests that SETD7 promotes apoptosis, supporting the results of a study by Hu *et al.* (2013) in which SETD7 was associated with the induction of apoptosis of U266 multiple myeloma cells in response to treatment with berberine (171). The methylation of pRb at K873 by SETD7, which is necessary for pRb normal function, including inhibition of cell entry into S-phase of the cell-cycle and stimulation of apoptosis by interacting and controlling E2F1 (129), can also explain the observed cell number increase. In addition, E2F1 is also methylated by SETD7 at K185 which enhances E2F1-mediated apoptosis (113). Our results are also in agreement with the inhibition of STAT3 by SETD7 (178), since STAT3 is a transcription factor activated downstream of EGF that positively regulates anti-apoptotic and proliferative genes (35, 36, 179, 180). In fact, we also showed that there is a correlation between the expression of STAT3 and SETD7 after EGF depletion. However, (R)-PFI-2 had no significant effect over STAT3 protein levels.

On the other hand, SETD7 was also reported to have no effect over the apoptosis rates of human hepatocarcinoma cells (61), which does not corroborate our hypothesis. In addition, SETD7 role in cell proliferation is also controversial. Opposing evidence shows that SETD7 can either promote (61, 172) or inhibit (62, 63, 139) cell proliferation. Furthermore, ERα is methylated and stabilized by SETD7 (110), which could indicate that SETD7 positively regulates cell proliferation. In fact, SETD7 knockdown or inhibition by cyproheptadine reduces the amount of ERα in MCF7 cells (but not its mRNA expression levels) due to ERα destabilization and subsequent degradation (177). Nevertheless, we did not find an association between ERα and SETD7 protein increase.

EGF acts on a receptor tyrosine kinase, EGFR, which then activates multiple intracellular signaling pathways involved in cell survival and proliferation, including the PI3K/AKT, JAK/STAT, MAPK pathways (35, 36, 190). In order to verify if the observed negative regulation of SETD7 by EGF is mediated by the MAPK pathway we decided to study the effect of the MEK1/2 inhibitor U0126 [which was shown to diminish the capacity of HC11 cells to proliferate in response to EGF (190)], on SETD7 expression. As expected, SETD7 increased in cells treated with U0126, supporting our hypothesis that EGF downregulates SETD7 through the MAPK pathway. Moreover, the same outcome could be observed even in cells deprived of EGF, with a significant increase in SETD7 protein levels in cells treated with U0126 in comparison to controls. These results may indicate that this effect may be independent of EGF and give emphasis to MAPK pathway as a potential regulatory pathway for SETD7 gene expression.

Next, in order to study SETD7 expression patterns during cellular differentiation, HC11 cells were exposed to a cocktail of lactogenic hormones. Since SETD7 protein levels are increased after 6h DIP treatment but this is not maintained we hypothesize that SETD7 may be required during the early stages of differentiation. In corroboration with our findings, a recent study showed that SETD7 is downregulated in human embryonic stem cells and is then induced during differentiation. Additionally, they also found that SETD7 knockdown resulted in differentiation defects (200).

We also analyzed HOXB2 protein levels in HC11 cells either treated with or without EGF or DIP medium. HOXB2 is an inducer of differentiation and well-known to inhibit breast cancer cells' growth (194) that may be regulated by SETD7 since H3K4 methylation is known to be present at the promoters of HOX gene clusters. In fact, several other H3K4 methyltransferases have been established as positive regulators of HOX genes (7, 201), and in this study we expected to find similar results for SETD7. However, we did not find an association between HOXB2 and SETD7 increase in both cells depleted of EGF (except after 16h) or treated with DIP medium, which suggests that there are other factors modulating HOXB2 protein. Nonetheless, our results show that HOXB2 exhibits a clear tendency towards a higher levels in cells treated with DIP medium which, since HOXB2 is overexpressed during cell differentiation (194), indicates that we successfully caused HC11 cells to differentiate by adding lactogenic hormones to the medium.

Since previously we found a correlation between STAT3 and SETD7 protein levels after EGF depletion, we also felt impelled to investigate if the same could be observed after treatment with lactogenic hormones. In fact, a correlation between SETD7 and STAT3 increase could be observed and therefore, of all three SETD7 molecular targets here analyzed, STAT3 protein levels seems to correlate more strongly with SETD7.

Finally, we also explored the effect of (R)-PFI-2 and DIP medium on cell number. DIP medium alone (+DIP controls) caused cell number to decrease 58% comparatively to –EGF controls. However, relatively to +DIP controls, we observed a 70% increase in cell number after the addition of 8 nM of (R)-PFI-2, which suggests that (R)-PFI-2 can reverse the negative effect of DIP on cell number. Furthermore, having into account that DIP medium induces differentiation in HC11 cells, these results may indicate that (R)-PFI-2 negatively regulates cell differentiation and allows proliferation; therefore, these results suggest that loss of SETD7 methyltransferase activity could be implicated in the carcinogenic process.

6. Concluding Remarks

During this study we successfully linked SETD7 to the regulation of cell number and possibly of apoptosis, having found that cell number (but not cell proliferation) was lower in cells that expressed SETD7 in comparison with the ones treated with (R)-PFI-2. In fact, we found that EGF (a growth factor essential for mammary epithelial cells proliferation and survival) negatively regulates SETD7 and that this regulation may be mediated by the MAPK pathway. We also confirmed that lactogenic hormones, after an incubation period of 6h, are capable of inducing SETD7 expression which can indicate that SETD7 is involved in the initial stage of cell differentiation. However, more studies should be conducted to confirm these findings and to verify if the effects here reported can be observed in a different cell line and *in vivo*. The next logical step would be to analyze the expression of SETD7 as well as its molecular targets through RT-PCR and to confirm if SETD7 induces apoptosis of mammary epithelial cells through, for example, a TUNEL assay. An immunohistochemical analysis of SETD7 expression in mammary tissue is also mandatory. Thus, our future perspectives are to further clarify SETD7 expression and role in physiologic processes such as mammary gland proliferation, survival and differentiation and in breast cancer. We are certain that the study of SETD7 in normal mammary epithelial cells is vital for the understanding of alterations occurring in breast cancer. Furthermore, SETD7 is now known to methylate numerous histone and non-histone substrates, many of them acknowledged as important players in cancer. Although in this study we could not find a very strong correlation between SETD7 and HOXB2 and STAT3 expression levels (and none with ERα), we highlight the importance of studying SETD7 role in the regulation of the expression and function of its substrates, in both normal and transformed cells. Undeniably, the link between SETD7 and its substrates, as well as its role in cancer, is an exciting lead to follow. Indeed, we believe that due to its characteristics SETD7 has the potential to be of significant importance for cell's homeostasis and, thereby, a major player in breast cancer.

In summary, in this study we successfully established SETD7 as a regulator of mammary epithelial cell proliferation and opened the door for future studies of SETD7 role in cell proliferation and differentiation and in the regulation of multiple pathways.

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