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REGULAÇÃO DA PROLIFERAÇÃO PELA SETD7 NO CANCRO DA MAMA

Tese 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 do Departamento de Ciências Médicas da Universidade de Aveiro

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Dedico este trabalho à minha família e ao meu namorado por serem os grandes amores da minha vida

o júri/ The jury

Presidente/President

Prof. Doutora Odete Abreu Beirão Da Cruz e Silva
professor auxiliar c/ Agregação da Universidade de Aveiro

Prof. Doutora Luisa Alejandra Helguero
Professora Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro

Doutora Joana Vieira da Silva
Investigadora de Pós-Doutoramento do Instituto de Investigação e Inovação em Saúde e do Instituto de Biologia Molecular e Celular

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palavras-chave

Recetor de estrogénio α, SETD7 metiltransferase, degradação, proteossoma, modificações pós traducionais, proliferação, a, regulação, T47D cancro da mama, linha celular de HC11, EGF, FGF, R-PFI

resumo

O crescimento e progressão do cancro da mama é maioritariamente dependente da estimulação hormonal, nomeadamente pela hormona ovariana estrogénica. Esta hormona liga-se e ativa o recetor de estrogénio α (ERα) que culmina na transativação de genes que por sua vez gera uma resposta celular ao nível da proliferação e sobrevivência celular. Uma das terapias para cancros dependentes de estrogénio é a terapia hormonal. Porém, apesar de eficaz, a longo tempo gera mecanismo de resistência endócrina e até recorrência. A ativação de vias de sinalização dependentes de fatores de crescimento e outras vias de sinalização alteram a estrutura do ERα, a sua estabilidade e função. Estes mecanismos são os propostos para explicar a resistência endócrina. A estabilidade e atividade do ERαsão processos regulados por modificações pós traducionais bem como a degradação da proteína mediada pelo proteossoma. A metilação do ERαpela SETD7 parece contribuir para a estabilidade e atividade transcricional do recetor. Tal, traduzir-se-ia num aumento de sobrevivência celular. Contudo, resultados anteriores do laboratório indicam que a SETD7 inibe a proliferação celular de células epiteliais mamárias. Assim, este estudo tem como objetivo principal estudar a regulação do ERα pela SETD7 e vice-versa, bem como os efeitos desta regulação ao nível da proliferação celular mediada por diversos estímulos mitogénicos, e com foco nos efeitos dos estrogénios. Para tal, realizámos vários estudos utilizando um inibidor da atividade da SETD7, R-PFI. Neste estudo mostrámos que os níveis de SETD7 diminuem quando as células são estimuladas a proliferar. A inibição da atividade da SETD7 pelo R-PFI origina um aumento do número de células. Contudo, quando combinado com estrogénio, o aumento deixa de ser significativo devido a uma paragem no ciclo celular. Na presença de R-PFI, pudemos observar um incremento dos níveis proteicos de ERα idênticos aos que se verificam quando o proteossoma está inibido, sugerindo um controlo dos níveis proteicos de ERα pela SETD7 poderia estar associado ao controlo da degradação do ERα. Contudo, este aumento dos níveis de ER não se traduz num aumento de atividade do recetor, pelo que a SETD7 parece ser dispensável para a atividade do ERα. Os resultados obtidos sugerem um papel inibitório da SETD7 sobre o crescimento de células mamárias na ausência de estradiol. Por outro lado, os nossos resultados não estão de acordo com estudos já publicados, pelo que será necessário aprofundar os estudos para esclarecer qual a função da SETD7 na regulação da proliferação mediada pelo ERα.

keywords

Estrogen receptor α, SETD7 methyltransferase, degradation, proteasome, ERα post transdutional modifications, proliferation ,breast cancer, EGF, FGF, R-PFI

abstract

Breast cancer growth is dependent on stimulation by the ovarian hormone estrogen. Estrogen activates estrogen receptorα (ERα) which is a ligand-activated transcription factor. Estrogen-bound ERα transactivates genes which stimulate proliferation and survival. Hormonal therapy is an effective strategy to treat hormone-related breast cancer. However, it is associated with endocrine resistance and recurrence. Activation of growth factors signaling pathways and alterations on ERα protein have been propossed as resistance mechanisms. ERα protein stability and activation of transcription is regulated by post transcriptional modifications such as methylation and by proteasome degradation. SETD7 is a methyltransferase which targets histones and others non-histone proteins important for cellular proliferation, including ERα. Methylation of ERα seems to contribute to ERα stability and activity; this would lead to an increase in cell survival. However, previous results from our lab indicate that SETD7 inhibits mammary epithelial cell proliferation. Therefore, in this project, we intent to study the co-regulation between ERα and SETD7 as well as the subsequent effects on proliferation in response to different mitogenic stimuli, with focus on estrogen. For this purpose, we used a SETD7 activity inhibitor known as R-PFI. In this study, we show that SETD7 levels decrease when cells are stimulated to proliferate. Inhibition of SETD7 activity by R-PFI leads to an increase in cell number. However, when cells were co-treated with R-PFI and estrogen the increase was not significant once cells suffer a cell cycle arrest. In the presence of R-PFI, we show an increase of ERα protein levels, identical to the levels obtained when ERα degradation is impaired by blocking the proteasome, suggesting that SETD7 may control ERα protein levels at this level. However, this increase of ERα levels did not translate into an increase of ERα activity. Thus, SETD7 can be dispensable for ERα activity. Our findings suggest an inhibitory role of SETD7 in breast cells growth in the absence of estradiol. But, on the other hand, our results do not support studies previously reported in relation to ERα regulation. Therefore, further studies are needed to establish SETD7 function in ERα-induced proliferation.

INDEX

I. LIST OF ABBREVIATIONS

AdoMet	S-Adenosyl methionine
AF-1	Activating Function 1
AF-2	Activating Function 2
AI	Aromatase inhibitor
AP-1	Activating protein 1
APS	Ammonium persulphate
AREG	Amphiregulin
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
BTC	Betacullin
CBP	CREB-binding protein
CDK-7	Cyclin dependent kinase 7
CDK-4	Cyclin dependent kinase 4
Cys	Cysteine
DBD	DNA binding domain
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNMT1/3	DNA (cytosine-5) Methyltransferase 1/3
E1	E1-ubiquitin activating enzyme
E_2	17ß-Estradiol
E2	E2-ubiquitin conjugating enzyme
E3	E3-ubiqutin protein ligase
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EPR	Epiregulin
ERα	Estrogen Receptor α
ERD	Estrogen receptor down-regulators
ERE	Estrogen responsive elements
Erk	Extracellular-signal regulated kinase
ER	Estrogen Receptor
ERß	Estrogen Receptor ß
ESR1	Estrogen Receptor α gene
FACS	Fluorescent-Activated Cell Sorting
FGF_2	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FOXO3	Forkhead Box O3

G9a	Euchromatic histone-lysine N-methyltransferase 2
Gli-1	Gli-1 zinc finger protein
GPER	G-protein estrogen receptor
GSK	Glycogen synthase kinase
H3,4	Histone 3,4
H3K4me	Histone 3 methylation on lysine 4
HB-EGF	Heparin binding EGF
HDAC	Histone deacetylase
HER2	Human Epidermal Growth Factor 2
HSP	Heparin sulphate proteoglycan
IGF	Insulin growth factor
IGFR	Insulin growth factor receptor
JAK/STAT	Janus Kinase/Signal transducer and activator of transcription
Jub/WTIP	Ajuba family protein
K	Lysine
LBD	Ligand binding domain
LHRH	Luteinizant hormone-releasing hormone
Lys	Lysine
MAPK	Mitogenic activated protein kinase
MeCP2	Methyl CpG binding protein 2
MEK	Erk kinase
MISS	Membrane-induced steroid signaling
MLL2	Mixed lineage leukemia-2
MMP	Matrix metalloproteinase
mTOR	Mammalian target of rapamycin
MUC1	Mucin 1
NF-kB	Nuclear factor kappa B
NRG	Neuregulin
RIP140	Nuclear repressor interacting protein 1
NSD-1	Nuclear receptor binding SET domain containing protein 1
NuRD	Nucleosome remodeling and deacetylase
PBS	Phosphate buffer serum
PDK1	Protein dependent kinase 1
PIN1	Protein interacting with never in mitosis A
PI3K/Akt	Phophoinositide 3 kinase/ Protein kinase B
PI3P	Phophoinositide 3 phosphate
PIAS	Protein inhibitor of activated signal transducer and activator of transcription
PKA	Protein kinase A

PKC	Protein kinase C
PLCγ	Phospholipase γ
PR	Progesterone Receptor
PSI	Proteasome inhibitor
PTEN	Phosphatase and tensin homolog
PTM	Post transcriptional modifications
Rb	Retinoblastoma
RIZ-1	Rb-interacting Zinc-finger protein 1
R-PFI	(R)-PFI-2
RT	Room temperature
RTK	Receptors tyrosine kinase
RUNX	Rnt-related transcription factor
SAM	S-adenosylmethionine
WW45	Human Sav1 protein
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SERM	Selective estrogen-receptor modulators
SET	Su(var)3-9-enhancer of zeste-trithorax
SETD7	SET domain containg protein 7
SIRT1	Sirtuin-1 protein
SMYD	SET and MYND protein family
SP-1	Stimulating protein 1
Src	Steroid Receptor Coactivator
STAT	Signal transducer and activator of transcription
T-Erk	Total Erk protein
TAED	TEA domain family member
TAF10	TATA-box-binding protein associated factor 10
TAM	Tamoxifen
TAZ	Taffazin
TEMED	Tetramethylenediamine
TGF-A	Transforming growth factor A
Ub	Ubiquitin
Ubc	Ubiquitin conjugating-enzyme
UPP	Ubiquitin proteasome pathway
WB	Western Blot
YAP	Yes-associated protein

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1. STATE OF THE ART

1.1. Introduction

Breast cancer is considered the most common type of cancer affecting women and it is the second highest cause of female death. Worldwide, it is estimated that 1,4 million women per year are diagnosed with breast cancer, while 458.000 die from this disease(1–4). It is considered the main cause of women death in most developed countries(4,5). Breast cancer is more common after menopause. Most breast cancer subtypes are ovarian hormone-related and hormone replacement therapy containing estrogen increases the risk of breast cancer (6–8).

Estrogen receptor α (ERα) is expressed in approximately 75% of all breast cancers(9). ERα belongs to the nuclear receptor superfamily of transcription factors. When activated by its ligand, estradiol, ERα stimulates transcription of many target genes, leading to breast tumor cell proliferation. Nowadays, the presence of ERα in breast tumours is used to diagnose patients who could benefit from hormonal therapy(10). Because of the mechanism of action of ERα, most treatment options involve the blockage of ERα´s estrogen binding site by competitive antagonists (such as tamoxifen) or by blocking estrogen synthesis with aromatase inhibitors(11). Unfortunately, approximately 50% of ERα-positive breast cancers become resistant to this therapy. Endocrine-resistance remains a major challenge for effective treatment of $ER\alpha^+$ breast cancer (12). ERα protein phosphorylation in specific aminoacids can induce resistance to tamoxifen (13,14). But, further studies in this area are needed clearly to understand the mechanisms responsible for the loss of estrogen responsiveness and subsequently the acquisition of endocrine resistance. Several post transcriptional modifications (PTM) such as methylation, acetylation and phosphorylation, contribute to ERα stability and activity (15,16). Hence, enzymes capable of modifying ERα post transcriptionally can regulate response to therapy. SET domain containg protein 7 (SETD7), is a lysine methyltransferase initially thought to methylate histone H3 (17). However, SETD7 methylates many others proteins, most of which regulate the cell cycle both by stimulation or inhibition of its progression. Recently, SETD7 was shown to methylate ERα (18). Little is known about the potential of this methyltransferase and its role in breast carcinogenesis and disease progression.

The main goal of this work was to understanding how SETD7 regulates proliferation through EGF effect and ERα protein levels and activity in breast cancer cells and non-malignant mammary epithelial cells.

1.2. Classifications of breast cancer subtypes

Breast cancer exhibits distinct phonotypes with different treatment options and different responses associated. (19).

The histopathological classification of breast cancer subtypes takes into consideration the expression or absence of three proteins: ERα, progesterone receptor (PR) and Receptor tyrosine-protein kinase erbB-2 (HER2) expression (**Table 1**). This classification related to the breast cancer phenotype have significant differences in overall survival and responsiveness to treatment. For example, the expression of ERα and PR, and the absence of HER2 protein overexpression is correlated with a better prognosis (20).

ERα positive	PR positive	HER2 positive	Triple Negative Breast Cancer
PR^-/PR^+	$ER\alpha^+/ER\alpha^-$	PR^-/PR^+	$ER\alpha^-$
$HER2^+/HER2^-$	$HER2^+/HER2^-$	$ER\alpha^-/ER\alpha^+$	PR^-
			$HER2^-$

Table 1: Histopathological classification of breast cancer. This classification is based on 3 biomarkers classically used; ERα: Estrogen Receptor alpha, HER2: PR: progesterone receptor;

1.3. Estrogen signaling and breast cancer

Estrogen is a potent morphogen which stimulates cellular proliferation and differentiation, mainly in breast tissue (21). The most relevant estrogen produced by human body is 17ß-estradiol (E_2). E_2 effects are mediated through two G-protein estrogen receptors (GPER): ERα and ERß, both belonging to the nuclear receptor superfamily of transcription factors(22). ERα was first discovered in the late 1950 and ERß in 1998. For this reason, ERα is the most extensively studied and currently the only one of the two receptors considered in the clinics.

ERα and ERß have distinct, nonredundant roles in the immune, cardiovascular and sketal systems. On the promoter of some genes, ERα and ERß can have opposite effects, suggesting that the overall proliferative response to estrogen is the result of a balance between ERα and ERß signaling(23–26).

ERs contain an amino-terminal region that harbours the ligand independent activating function (AF-1), a central DNA binding domain (DBD) and a carboxyl-terminal hormone binding domain (LBD) which contains the ligand-dependent activation function (AF-2). The hinge domain determines functional synergy between AF-1 and AF-2 in response to ERα agonists and antagonists to achieve full transcriptional activity of ER(27).

Transcriptional activation is mediated by the AFs, the constitutively active AF-1 located at NH_2 terminus of ERα and the ligand-dependent AF-2 that resides in the COOH-terminal LBD. E_2 binds within the hydrophobic pocket of the LBD and it is wrapped inside by helix 12. The subsequent conformational change activates AF-2.

The central and most conserved domain, DBD, is involved in DNA recognition and concomitant binding whereas ligand binding occurs in COOH-terminal on LBD. The NH_2-terminal domain is the most variable domain in sequence and length between the two ERs (28,29).

There are four models that explain all ERα actions: 1) classical, direct pathway, 2) tethered, 3) non-genomic and 4) ligand independent (Figure 1).

In the classical direct pathway, E_2 binds to ERα in the cytoplasm, and ERα then dimerizes and translocates to the nucleus, where interacts with estrogen responsive elements (ERE) on the DNA sequences in target genes. The conformational changes in ERα after E_2 binding allow the recruitment of coregulators as coactivators or corepressors such as p160 family steroid receptor coactivator 1,2 and 3 (SRC1, SRC2, SCRC3), Rb-interacting Zinc-finger protein-1 (RIZ-1), Euchromatic histone-lysine N-methyltransferase 2 (G9a), nuclear receptor-binding SET domain containing protein-1 (NSD-1), CREB-bindind protein (CBP)/p300, RIP140 and mixed lineage leucemia-2 (MLL2) which acts via AF-1 and AF-2 domains(18,30). Some of this coregulators complexes contain histone-modifying enzymes proteins as histone acetyltransferase (HAT) which disrupt electrostatic bonds on DNA and increase access of transcription factors to the promoter, as well as histone methyltransferases (HMT) which alter local chromatin structure and facilitate the recruitment of the transcription machinery as components of the RNA polymerase II complex to activate target genes (31,32).

In the tethered pathway, ERα does not bind DNA directly, but after ligand activation interacts with other transcription factors including stimulating protein 1 (SP-1), activator protein 1 (AP-1) and nuclear factor kappa B(NF-kB) which bind to their target DNA sequences (Figure 1). This pathway regulates the expression of proteins in cell division, survival and angiogenesis (33).

The non-genomic pathway, also known as membrane induced steroid signaling (MISS), mediates rapid effects. MISS involves the lipidation of ERα located close to membrane. Upon E_2 activation, ERα interacts with protein kinases (Src and PI3K). In addition, there is a protein known as G-coupled protein α_I which can also bind E_2. Although this is not the ERα we refer to in this work, signaling through E_2-activated G-protein can activate protein kinases cascades (Akt, PKA, ERK1/2) and endothelial NO synthase (34–36).

The ligand independent mechanism, occurs when ERα is activated by AF-1 phosphorylation catalysed by activated kinases like protein kinase A (PKA), protein kinase C (PKC) and mitogen activated pathway kinase (MAPK), due to the crosstalk with growth factor receptor tyrosine kinase, involving for example insulin growth factor receptor (IGFR) and epidermal growth factor receptor (EGFR). ERα activated in this manner can regulate transcription of target genes (37–41).

The current acceptance is that that E_2 plays a main role in mammary tumorigenesis. Long term exposure to estrogen hormone is associated with an increased breast cancer risk. Furthermore, many studies have reported a strong correlation between elevated estrogen levels and breast cancer, involving a hiperactivation of ERα(42).Researchers hypothize that E_2 acting through ERα stimulates cell proliferation that could originate

mutations arising from replication errors during phase S of DNA synthesis. Over a period of E_2 exposure, a sufficient number of mutations accumulates and could induce a neoplastic transformation (43,44).

ERα can interact with cyclin D1 through the PKA-mediated pathway, in response to E_2, to promote G_1 to S transition by activating cyclin D1-regulated cyclin-dependent kinase 4 (CDK-4). Cyclin D1 is also responsible for enhancing ERα transcription both in a ligand dependent and independent manner. It was reported that mammary epithelial overexpression of cyclin D1 is related to mammary carcinoma (45,46). Moreover, it was reported that the ERα gene (ESR1) can be amplified in breast cancers and this is correlated with high ERα expression levels and better response to endocrine therapy (47).

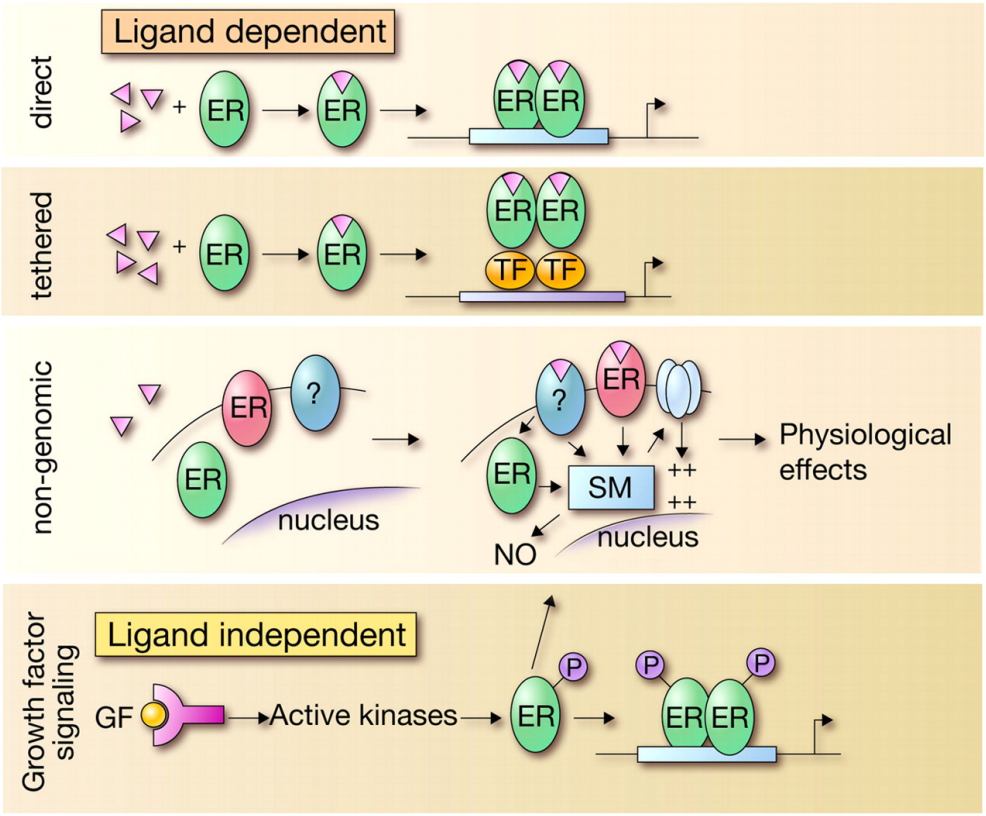

Figure 1: Molecular pathways of estrogen signaling. There are four pathways involved on estrogen-mediated signaling. Ligand-dependent signaling is subdivided in direct pathway tethered pathway and non-genomic pathway and ligand-independent pathway involves a crosstalk with growth factor signaling. ER, estrogen receptor; SM, second messengers; TF, transcription factors; NO; nitric oxide; GF, growth factors. Adapted from (37)

1.4. Receptors type Tyrosine Kinase in breast cancer

Receptors tyrosine kinase (RTK) are a family of cell surface receptors, which act as receptors for growth factors, hormones, cytokines and extracellular signaling pathways. RTK activation by autophosphorylation activates signaling pathways regulating cell proliferation, differentiation and apoptosis. The RTKs involve several subfamilies such as EGFR, fibroblast growth factor receptors (FGFR), IGFR and HER-2 (48). Activation of RTKs triggers a cascade of phosphorylation events involving downstream kinases which leads to the phosphorylation of target transcription factors and transcription of specific genes (49). Upon ligand binding, RTK are auto-phosphorylated, which results in the activation of Ras/Raf. Then, Raf phosphorylates Erk kinase Mek1/2 which in turn phosphorylate Extracellular-signal regulated kinase 1/2 (Erk1/2). These pathway is known as MAPK (50). In addition to MAPK, also Phophoinositide 3 kinase/ Protein kinase B (PI3K/Akt) pathway is activated by RTK autophosphorylation and it results in the activation of Akt and subsequent activation of mammalian target of rapamycin (mTOR). Activation of MAPK leads to translocation of transcription factors to the nucleus as c-MYC, influencing cell cycle, while PI3K/AKT result in anti-apoptotic signaling, cell growth and proliferation, motility and adhesion. RTK also activates the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway and the phospholipase Cγ (PLCγ) pathway (Figure 2).

In summary, RTK signaling leads to transcriptional activation of genes involved in angiogenesis, protein synthesis, cell proliferation and survival, all biological processes which are altered in cancer cells. In fact, RTKs mutations are often associated with tumorigenesis (48).

1.4.1. Epidermal Growth Factor family and its receptor

The EGF family of growth factors comprises potent morphogens such as EGF, transforming-growth factor α (TGF-A), heparin-binding epidermal growth factor (HB-EGF), amphiregulin (AREG), epiregulin (EPR), betacellulin (BTC) and neuregulins (NRGs).

EGFR is a RTK present in epithelial and stromal cells, that belongs to the ErbB family. When a ligand binds to extracellular region of EGFR, the receptor dimerizes, turning on kinase activity following by autophosphorylation at multiple tyrosine residues in order to recruit various substrates that activate multiple signaling pathways involved on cell proliferation, motility and survival such as PI3K/AKT, JAK/STAT, MAPK kinase and PLCγ (51,52).

EGF, EGFR and EGFR regulators are positively correlated with breast cancer cell proliferation (53). EGFR gene is mutated in many epithelial tumours (54). In case of ERα-positive breast cancer, EGFR mediates estrogen proliferative effect and promotes invasion of breast cancer epithelial cells (55). Moreover, EGFR overexpression in breast cancer is associated with large tumor size and a more aggressive phenotype, and can

be involved in resistance to endocrine therapy (55). AREG is transcriptionally regulated by ERα and a key mediator of E_2-driven epithelial proliferation in mammary gland ductal morphogenesis during puberty (56). Several studies suggest that AREG can play a role in human breast cancer initiation and progression and its expression is associated with aggressive disease (57).

HER2 gene and protein overexpression was reported in 30% of invasive breast cancers. HER-2 gene amplification in breast cancer potentiates cell proliferation, angiogenesis and tumor invasiveness (58). The specific ligand binding of HER2 was not been discovered, however, HER-2 exerts its effects by heterodimerization with other Erb receptor family members including EGFR.

1.4.2. Fibroblast Growth Factor (FGF) family and its receptors

FGF is a family of growth factors that mediate cellular responses by activating RTKS designated FGFR1, FGR3, FGFR3 and FGFR4. FGFs act in concert with heparin or heparin sulphate proteoglycan (HSP) in order to activate their receptors and to induce cellular responses. The binding of FGF_2 and HSP to FGFR leads to receptor dimerization, activation and autophosphorylation of tyrosine residues and following recruitment of effector molecule that lead to stimulation of intracellular signaling pathways such as MAPK and PI3K/Akt (59,60). FGFR signaling can play a relevant role in development and progression of breast cancer. Mutations gain of function in FGFR were identified in breast cancers (60,61). FGFR amplifications and mutations in breast cancer are associated with poor prognosis. Furthermore, it was shown, in MCF7-cells, that FGFR3 can induce resistance to tamoxifen mediated by enhanced activation of PLCγ signaling (62).

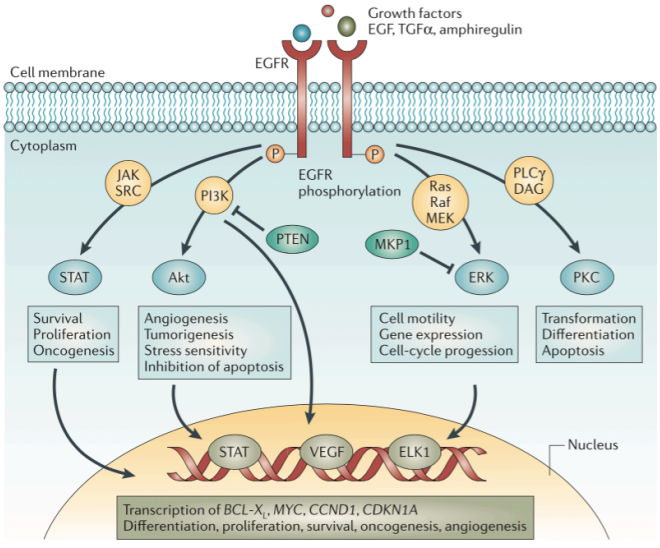

Figure 2: Pathways involved in response to TRK activation. The binding of growth factors to their receptors leads to TRK activation by autophosphorylation. Subsequently, it triggers the activation of downstream signaling pathways as JAK/SRC, PI3K, Ras-Raf-MEK (MAPK pathway) and PLCγ. Activation of these pathways result in expression of genes as BCL-X, MYC, CCND1 and CDKN1A important for differentiation, proliferation, survival, oncogenesis and angiogenesis.
Taken from (63)

1.5. Hippo pathway and its relevance to breast cancer

Hippo pathway is a conserved signaling pathway involved on integration of cytoskeletal changes with extracellular environment (64). This signaling pathway negatively regulates cell number by promoting cell death and differentiation and inhibiting cell proliferation. The coordination of cellular responses mediated by Hippo pathway may contribute to development, tissue homeostasis and tumorogenesis (65–67).

The Hippo pathway consists in a serine kinase cascade that acts on a transcriptional complex to regulate gene expression. The MST1/2 activates another kinase, LATS1/LATS2; which in turn leads to phosphorylation of the transcription co-factors Yes-associated protein (YAP) and tafazzin (TAZ), human Sav1 protein (WW45) and Mob are regulators that interact with kinases components of Hippo pathway.

The phosphorylated form of YAP/TAZ interacts with 14-3-3 cytosolic proteins resulting in cytoplasm sequestration and an inhibition of target gene transcription. YAP/TAZ may be further phosphorylated by Casein kinase 1δ/ε leading to recruitment of a E3 ubiquitin ligase leading to proteasome-mediated degradation (68,69). When upstream kinases MST1/2 and LATS1/2 are inactive, YAP/TAZ remains non-phosphorylated and can be translocated into the nucleus to mediate transcriptional activation by interacting with DNA-binding transcription factors such as TEA domain family member (TAED), Smad, RNT-related transcription factor (RUNX) to regulate genes expression involved in cell proliferation, apoptosis, development and differentiation (67,69). An important role of YAP/TAZ-induced proliferation might be transcriptional activation of cell-cycle genes in a TEAD- dependent manner. Several studies showed that YAP plays a key role in the regulation of S-phase entry and progression as well on G2–M phase, through cyclin D1 transcriptional activation(70). Dysregulation of Hippo pathway leads to aberrant activation of YAP that contributes to tumorigenesis in several tissues, including breast tissue. Thus the components of Hippo signaling can be considered tumour suppressors once they limit cell proliferation (71).

1.5.1. Modulation of Hippo pathway by EGF

Both Hippo and EGFR signaling control cellular growth, and when dysregulated, contribute to abnormal cell proliferation. Several growth factors including EGF and Insulin growth factor (IGF) stimulate nuclear accumulation of YAP by a downstream effect on PI3K and phosphoinositide dependent kinase 1 (PDK1).

Ajuba protein can be considered a regulatory mechanism of EGFR and Hippo pathway (72). Mammalian contains three Ajuba family proteins Ajuba (Jub), LIM domain containing 1 and Wilms tumor protein 1-interacting proteins (WTIP)(73).

In the presence of growth factors, activation of the RAS-MAPK phosphorylates Ajuba protein Jub/WTIP, which binds and inhibits the activity of WW45-LATS complex leading to nuclear transactivation of YAP where it can exert its transcriptional activity leading to increased cell proliferation (Figure 3).

In the absence of growth factors, RTK is not phosphorylated and consequently PI3K remains inactive. PDK1 forms a complex with Hippo components in order to phosphorylate and retain YAP in cytosol. YAP can't be translocated to nucleus and proliferation mediated by YAP is turned off.

Growth factors can also activate PI3K and recruit PDK1 to membrane resulting in PDK1-MST1/2-Sav1-LATS1/2 dissociation which drives YAP translocation and subsequently proliferation-mediated YAP (74). Furthermore, it was reported that activation of PI3K by EGFR mediated YAP gene expression and subsequently nuclear translocation to interact with TEAD transcription factor in order to upregulate genes involved on connective tissue growth factor and AREG genes (75).

Therefore, there is a positive feedback mechanism for regulation of these two pathways (72). In breast cancer cells, EGFR activation contributes to YAP-mediated cell proliferation and migration (76,77). In MCF-10A non-malignant mammary cell line, EGF treatment inhibited Hippo pathway (77).

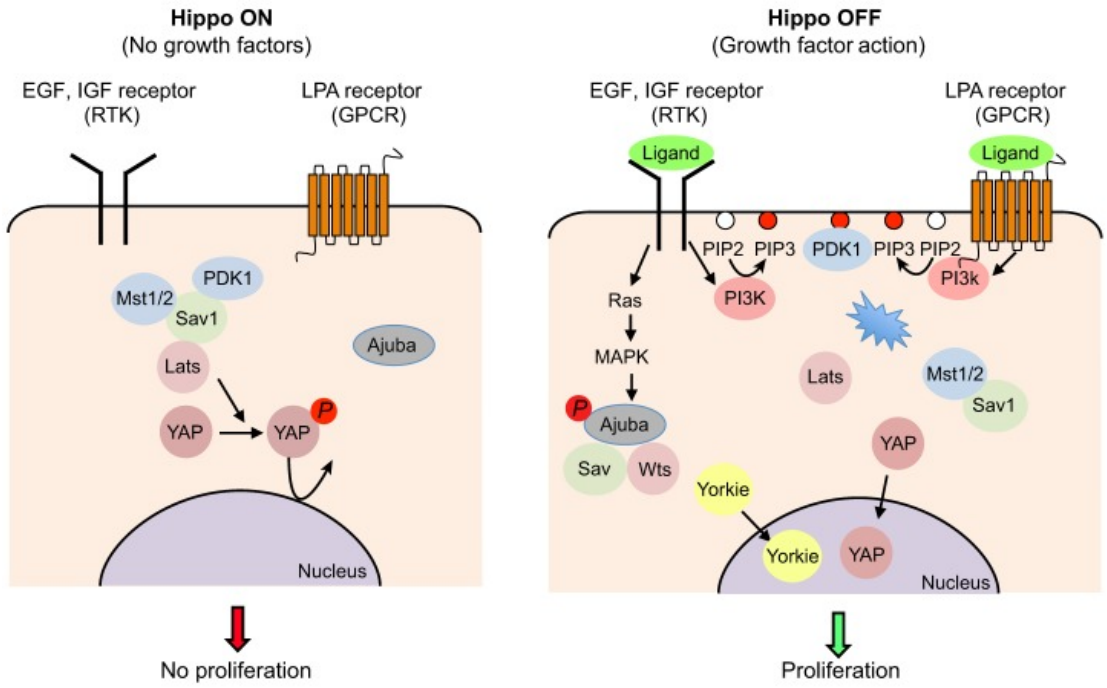

Figure 3: Regulation of cellular proliferation mediated by Hippo pathway in the absence or presence of growth factors. In the absence of growth factors, RTK is not phosphorylated and PI3K isn't active. PDK1 forms a complex with Hippo components in order to phosphorylate and retain YAP in cytosol. YAP can't be translocated to nucleus and proliferation mediated by YAP is turned off. In the presence of growth factors, RTK active phosphorylate downstream proteins involved on PI3K and MAPK; Ajuba proteins, phosphorylated by MAPK, form a complex with Sav and Wts hippo proteins. YAP is not phosphorylated by hippo effectors and it is translocated to nucleus where activates expression of proliferative genes and inhibits apoptotic gene expression. Taken from (74)

1.6. Regulation of Estrogen Receptor α expression and function

To assure proteostatic regulation of ERα, the spatiotemporal abundance of this protein must be strictly controlled in the cells. The expression and activity of ERα can be regulated at pre- and post-transcriptional levels at three distinct levels: protein expression, activity and degradation.

Epigenetic mechanisms contribute to transcriptional regulation of ERα expression. The activity of ERα protein is regulated through post transcriptional modifications (PTM) including methylation, acetylation, phosphorylation, sumoylation and ubiquitination. The E_2 binding to ERα triggers PTMs to promote target gene transactivation and subsequent ubiquitin-mediated ERα proteolysis.

1.6.1. Control of ERα expression

Epigenetic mechanisms regulate estrogen signaling through and ERα gene expression and protein levels. Those epigenetics mechanisms include DNA methylation, histone post translational modifications and RNA silencing by non-coding RNAs and contribute to regulate ERα expression (78–80).

Loss of expression of ERα is explained by DNA hypermethylation. DNA-methylation-mediated promotor of ESR1 silencing is associated with tumorigenesis.

In fact, methylation of ERα promoter by Dnmt3 induce recruitment of methyl-CpG binding protein 2 (MeCP2) to complete ERα repression and silencing by further methylation (81). Furthermore, overexpression of HDAC1 impairs ESR1 expression in MCF-7 cells (82).

Several evidences show that ERα gene CpG islands are hypermethylated in ERα-negative human breast cancer cells and unmethylated on ERα-positive (83). DNMT1 is a methyltransferase responsible for the methyl transference group to cytosine bases to CpG islands in ERα promotor, inhibiting ERα transcription (84,85).

The “switch” in histone modifications resulting in chromatin remodelling can contribute to transcriptional silencing or activation of ERα. Specifically, methylation in histones H3 or H4 can contribute to activate or repress genes transcription. Transcriptional activated ERα is characterized by the presence of acetyl groups on H3 and H4, which promotes an open conformation leading to ERα gene expression. The removal of acetyl groups by HDAC the chromatin becomes condensed and ERα is transcriptional inactive.

RNA silencing consists on mRNA post transcriptional degradation induced by small non-coding RNAs. miRNAs are a class of small nucleotides that negatively regulate gene expression by inhibiting translation of target RNAs or inducing mRNA degradation (86,87). miRNAs can influence estrogen-regulated gene expression by directly reducing ERα mRNA stability or translation. Studies in MCF-7 cell line reported that transfection with an expression vector for miR-206 reduced both mRNA and proteins level of ERα (88).

Nuclear hormonal pathways are also involved in ERα gene expression. Some studies show that progesterone and androgens also regulate ERα expression(89,90). E_2 induced ERα turnover mediated by ubiquitin ligase 26S proteasome is linked to ERα transcription but this subject will be further explored in posterior sections.

1.6.2. Regulation of ERα activity

The existence of ERα PTMs such as phosphorylation, palmitoylation, acetylation, ubiquitination, methylation and sumoylation regulate ERα activity after and before its binding to the DNA in a ligand-dependent or independent manner (Table 2).

These PTMs on ERα protein determine the binding of coactivators and corepressors to the receptor, and therefore affect its transcriptional activity. PTMs located within DBD and AF-1 can condition non-classical signaling of ERα whereas HBD/AF-2 PTM can affect the classical pathway of ERα. Residues in the hinge domain, localized between DNA binding domain and hormone binding domain, are also commons targets for PTMs that affect ERα activity through alteration of receptor stability or regulation of estrogen-dependent gene transcription (91).

ERα phosphorylation is necessary for receptor dimerization and recruitment of specific transcription factors chromatin remodelling enzymes that lead to an enhancement of cell proliferation and survival (92). Acetylation of ERα is associated with DNA binding and negative regulation of ligand-induced transcriptional activity (92). Lysine methylation is found in ERα (17,93). Methylation of ERα is associated with receptor stability and transcriptional activity. ERα is methylated at K266 by SET and MYND protein family 2 (SMYD2) and at K302 by SETD7 methyltransferase. SETD7 methylation of ERα is involved on stabilization and enhancing the transcriptional activity of ERα (18,94). On the other hand, SMYD2-mediated methylation allows the attenuation of the chromatin recruitment of ERα to prevent ERα target gene activation under an estrogen-depleted condition (32). Some of methylated residues as K302 may also be modified by acetylation, suggesting a coordinated regulatory mechanism to take place at these sites (92). ERα palmitoylation on Cys 447 allows to ERα interact with other signaling pathways as MAPK and PI3K (95). ERα sumoylation consists of covalent attachment of SUMO to a residue in order to facilitate interaction with other proteins. The main sumoylation residues in ERα are K266 and K268, present on hinge region of steroid receptor. The process of ubiquitylation usually targets a protein for degradation via proteasome pathway or affect protein-protein interactions and subcellular localization (96,97). In fact, ubiquitination of ERα in inactive state allows clearance of misfolded ERα, and after ligand binding, it provides a mechanism of promoter clearance to allow new rounds of transcription (98).

Table 2: Post translational modifications (PTM) on estrogen receptor α and their biological effect

ERα domain	Residue	TPM/Enzyme	Effect on ERα	References
ACTIVATING FUNCTION-1	S46/47	Phosphorylation by PKC	Ligand-independent activation of ERα	(14)
	Y52	Phosphorylation by Src-like non-RTK	ERα stability and activity	(99)
	S102	Phosphorylation by Erk1/2	Ligand-independent transcription of ERα	(100)
	S104	Phosphorylation by Erk 1/2	Important for ERα activity and its promote tamoxifen resistance	(101)
	S106	Phosphorylation by Erk1/2	Important for ERα activity and promote tamoxifen resistance	(101)
	S118	Phosphorylation by CDK-7 or Erk1/2 Phosphorylation by GSK3	Sensivity of the ERα to the ligand Ligand-independent activation Stabilization of ERα and ligand-dependent transcriptional activity of ERα	(92,102)
	S167	Phosphorylation by CDK-7	DNA binding and co-activators binding Ligand-dependent activation	(14,92)
DNA BINDING DOMAIN	S236	Phosphorylation by PKA	ERα dimerization and DNA binding	(92)
	K266	Acetylation by histone acetylase p300	DNA-binding and ligand-independent activation	(33)

HINGE DOMAIN		Sumoylation by PIAS1, PIAS3, Ubc9	Stimulation of ligand-dependent ERα activity	(92)
		Methylation by histone methyltransferase SMYD2	Attenuation of chromatin recruitment of ERα. Prevent ERα target gene activation under an estrogen-depleted condition.	(32)
	K268	Acetylation by histone acetylase p300	DNA-binding and ligand-dependent activation	(33)
		Sumoylation by PIAS1 PIAS3 and ubiquitin conjugating-enzyme	Stimulation of ligand-dependent ERα activity	(92)
	K294	Phosphorylation by unknown enzyme	Ligand-dependent ERα transcriptional activity	(14)
	K299	Acetylation by p300	DNA-binding and ligand-dependent activation	(92)
	K302	Acetylation by histone acetylase p300	Inhibition of ERα activation	(92)
		Methylation by SETD7	Stabilization of ERα and efficient recruitment of ERα to its target genes	(33)
		Ubiquitylation by BRCA1	Induction of proteasome-	(103,104)

			dependent degradation of ERα	
	K303	Acetylation by histone acetylase p300	Inhibition of ERα activation. Attenuation of ERα-driven transcription	(33)
	S305	Phosphorylation by PKB/Akt or PKA	Regulation of K302/K303 acetylation	(92)
HORMONE BINDING DOMAIN	C447	S-Palmitoylation	Plasma membrane localization and non-genomic ERα signaling	(95,105)
	Y537	Phosphorylation by Src family	Inhibition ERα dimerization, ligand-dependent activation and ER activity	(106)
	S559	Phosphorylation by CK2	ERα binding to coregulators Inhibition of ligand-independent activation of ERα	(14)

1.6.3. Regulation of ERα stability

The ubiquitin proteasome pathway (UPP) is considered the major system for protein degradation specially for proteins with a short life span. This system can also play a role as directing the subcellular localization of proteins (107). The UPP is important in a wide range of cellular processes such as cell cycle regulation, signal transduction, differentiation, embryogenesis and development, apoptosis, and carcinogenesis (108).

A common feature of the proteasome-mediated protein degradation is the covalent attachment of ubiquitin to lysine residues of target proteins followed by the formation of polyubiquitin chains. Those ubiquinated proteins are then recognized and degradated by 26S proteasome, a protease complex or by the lysosome (109).

Protein ubiquitination involves three types of enzymes: E1-ubiquitin activating enzyme (E1), E2 ubiquitin-conjugating enzymes (E2) and E3 ubiquitin-protein ligases (E3). The first step of this cascade is associated with

activation of ubiquitin in an ATP-dependent manner (Figure 4). The activated ubiquitin forms a thioester bond between the C-terminal glycine residue of ubiquitin and a cysteine residue of E1. Then, ubiquitin is transferred from E1 to one enzyme of E2 complex. The ubiquitin can be transferred in two ways: directly from the E2 to the target protein or indirectly from E2 to target proteins proceeds through an E3 intermediate. E3 covalently ligates ubiquitin to Lys residues on the substrate protein and mediates substrate specificity. This protein-ubiquitination is repeated by a several times and can lead to the formation of a polyubiquitin chain bind to the target protein. Ligation of polyubiquitin can serve as tag to target proteins for promote the assembly of signaling complexes or proteasomal degradation. In the 26S proteasome, the ubiquinated protein binds to the 19S regulatory component and the substrate is subsequently degraded by 20S core particle. The fragments generated are hydrolysed to aminoacids(110).

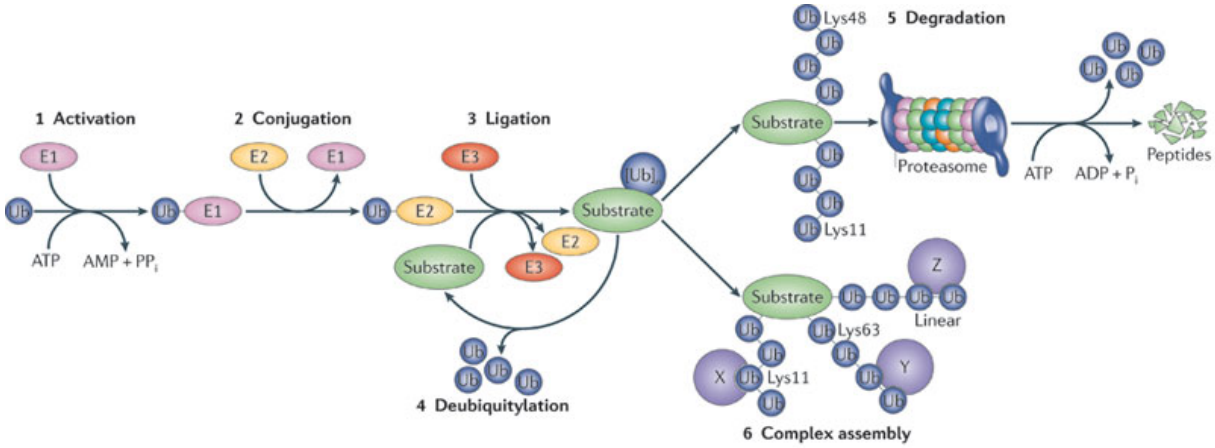

Figure 4: Enzymes and several reactions of the ubiquitin cascade system. X, Y and Z indicate ubiquitin-binding proteins. Pi, inorganic phosphate; PPi inorganic diphosphate; Ub, ubiquitin. Adapted from (111)

Ligand induced ERα degradation is important to regulate physiological ERα protein levels (112). The UPP allows the rapid clearance of ERα to maintain the balance of ERα in response to hormonal stimulation. Degradation of the ERα-E_2 complex at each round of transcription a way for the cell to adjust transcription activation in response to rapid changes in E_2 concentration (113). Therefore, E_2 stimulation induces ligand-mediated ERα degradation by UPP in order to control the intensity and duration of E_2-ERα signaling. UPP degradation is also responsible for regulate transcriptional activity of ERα. In fact, some studies purposed that E_2-ERα signalling allows the recruitment of coactivators but also E3-ubiquitin ligases with the aim of its own downregulation (114).

E_2 forms a stable complex with ERα. This complex sustain transcription for extended periods of time. In the absence of E_2, the half time of ERα is approximately 5 days, but in the presence of E_2 the life time reduces substantially to 3 hours (115). ERα turnover results from the formation of polyubiquitin chains on receptor lysines and its subsequent proteasomal degradation through UPP. Hinge region lysines are responsible for promote ligand-induce receptor turnover (116).

As in the presence of estradiol ERα protein is considered a short life protein, it is possible that receptor can be a target of UPP. In fact, some studies show that, *in vitro*, ER α is degradated in UPP-dependent manner following by proteolysis in proteasome (117).

Recent evidences show that transactivation and quality control of ERα requires estrogen-dependent receptor ubiquitination and degradation and that its turning off ERα-mediated transcription also requires ubiquitination and degradation (98,109,118).

Ubiquitin-dependent degradation of ligand-bound receptor molecules is thought to be critical for promoter clearance and additional rounds of transcriptional response to estrogen. This mechanism is necessary for sustained and controlled transcription at a given promoter take place (92).

Many proteins are responsible for maintain ERα stability by inhibiting its degradation via UPP, including ERα coactivators as mucin-1 (MUC1) and protein interacting with never in mitosis A (PIN1) (119). MUC1 knock out MCF7 cells leads to ERα levels decrease, suggesting that MUC1 confers ERα stability (120). PIN1 associates with ERα to prevent its interaction with E3 ligase (121). Other proteins involved on ERα stability are glycogen synthase 3 (GSK3), Rb and SETD7 (94,122).

1.7. Breast cancer therapy and mechanism of endocrine resistance

Hormonal therapy works by decreasing the amount of estrogen in the body or blocking estrogen signaling in case of breast cancer (Figure 5). The main types of hormonal therapy that may be used are selective estrogen-receptor modulators (SERMs), aromatase inhibitors (AI), estrogen-receptor downregulators (ERDs) and luteinizing hormone-releasing hormone agents (LHRHs). SERMs block the effect of estrogen in the breast tissue (123).

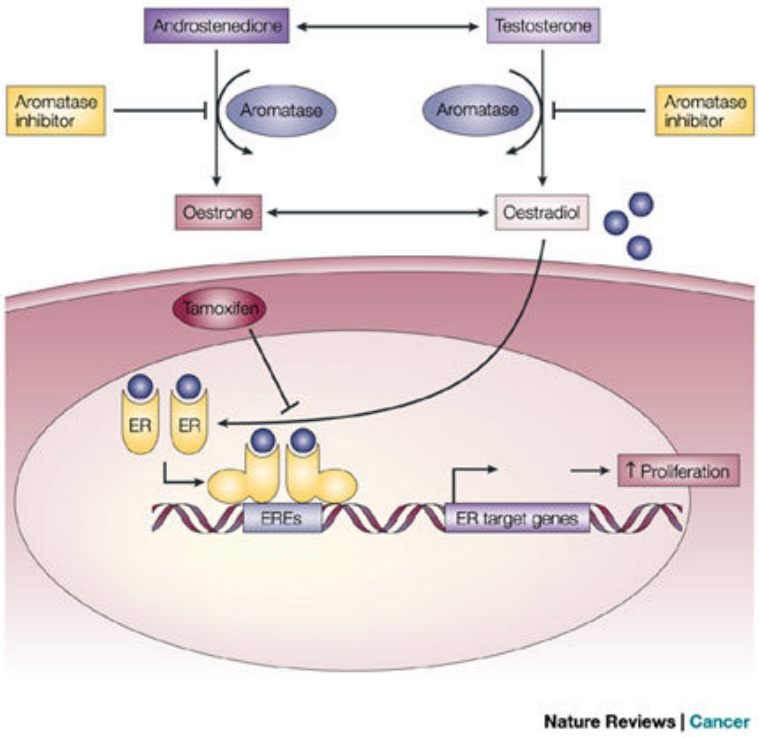

Figure 5: Cellular mechanism of aromatase inhibitors and tamoxifen. Aromatase inhibitors and Tamoxifen are therapies responsible for decrease in ERα signaling pathway through aromatase inhibition and ERα activity respectively. Adapted from (114)

It is widely accepted that growth factor receptor pathways interact via cross-talk with ERα and thereby affect cell growth and resistance (124). Therefore, it is possible that up-regulation of RTK pathways during tamoxifen treatment lead to loss of E_2 dependence and tamoxifen resistance. Overactivation of EGFR and consequently activation of MAPK can contribute to transcriptional repression of ERα gene, resulting in endocrine resistance. (125). Co-activators and co-repressors have important roles in mediating transcriptional activation by ERα. Alterations in co-regulators expression may be related with therapy-resistance. Co-repressors are usually recruited when an antagonist such as tamoxifen binds to ERα. They form a repressor complex with histone deactetylases (HDAC), facilitating chromatin condensation and inhibition of gene transcription. Several data

suggest that progressive reductions in co-repressor activity in response to tamoxifen therapy may contribute to resistance (102) .

Overexpression of HER2 gene is present in approximately 25% of breast cancer. HER2 confers a more aggressive phenotype. Trastuzumab is a monoclonal antibody using for HER2 positive breast cancer. Trastuzumab binds to a extracellular domain of HER2/neu and reduces signaling downstream as PI3K and MAPK cascade (126). Cells treated with trastuzumab suffer a cell cycle arrest during G_1 phase of the cell cycle through downregulation of Akt activation. Trastuzumab inhibits HER2 cell growth through prevention of dimerization, downregulation of HER2R, and induction of antibody cellular cytotoxicity (127,128).

Despite successful results in HER2 breast cancer, some patients suffer of tumor recurrence after an adjuvant treatment reflecting the existence of mechanisms of resistance to trastuzumab. Inactivation of target receptor through truncated HER2 receptors lacking extracellular trastuzumab-binding domain, overexpression of alternative RTKS and alterations on downstream pathways as MAPK and PI3K/Akt are mechanisms involved on immune resistance (129). Mutated HER2 isoform promotes the continuous activation of oncogenic signaling and avoid trastuzumab's effects. Overexpression of alternative RTKs is a compensatory mechanism for HER2 inhibition pathway. In some studies, it was reported that increased signaling from HER family as EGFR, HER3 or HER4 through EGF, BTC, Heregulin can compensate for the inhibition of HER2 signaling mediated by trastuzumab (130,131). Furthermore, overexpression of IGFR reduces trastuzumab effects (132).

One of the most established mechanism of trastuzumab resistance is the loss of phosphatase and tensin homolog (PTEN). PTEN is a tumor suppressor that regulates intracellular levels of phosphoinositide 3 phosphate (PI3P) and Akt signaling pathway (133). In fact, patients with PTEN loss in breast cancers have poor responses to trastuzumab therapy (134,135). Other active mutations on downstream pathways as RAS mutations in MAPK are also related with trastuzumab resistance (136).

1.8. SETD7, a lysine methyltransferase

SETD7 is a lysine methyltransferase that comprises a Su(var)3-9-enhancer of zeste-trithorax (SET) domain in its C terminus. SET proteins can be classified according to the lysine residue that they target on histones H3, H4 and H2A. The evolutionarily conserved SET domain occurs in the most protein known to possess histone lysine methyltransferase activity. Human SETD7 contains a N-terminal ß-sheet domain as well as the conserved SET domain. Two residues presented on C terminus of the protein appear to be essential catalytic activity. The cofactor S-Adenosyl methionine (AdoMet) binds also to this domain. AdoMet is the principal methyl donor in the methyltransferase reaction (137,138).

High resolution crystal structure identifies SETD7 as a ternary complex, where peptide substrates and co-factor binds on opposite surface of the enzyme. Substrate binds to the active site of enzyme through recognition of consensus sequence and AdoMet insert its side chain into a narrow tunnel (139). SETD7 tunnel pass through the enzyme and connects the peptide and cofactor binding surfaces. In this tunnel, the alignment of methyl group allows the transference from AdoMet to the amine in Lysine (140). Further analysis showed that SETD7 function as a monomethytransferase (139).

SETD7 substrates are important for a wide range of biological processes such cell cycle regulation, DNA damage response, RNA Polymerase II dependent gene transcription, chromatin modulation, cellular differentiation and regulation of developmental pathways(141). Consequently, its acceptable consider its role on several physiological and pathological processes, as immune and metabolic pathways and cellular oxidative stress (142–146). Recent reports demonstrated that SETD7 is involved on cellular differentiation as well as cell cycle, apoptosis and metabolism (146–148).

SETD7 is specifically known by monomethylated Lysine 4 of Histone 3, a specific tag for epigenetic transcriptional activity (149). SETD7 plays a role on maintenance of transcription and euchromatine structure at islet-enriched genes (145).

In relation to tumorogenesis, SETD7 is required for Wnt-driven intestinal tumorogenesis and regeneration. This enzyme is a part of a complex containing YAP, a hippo target, and ß-catenin. SETD-7-dependent methylation of YAP facilities Wnt-induced nuclear accumulation of ß-catenin. These signaling pathways are frequently disrupt on cancer development (150). SETD7 was also reported as a mediator of YAP cytosolic retention, as mentioned in the previous sections, this contributes to inhibit YAP-nuclear-mediated transcription (151). Other studies showed that depletion of SETD7 enhanced the expression of Wnt/ß-catenin target genes as c-Myc and cyclin D1 to promote cancer cells proliferation (152).

Knock down studies reported that when SETD7 expression decrease, breast cancer cells proliferation, migration and invasion increase. These effect is mediated by Gli-1 (153). In case of gastric cancer, data suggest that SETD7 have tumor suppressor functions and depletion of SETD7 can be associated to gastric cancer progression

(154). In breast cancer, SETD7 was associated with a poor outcome. A recent study published that SETD7 positively regulates cell number by promoting proliferation and preventing apoptosis in breast cancer cell lines through regulation of redox response (155).

1.8.1. SETD7 Histone Targets

It was showed that SETD7 methylates H3K4 *in vitro* and *in vivo* (156). H3 methylation at K4 (H3K4me) is presented at actively transcribed genes. H3K4me enhances transcriptional activation by preventing chromatin condensation. Methylation mediated by SETD7 prevents the association of complex that regulates gene expression at the level of chromatin, nucleosome remodeling and deacetylase (NuRD)complex, with H3 tail, suggesting that methylation of histone tails can have distinct effects on transcription depending on its location and enzymes involved on PTM (157). H3K4me inhibits the H3K9 methylation, which is a repressive genetic marker associated with heterochromatin (158). Thus, SETD7 H3K4 methylation is associated with an active genetic state.

H2A and H2B are also SETD7 targets, however little is known about the biological effects of these modifications on cell context. H2B is methylated exclusively at K15 whereas H2A appears to anchorage several methylation sites (159). H1.4 can also be methylated by SETD7. PARP1-mediated ADP –ribosylation of H3 was shown to promote SETD7-dependent methylation of linker histone variation H1.4. Methylation of H1.4 influences directly H1 binding to DNA and, likewise, interfere in chromatin compactation and genetic expression of H1 target genes (146).

1.8.2. SETD7 Non-Histone Targets

SETD7 was shown to have a very broad target specificity in vitro, including transcriptional regulators involved on cell cycle regulation, proliferation, apoptosis, protein degradation such as TATA-box-binding protein associated factor 10 (TAF10), p53, Retinoblastoma(Rb), ERα, Signal transducer and activator of transcription3 (STAT3). SETD7 was also reported to methylate MeCP2, a nuclear protein that recognizes and binds methylated DNA, functioning as a promoter or a repressor, depending on the cell context. Higher expression of MeCP2 was found in neoplastic breast tissue, especially in ERα positive breast cancer (156).

p53 is a tumor suppressor, coordinator of the cellular response to stress signals and transcriptional repressor of genes involved in cell proliferation, as c-Myc (160). p53 methylation SETD7 methylates p53 resulting on p53 stabilization, nuclear export inhibition and transcriptional activation of p53 target genes. That lead to an increasing in p53-mediated G_2/M arrest and apoptosis. Upon stabilization, p53 activates numerous genes involved in cell-cycle arrest, DNA repair, or self-regulation (161,162). In response to DNA damage, sirtuin 1 (SIRT1) methylation catalysed by SETD7, inhibit SIRT1-p53 association, enhances p53 acetylation and lead to subsequently p53 transactivation. That culmines on apoptotic cell death (163).

However, recent studies indicate that SETD7 is dispensable for p53-mediated transcription response to DNA damage (164). So, further studies are needed to clarify the biological role of SETD7 methylation on p53.

TAFs are transcription factors, part of TAFII complex, that binds gene promoters and regulate gene transcription initiation by RNA Polymerase II. SETD7 has a stimulatory effect over TAF10.SETD7 methylation is necessary for increasing TAF10 affinity by RNA Polymerase II recruitment(165).

Forkhead Box O3 (FOXO3) is an activator of genes involved on cellular regulatory pathways including stress resistance and cell cycle arrest. FOXO3 methylation by SETD7 at K271 leads to an increase in its transcriptional activity (166).

Retinoblastoma tumor suppressor protein (pRb) is monomethylated at K873 by SETD7, which is necessary for its function on cell cycle arrest, transcriptional repression and differentiation (167).

E2F1 is a transcription activator important for progression through the G_1/S transition on cell cycle, also (168). SETD7 methylation destabilizes E2F1 which prevents E2F1 accumulation during DNA damage and the activation of pro-apoptotic target genes (169).

STAT3 is also methylated on K140 by SETD7 in response to IL-6 signaling. This methylation a negative regulatory event, because its blockade greatly increases the steady-state amount of activated STAT3 and the expression of many STAT3 target genes. STAT3 is a transcription factor activated in response to cytokines and growth and, when methylated by SETD7, inhibits it binding to DNA promoters genes(170).

DNA (cytosine-5) Methyltransferase 1 (DNMT1) is also a SETD7 target protein. Methylated DNMT1 peaks occur during S and G_2 phases of the cell cycle (171,172). This DNA cytosine-5-methyltransferase 1 is involved on the specific DNA methylation at CpG residues, normally involved in transcriptional repression. Methylation at K142 promoted by SETD7 decreases DNMT1 levels by facilitating its polyubiquitination and subsequent proteasome-mediated degradation, demonstrating that signaling through SETD7 represents a regulatory mechanism of DNMT1 enzyme turnover (172).

ERα is the last and more important non-histone target referred in this monography. *In vitro* assays found that monomethylation of lysine 302 in the ERα hinge region is specifically target by SETD7 (94). Posterior studies revealed that SETD7-mediated methylation of K302 can be impaired by the mutation of neighbouring K303 residue (116). Structural studies show two salt bridge between ERα and SETD7 residues (173). SETD7 knock-down assays reveals that underexpression of SETD7 decreases the steady state level of ERα protein but not mRNA(18). This result suggests that K302 methylation modulates ERα stability. Consistent with these findings, posterior studies reported that estrogen-mediated transcription is inhibited when SETD7 expression is impaired, suggesting that SETD7 is important for ERα activity (18).

In summary, SETD7-mediated methylation stabilizes ERα and is necessary for the efficient recruitment of ERα to its target genes and their subsequently transactivation. Moreover, SETD7-mediated K302 methylation stabilizes ERα by directly competition with ubiquitylation enzymes for the same lysine residue (33).

2. AIMS OF THE STUDY

E_2 stimulates mammary epithelial cell proliferation in breast cancer (174,175). As result, hormone therapy is a common treatment for estrogen-receptor positive breast cancer (47,168,169). However, effectiveness of treatment is limited by endocrine resistance. One of the resistance mechanisms to endocrine therapy is the decrease or loss of ERα expression and/or activity, as well as loss of estrogen dependence even when ERα is still expressed. Multiple molecules have been implicated in endocrine resistance, as ERα itself, coregulators and factors that deregulate ERα stability, activity and response to endocrine therapies (116,170,171). ERα activity and protein levels are regulated by post translational modifications (PTM) (86). SETD7 methyltransferase is a protein that methylates ERα, which contributes for ERα stability, by blocking the ubiquitination of the same residue, and consequently preventing ERα degradation by the proteasome. As result, estrogen-mediated proliferation is enhanced (26,166). SETD7 is also involved in regulation of proliferation once several checkpoints of cell cycle are SETD7 targets (172).

The major goal of this study is to understand if SETD7 modulates the response to E_2 mediated by ERα in mammary epithelial cells and in breast cancer cells, and to establish if this regulation could affect cell proliferation.

The specific aims of this thesis are:

- Understand if SETD7 protein levels are regulated by different mitogens, with focus on the effect of estrogens
- Analyse if SETD7 affects proliferation mediated by ERα
- Study if SETD7 influences ERα protein levels
- Study if SETD7 affects ERα activation by analysing the relative expression of an ERα target protein (RIP140)

3. MATERIALS AND METHODS

3.1. Cell Culture

3.1.1. Agonists and inhibitors

R-PFI-2 (R-PFI) is a potent highly selective and active inhibitor of SETD7 activity with an IC50 value of 2.0±0.2 nM. This inhibitor competes with SETD7 substrate by occupying the substrate peptide binding groove of SETD7 as well the catalytic-binding domain, and by direct contact with donor methyl group of co-factor S-adenosylmethionine (SAM). R-PFI only binds to SETD7 after the binding of its cofactor SAM to the protein (151). R-PFI was diluted in dimethyl sulfoxide (DMSO). The same volume of DMSO was used as negative control for R-PFI condition. R-PFI was a kind gift from Dr. Peter J. Brown, University of Toronto.

The proteasome inhibitor N-[(Phenylmethoxy)carbonyl]-L-isoleucyl-L-α-glutamyl-tert-butyl ester-N-[(1S)-1-formyl-3-methylbutyl]-L-alaninamide (PSI) inhibits chymotrypsin-like activity of the proteasome. It was dissolved in DMSO and the same volume of DMSO was used as negative control for PSI condition.

EGF and FGF_2 are growth factors that stimulate the growth of cells through their receptors tyrosine kinases. Both of growth factors were diluted in dH_2O. EGF was used at a concentration of 10ng/mL and FGF_2 at 50ng/mL.

E_2 is a steroid hormone that is soluble in absolute ethanol. E_2 was dissolved 1000X concentrated and used at a concentration of 10nM. The same volume of absolute ethanol was used as control for E_2 treatment.

3.1.2. Cell lines

In this project, HC11, T47D and MC4L2 cell lines were used as experimental model.

HC11 cells (ATCC CRL_3062, American Type Culture Collection, Massanas, VA, USA) express ERα and were derived from murine epithelial mammary cells. HC11 cells were grown in complete growth medium containing RPMI 1640 medium (Gibco by life technologies Ca, USA), 1% glutamine, 100 µg/mL gentamicin (Gibco), 10% Fetal Bovine Serum (Gibco), 10 ng/mL EGF and 5µg/mL insulin medium (Sigma, Aldrich, St. Louis, MO, USA).

T47D (ATCC HTB-133) cells are human breast cancer cells. This cell line is characterized by its epithelial, adherent morphology and the expression of ERα. T47D cells were cultured in RPMI 1640 medium supplemented with 10% of FBS and 1% PEST.

MC4L2 is a murine mammary ductal carcinoma cell line. MC4L2 cells express ERα (176). These cells grown in DMEM -F12, 1% glutamine, 5mg/mL insulin and10% of FBS.

All cell lines were grown in 37ºC and 5% CO_2 in T-75 culture flaks. The culture medium was renewed every 2-3 days and the cells were trypsined once the culture reached about 80% confluence. Trypsinization was performed using 2-3 mL trypsin-EDTA (Tryple Express Gibco by Life Technologies) for 2-4 minutes at 37ºC.

3.2. Cell Counting

Cells were seeded in a 24 well-plate with a cellular concentration of $5x10^3$ cells/mL. The treatments were given when they reached 50% confluence and were renewed 48h later. The cells were counted the following day (day 3 of treatment). For this purpose, the experimental medium was removed and the cells were washed with 500 µL/well of sterile Phosphate buffer serum(PBS) (137 mM NaCl, 2.7 mM KCl, 1.8 mM $KHPO_4$ and 10 mM Na_2HPO_4 at pH 7.4). The cells were incubated in 200µL/well of pre-warmed trypsin-EDTA for 2-4 minutes and incubated at 37ºC, 5% CO_2 until complete detachment from the plate. Then, 10 µL of FBS were added to each well to stop trypsin reaction and the cells were counted using a Neubeauer counting chamber under a phase contrast microscope. Each treatment was carried out in quadruplicate. All experiments were repeated at least twice.

3.3. Preparation of protein extracts

To prepare cell lysates, the growth medium was removed and the cells were washed twice with ice cold PBS. The cells were gently scraped off the culture dish and transferred to a 1.5 mL microtube and placed on ice. Then they were centrifuge at 14000 rpm for 1 minute. The supernatant was discarded and the pellet was stored at -70 ºC. To extract the total proteins, the pellets were placed on ice and suspended in 100 µL/$1x10^6$cells of RIPA lysis buffer (150 mM NaCl, 1% Triton X-100, 5mM EDTA, 1% sodium deoxycholate, 0.1% SDS, 25 mM Tris-HCl pH 7.6) plus proteinase inhibitor (Roche; dilution:1:50), 1mM of DTT and a phosphatase inhibitor Na_2VO_4 (1:1000). The resulting suspension was vortexed and incubated on ice for 10 minutes and then vortexed again and placed on ice for others 10 minutes. Afterward, the samples were centrifuged at 14000 rpm for 15 minutes at 4ºC and the supernatant was transferred to a new microtube and stored at -70ºC.

3.4. Protein quantification

Protein was quantified by a colorimetric BCA assay for protein quantification (BioRad, California, USA). Bovine Serum Albumine (BSA) was used as protein standard. The curve comprised concentrations 0.3, 0.6, 1.25 and 2.5 mg/mL BSA using a stock solution of BSA 10mg/mL (Sigma). Two wells were prepared with 5µL each of distilled water (dH_2O) to obtain the blank value. The samples were diluted 1:4 in dH_2O. Then 25µL of Bio-Rad DC TM Protein Assay Reagent A plus Bio-Rad DC TM Protein Assay Reagent S (in a proportion of 1mL of A: 20 µL of S) were added to each well. Finally, 200 µL Bio-Rad DC TM Protein Assay Reagent B were added to each well. The plate was covered with parafilm and incubated for 15 minutes at room temperature. After incubating, the absorbance of the plate was read on the spectrophotometer at 750 nm. Standard curve was estimated by plotting absorbance as a function of concentration for the standards. Protein concentration of each sample was determined by extrapolation of standard curve values using linear regression.

3.5. Western Blot

The volume corresponding to 20µg protein was mixed with the same volume of Laemmli buffer 2x (60mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue and 16% β-mercaptoethanol) and the final volume of all samples was adjusted with dH_2O. During this process, samples were always kept on ice. After a short spin, samples were denatured at 100ºC for 5 minutes and then placed on ice.

The proteins were separated according their molecular size by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). Stacking and running gels were prepared according to the percentage required of acrylamide:bisacrylamide. Ammonium persulfate (APS) and tetramethylenediamine (TEMED) were added to start gels polymerization. Protein separation was performed at 200V in running buffer (192 mM glycine, 25 mM Tris, 3.5 mM SDS) using a Bio-Rad Mini-Protean Electrophoresis System. Then, the proteins were transferred from the gel to the nitrocellulose blotting membrane (GE Healthcare, Life Science) with the Trans-Blot Turbo System (Bio-Rad) for 30 minutes at 25 V and 1.0 A using transfer buffer (192 mM glycine, 25mM Tris, 20% Methanol). After transference, the membrane was stained with Ponceau S solution to check if the transference had been correctly performed. Then the membrane was washed with dH_2O until the bands resulting from Ponceau staining were no more visible, to be posteriorly blocked in a blocking solution [5% dry-milk in Tween-TBS (20mM Tris, 150 mM NaCl, pH 7.6 and 0.05% Tween-20)] for 1 hour. Then, the membrane was incubated with the primary antibodies anti-SETD7 (ab14820 abcam; dilution 1:1500), anti-ERα (sc-542, Santa Cruz Biotechnology; dilution 1:300)) anti-RIP140 (Santa Cruz Biotechnology; dilution 1:300), anti-Erk1/2 (Gentex,BD Transduction; dilution 1:1000) and anti-αTubulin(NB600-506, Nouvs Biologicals, CO, USA; dilution 1:5000) in Tween-TBS overnight. After incubation with primary antibodies, the membrane was washed three times with Tween-TBS for 10 minutes each and incubated with secondary antibodies anti-mouse IgG (Sigma, dilution 1:10.000) or anti-rabbit IgG (Sigma, dilution 1:10.000) also diluted in Tween-TBS for 1 hour at room temperature in agitation.

To assure that equal amounts of protein were loaded into the wells and validate all variations in target protein, we used a housekeeping protein reference, α-tubulin or total Erk1/2.

After secondary antibody incubation, the membrane was washed twice with Tween-TBS for 10 minutes and once with TBS (20mM Tris, 150 mM NaCl, pH 7.6) for another 10 minutes. Detection was performed using a chemiluminescent detection assay WesternBright ECL kit (Advansta). Finally, the immunodetection was performed using ChemiDoc Image System software (Life Science, Bio-Rad) and the relative intensity of bands was determined using ImageLab (Bio-Rad software).

3.6. BrdU ELISA

Bromodeoxyuridine enzyme-linked immunosorbent assay (BrdU ELISA) is a colorimetric immunoassay that allow us to quantify cell proliferation, based on the measurement of BrdU incorporation during DNA synthesis in S phase of cell cycle. For this assay, the colorimetric kit Cell Proliferation ELISA, BrdU (kit of Roche) was used. The cells were plated in a 96-well plate in a final concentration of $2x10^4$ cells/mL. When the cells reached a 60-70% confluence the treatments were given to cells. Each treatment was carried out in quadruplicates. After 48 hours, the treatments were renewed and the cells were incubated with 10µM BrdU (Roche, Mannheim, Germany) and incubated overnight at 37ºC, 5% CO_2. Then 100 µL of FixDenat solution were added to each well and the plate was incubated for 30 minutes at room temperature. After removal of FixDenat the cells were incubated with 70µL per well of anti-BrdU-POC working solution in a dilution of 1:1000 for 1 hour at room temperature. Then, the plate was rinsed three times for 5 minutes each with 200µL of PBS and 70µL of substrate solution were added to each well. The cells were incubated at room temperature until colour development was sufficient for a colorimetric detection from 5 to 20 minutes. The absorbance was read at 370 nm and 492 nm (reference wavelength).

3.7. Indirect Immunofluorescence assay

$5x10^3$ cells/mL were seeded in a 24-well plate and grown on sterile glass microscope coverslides until they reached 70% confluence. Then, the treatments were given to each well and the cells were incubated with these treatments for additional 24 hours. Following incubation, cells were washed twice with sterile PBS and fixed with 4% buffered formalin for 10 minutes at room temperature. After two washes with PBS, cells were stored at 4ºC with 1mL of PBS per well. After storage, the cells were washed twice in PBS for 3 minutes and permeabilized with 0,5% Triton X-100 in PBS (250µL/well) for 10 minutes. After washed three times with PBS for 3 minutes, the cells were incubated with 250 µL blocking solution (0,01% Tween and 15% FBS in PBS) for 60 minutes. Following blocking, cells were incubated overnight at room temperature with 250µL/well of the primary antibody anti-Ki-67, (abcam; dilution 1:1000) in PBS overnight. The next day, cells were rinsed three times in 1 mL of PBS/well for 3 minutes and incubated for 1 hour with the secondary antibody anti-mouse (Sigma; dilution 1:400) in blocking solution+DAPI (Sigma-Aldrich, Sto Louis, MO, USA; dilution 0,1µg/mL) in PBS and 6 drops of Caspase Kit (ThermoFischer).

Then, cells were washed with 1mL of PBS/well and mounted in Prolong Gold system (Life Technologies, CA, USA). The slides were allowed to dry 24h at room temperature, in the dark and posteriorly analysed under an inverted microscope (Nikon, Eclipse Ti-U).

3.8. Fluorescence-Activated Cell Sorting Assay

For this assay, the cells were plated on a 3cm diameter plate in a concentration of $2x10^5$ cells/ mL. One plate was used as negative control with unstained cells and another plate was considered the initial time point, with cells not arrested. After the cells reached a confluence of 70-80%, their cell cycle was arrested with serum-free medium for 24 hours. Following 24h arrest, the cells were incubated for 12h and 24h with 10ng/mL EGF. Then, the growth medium was collected in a conical tube and the cells were rinsed with PBS before the addition of 1mL pre-warmed Trypsin-EDTA for 2 minutes at 37ºC. After the complete detachment, the cells were placed on the conical tube with 1mL of RPMI supplemented with 10% of FBS to stop trypsin reaction. The plate was also washed with 1mL of 10% FBS medium and both fractions were pooled. Subsequently, the conical tube with cells was centrifuged at 2500 rpm for 10 minutes and the supernatant was carefully removed. After wash the pellet in 1mL of PBS, the tube was centrifuged again at the same conditions and all the volume of supernatant was removed. After that, the pellet was gently resuspended in 300µL of ice cold PBS to obtain a single cell suspension. Then, the suspension was vortexed softly while 700µL of ice cold absolute ethanol were added and the cells were stored at 4ºC overnight. On the next day, the cells were spinned at 2500 rpm for 10 minutes and washed in 200µL of staining buffer (0,5% Triton in PBS). Then cells were ressuspended in 1mL of staining buffer. Cells were staining with 20µL of propidium iodide (20µg/mL) and incubated for 30 minutes at room temperature. Once stained, the cells were analysed in a flow cytometer under 400 events per second.

3.9. Statistical Analysis

All data was analysed by GraphPad software. Differences between control and treatments was analysed using one-way ANOVA and Tukey post-test or t-tests in the case of comparison between control with only one treatment. Differences between two groups were considered significant if p value < 0,05.

4. RESULTS

Previous work carried out into the lab showed that EGF treatment reduces SETD7 mRNA and protein levels in HC11 non-tumorigenic mammary epithelial cell line (177). This effect correlated with increased cell number. Therefore, we concluded that methylation of specific proteins by SETD7 could be one explanation for the reduction of cell population growth in cells without EGF as compared to those grown with EGF. Consequently, in cells stimulated with EGF, SETD7 reduction would correlate with lower methylation of target proteins and result in increased cell number (Figure 6). Since in this work we aim to study if SETD7 can influence cell growth through targeting ERα signaling pathway, and growth factors can influence ERα protein levels and activation degree, we first studied if EGF influenced ERα protein levels (178,179).

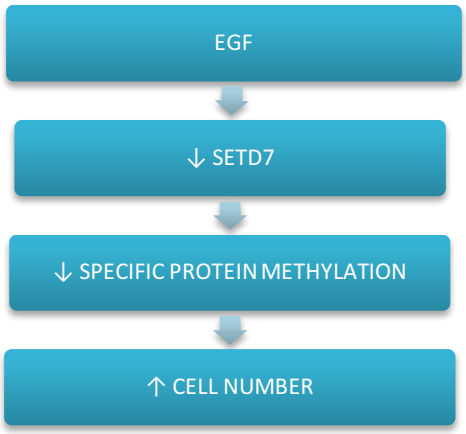

Figure 6: Regulation of EGF mediated by SETD7 in cell number

4.1. EGF regulation of ERα and SETD7 protein levels

It was previously reported that SETD7 methylates and contributes to increase ERα protein stability and, consequently, this induces of cell proliferation (17,18). With the intent of understanding if there is a correlation between ERα and SETD7 protein levels in response to EGF treatment, HC11 cells were stimulated with EGF for 24h and the levels of ERα were compared with the untreated control, as well as our previous blots of SETD7 (Figure 7).

As can be seen, ERα expression in HC11 cell line increased when the cells were stimulated to proliferate with EGF while SETD7 protein levels decreased. Therefore, the results show an inverse correlation between SETD7 and ERα. Notably, others have shown that EGF reduces ERα protein levels, these differences may be related to differences on experimental conditions such as EGF concentration (authors used 0,4ng/mL of EGF) or incubation times, which is not agree with our results (178).

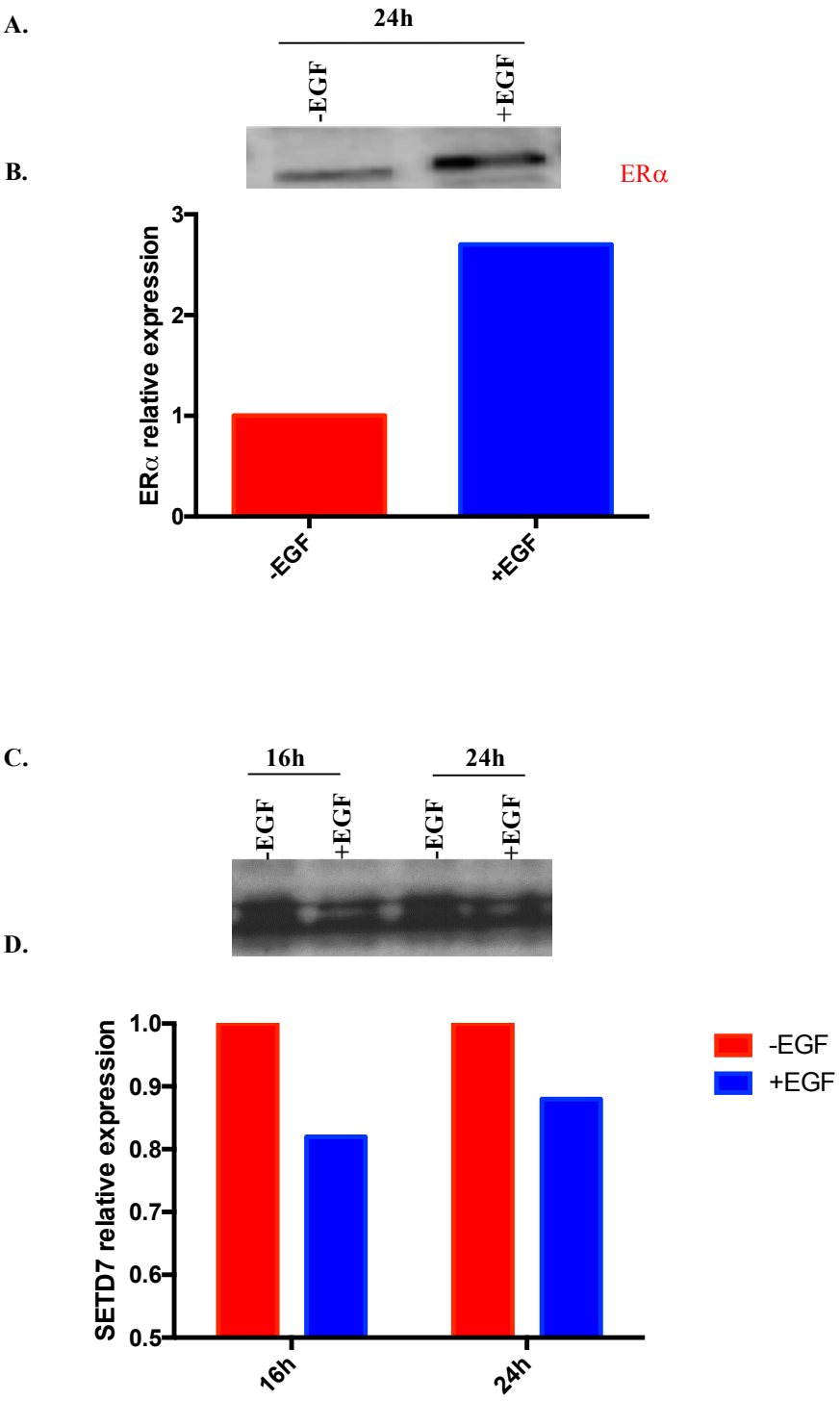

Figure 7: EGF effect on ERαand SETD7 protein levels. **A**. ERα protein levels in HC11 cells after 24h of incubation with and without EGF. 20 µg total protein was separated using a 7,5% SDS-Page. ERα protein levels analysed by WB **B.** Relative intensity after +EGF were normalizes to intensity in -EGF
C.HC11 cells were depleted of EGF for the indicated time intervals. Afterwards, SETD7 protein levels were analysed by Western Blot SETD7 protein levels in HC11. Figure obtained from previous work done in the lab. **D**. SETD7protein levels in HC11 cells after +EGF were normalized to intensity in –EGF.

4.2. Regulation of SETD7 protein levels by proliferative signals in breast cancer

In this part of the study, we aimed to analyse if other mitogenic signals besides EGF can influence SETD7 protein levels in breast cancer cells. We focused on E_2, which induces proliferation by direct activation of cell cycle genes as cyclin D1, and FGF_2 which stimulates proliferation indirectly through the activation of MAPK pathway (180,181).

T47D cells were incubated with 10nM 17ß-estradiol ($+E_2$) or the same volume of ethanol ($-E_2$) for 4h,7h, 16h and 24h. SETD7 proteins level decreased at 4h and 7h incubation with E_2 and again at 24h (Figure 8). These findings suggest that when cells were stimulated to proliferate with E_2, SETD7 proteins level decrease in an early-event. MC4L2 cell line was incubated with or without FGF_2 at a final concentration of 50ng/mL, for 24h. FGF_2 reduced SETD7 protein levels (Figure 9).

Therefore, we conclude that two factors that promote proliferation through different mechanisms seem to have similar effects on SETD7 protein levels in breast cancer cells at 24h. However, more studies are needed to establish if this is sufficient and necessary to promote proliferation induced by either E_2 or FGF_2.

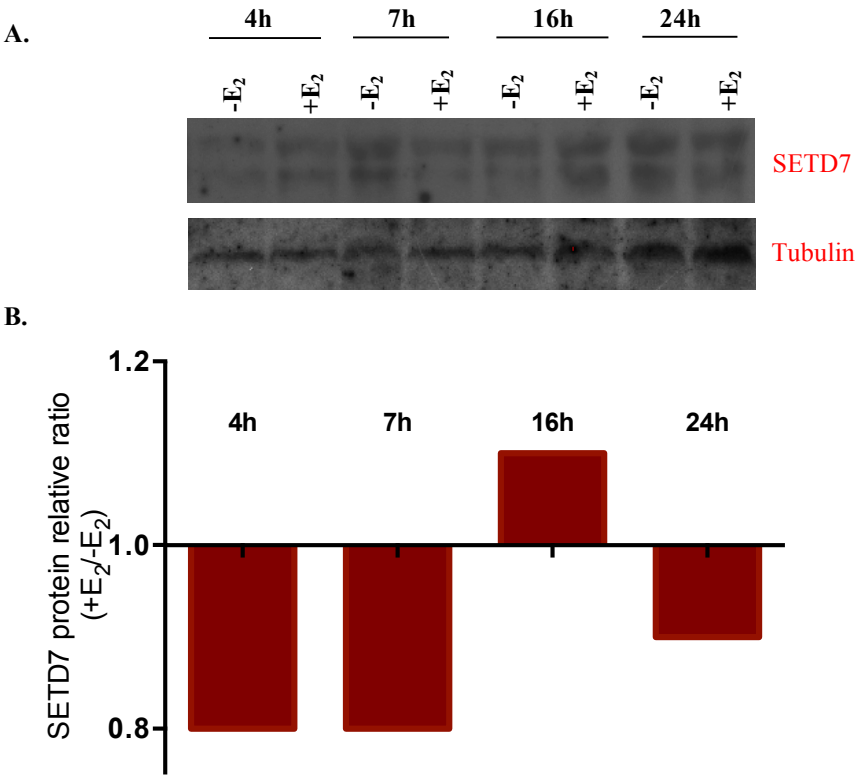

Figure 8:Effect of estradiol on SETD7 expression A. T47D cells were incubated with 10 nM of estradiol($+E_2$) or ethanol($-E_2$) for 2h, 4h, 7h 16h and 24h. Twenty µg of total protein was separated using a 10% SDS-PAGE and SETD7 and housekeeping tubulin protein levels analysed by WB **B.** Relative values of SETD7 band intensity of $+E_2/-E_2$ is in the graph when cells were incubated with 10nM E_2 and the same volume of ethanol in different time points.

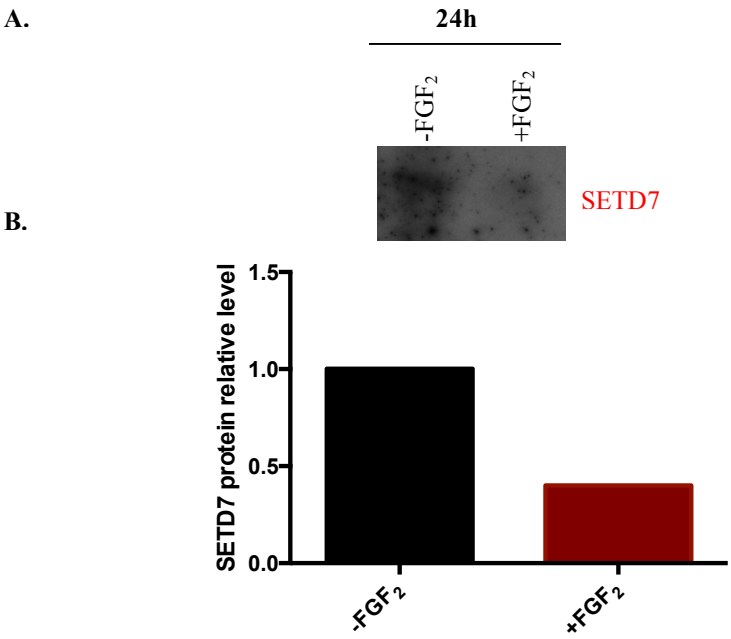

Figure 9: Regulation of SETD7 protein expression by growth factor A. MC4L2 cells were incubated with or without FGF_2 at a concentration of 10ng/mL for 24h. Twentyμg total protein was separated using a 10% SDS-Page SETD7 protein levels analysed by WB **B.** Relative values of SETD7 band intensity of $+FGF_2$ were normalized to intensity in $–FGF_2$

4.3. SETD7 inhibition by R-PFI resulted in to an increase of cell number

As previously reported, SETD7 protein level is negatively regulated by EGF and FGF_2. These growth factors, are also needed for the proliferation and survival of cells. This fact suggests that SETD7 may influence cancer cell proliferation and, consequently, cell number. To evaluate how this methyltransferase influences cell proliferation, a cell counting assay was performed.

T47D, HC11 and MC4L2 cells were grown in growth factor-free medium and, subsequently, treated with different concentrations of SETD7 inhibitor R-PFI (1nM, 8nM or 10nM) or the same volume of DMSO. Following SETD7 inhibition, cell number increased in all cell lines (Figure 10). However, each cell lines need different concentrations of R-PFI to achieve highest cell number. In T47D and MC4-L2, the maximal cellular number was obtained with a concentration of 1nM of R-PFI (Figure 10) and in HC11 this effect was obtained with 8nM of R-PFI (Figure 10). These results suggest that SETD7 is necessary to maintain a low proliferative state in all three cell lines once SETD7 inhibition increases cell number.

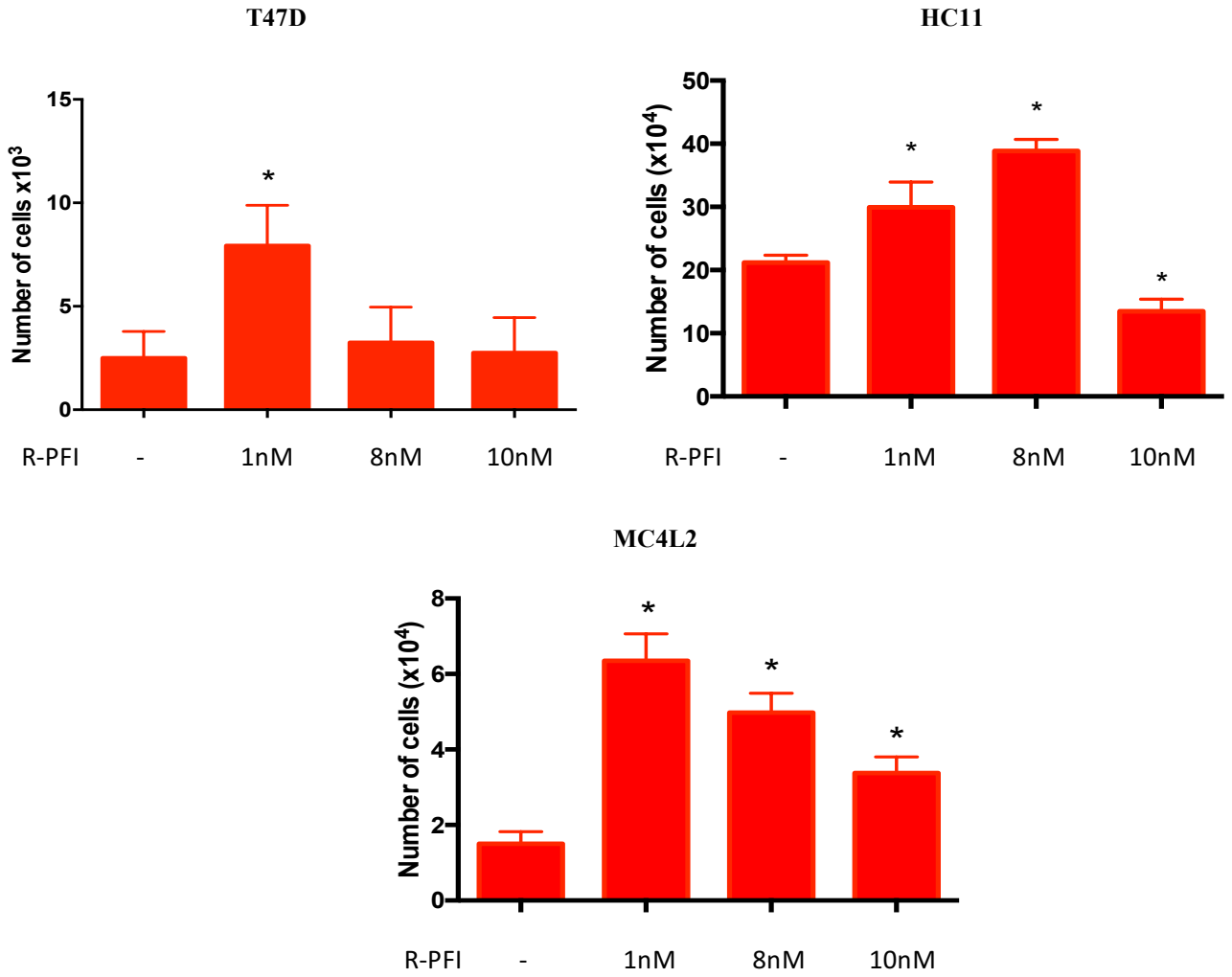

Figure 10:Effect of SETD7 inhibition on cell number in T47D, HC11 and .MC4L2. Cells were treated with R-PFI 1nM, 8nM and 10 nM for 3 days. Then cells were counted in a Neubauer chamber. The graph bars show the mean ± SD from each condition carried out in quadruplicates. Representative of at least 2 experiments. (*) indicates statically significant differences between treatment and control ($p<0.05$).

We were also interested to know if the effect observed after SETD7 inhibition was the same in the presence of EGF which itself induces a higher population growth rate of the cells. Cell counting assays were performed in non-cancer HC11 and cancer T47D cell line, with a concentration of R-PFI of 8nM for HC11 and 1nM for T47D. There was an increase in cell number in cells treated with R-PFI in comparison to control condition (Figure 11). These results suggest that in a high proliferative state, SETD7 inhibition contributes to the increase in cell number. However, the fold of cellular number was lower than in cells grown in the absence of EGF suggesting that SETD7 effect can be dependent of EGF (Figure 12).

In summary, in the presence or absence of EGF, SETD7 methyltransferase activity inhibition lead to an expansion in cell population. Thus, we conclude that SETD7 activity inhibits cell population growth which is

not corroborated by literature. Several studies report that SETD7 overexpression can promote cell proliferation in hepatocellular carcinoma cell line, which do not support our findings (182).

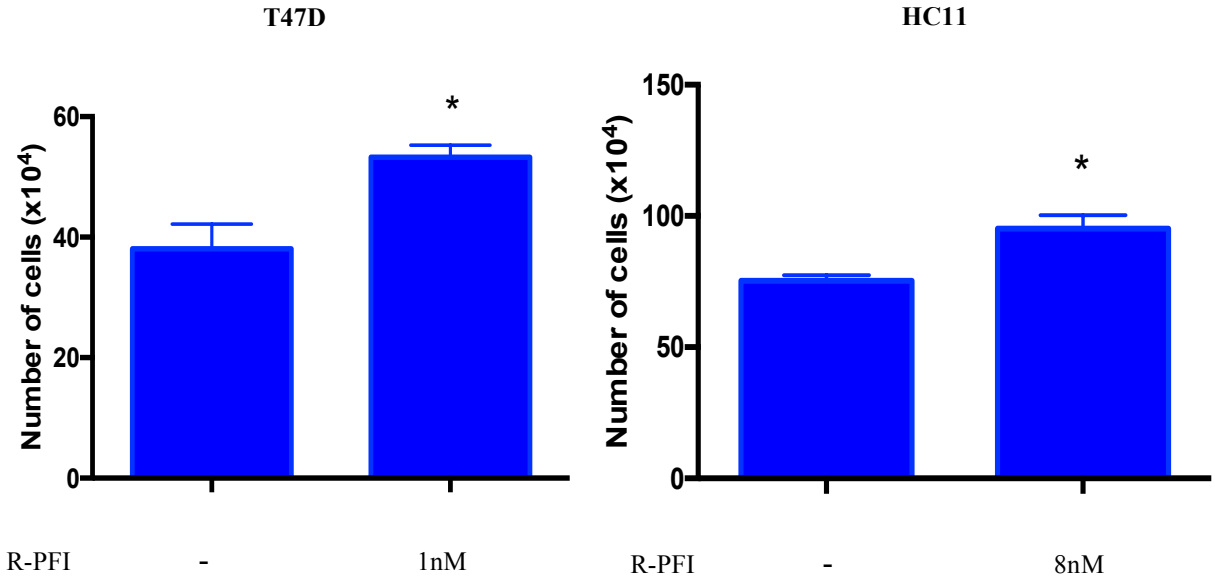

Figure 11:Effect of SETD7 inhibition in proliferating-stimulated cells. Effect of R-PFI 8nM on cell number in T47D and HERE respectively. After 3 days of treatment, cells were counted in Neubauer chamber. Mean ± SD from each condition was carried out in quadruplicates. Representative of at least 2 experiments. (*) indicates statically significant differences between treatment and control ($p<0.05$)

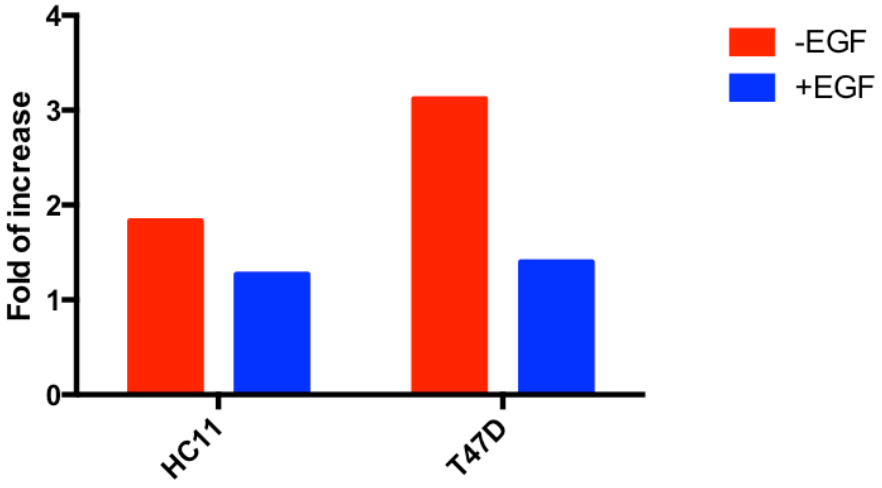

Figure 12: Fold of cellular increase in presence or absence of EGF. Fold was calculated as the median value of cells obtained for PFI 1nM in T47D and 8nM in HC11 in relation to the mean value of control in both cell types.

The combined effect of R-PFI and E_2 in cell number

T47D and HC11 cell lines express ERα which means these cells respond to estrogens and proliferate (22,183,184). In addition, others have shown that SETD7 in necessary to enhance ERα signaling(18). So, it would be expected that SETD7 inhibition would reduce ERα stimulation of proliferation. Thus, we studied what would happen to cell population growth if T47D and HC11 cells were co-treated with R-PFI and E_2. In both cell lines, cell number increased in response to E_2, which was considered a positive control. The same situation was verified when cells were incubated only with R-PFI. But, when cells were incubated with both R-PFI and 10nM E_2, there was no cumulative effect on cell number. In T47D cells the combined effect lead to similar levels as induced by E_2 alone (Figure 13A). This suggests that SETD7 inhibition did not reduce ERα signaling, but the stimulatory effect of R-PFI alone was lost. In HC11 cell line, the combined effect translated into no significant differences in relation to the control condition (Figure 13B), that is both E_2 and R-PFI effects were lost. In this case, it appears that SETD7 inhibition did reduce ERα signaling. Therefore, SETD7 inhibition alone seems to drive cell number population growth to higher levels than E_2 alone. However, contrary to what we observed in cells stimulated with the growth factor EGF, there is no additive effect when R-PFI is combined with E_2. Moreover, in breast cancer cells ERα signaling is maintained even if SETD7 is inhibited, but this is not observed in non-malignant cells.

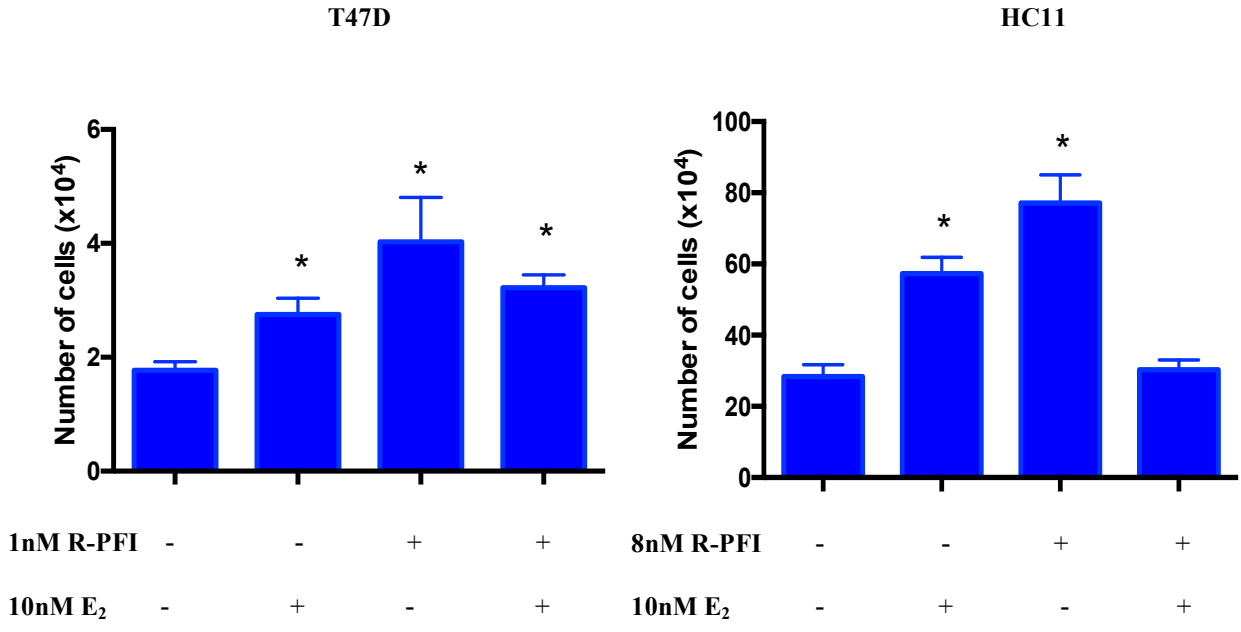

Figure 13: T47D and HC11 cell counting in EGF growth medium. Combined effect of R-PFI and E_2 on cell number in T47D and HC11 cell line respectively. After 3 days of treatment, cells were counted in Neubauer chamber. Mean ± SD from each condition was carried out in quadruplicates. Representative of at least 2 experiments. (*) indicates statically significant differences between treatment and control ($p<0.05$).

4.4. SETD7 regulates cell proliferation

In order to explore why the combined effect of SETD7 inhibition with R-PFI and the mitogenic stimulus with E_2 caused a decrease in HC11 cell number, in comparison to both stimuli alone we analysed if the combined treatment caused cell apoptosis and/or cell cycle arrest.

As a first approach, we intended to study R-PFI, EGF and E_2 effects on the cell cycle phases using Fluorescent activated cell sorting (FACS). For this purpose, we first carried out a set-up experiment to define the best time point to study the cell cycle. We began by incubating HC11 cells with EGF for 12 and 24 h (Supplementary information). However, we had problems with the 24h treatment groups. Nevertheless, we were able to observe an approximately 20% increase in cells in S phase already after 12h treatment. Unfortunately, the FACS machine broke and we could not complete our planed experiments. Therefore, the effect in cell proliferation was analysed with a BrdU colorimetric assay in HC11 cell line. As expected, E_2 and R-PFI alone stimulated proliferation while the combined treatment did not stimulate proliferation (Figure 14). Therefore, we can conclude that SETD7 inhibits cell proliferation, possibly being responsible for maintaining a low cell population growth rate. Moreover, activation of proliferation by ERα needs functional SETD7, because SETD7 inhibition reduced proliferation induced by E_2.

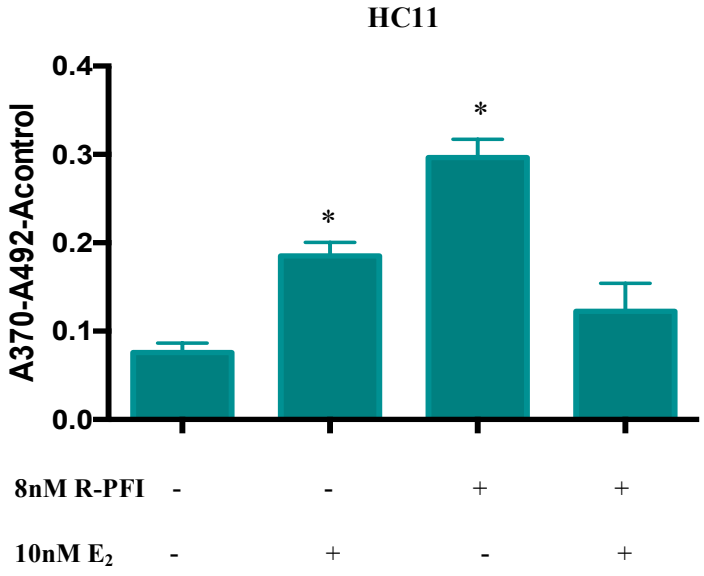

Figure 14: Effect of E_2 and R-PFI on HC11 cellular proliferation. Analysis of BrdU incorporation after 3 days of treatments with 10nM E_2, 8nM R-PFI or the same volumes of DMSO and Ethanol for control. Mean ± SD from each condition was carried out in quadruplicates. (*) indicates statically significant differences between treatment and control ($p<0.05$)

With the aim to confirm the BrdU results and analyse if SETD7 inhibition could have an effect on apoptosis, an indirect immunofluorescence assay was performed in HC11 cells, using two distinct markers: the marker for cell proliferation Ki-67, and the marker for apoptosis, active caspase-3. The antigen Ki-67 is a nuclear protein expressed in proliferating cells, present during cell cycle but absent in non-proliferative cells. These makes Ki-67 a good marker to compare, by immunoassays, proliferative state within a cell population (185–187). Capase-3 is frequently activated when the cell enters the programmed cell death pathway, catalysing the cleavage of several proteins. Because of that, caspase-3 is considered a specific hallmark of apoptosis (121–123). A positive control for apoptosis was obtained by starving HC11 cells in growth medium without serum and glutamine (Figure 15). 10nM E_2 induced an increase in proliferation, once the Ki-67 signal is higher than control condition and the apoptosis decreased. But, after combined 8nM R-PFI and10 nM E_2, we can observe a decrease on Ki-67 signal, and consequently in proliferation, in relation to E_2 treatment alone.

In conclusion, HC11 cells response to E_2 with an increase in cell proliferation. However, when ERα is stimulated and SETD7 is inhibited the proliferative signal activated by E_2 is lost. This explain the results obtained by cellular counting when HC11 are treated with both E_2 and R-PFI (Figure 13).

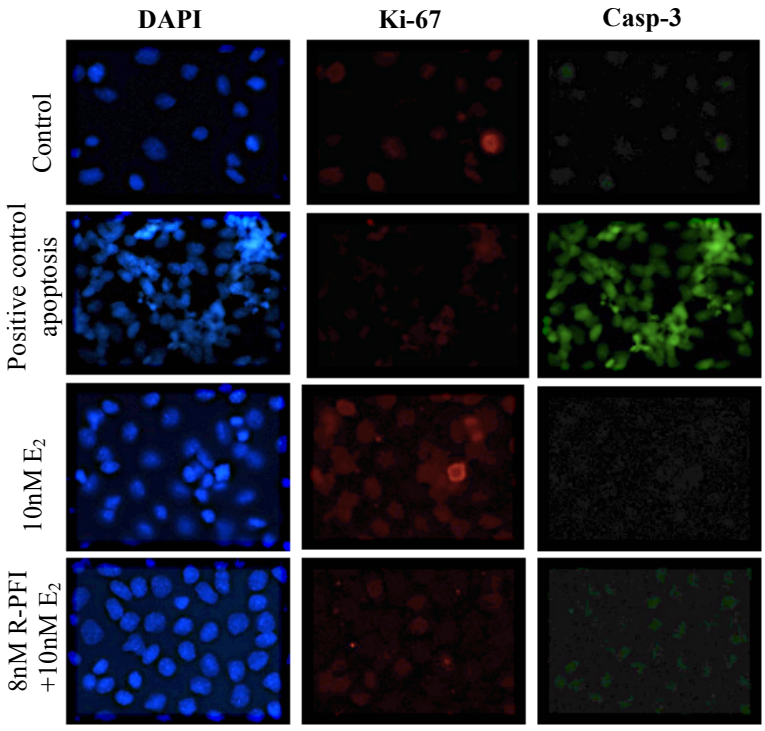

Figure 15: Effect of R-PFI and E_2 on cell cycle and aoptosis in HC11. Analysis of Ki-67 antigen marker (red) and Caspase-3-mediated apoptosis (green) by indirect immunofluorescence. In blue are cells nuclei stained with DAPI. HC11 were treated with 8nM R-PFI (or the same volume of DMSO), 10nM E_2 (or the same volume of ethanol) for 24hours.

4.5. SETD7 regulates ERα protein levels

With the aim to study how SETD7 regulates ERα protein levels in T47D breast cancer cell line, cells were co-treated with or without R-PFI at a final concentration of 1nM for 24h and 48h. 10nM proteasome inhibitor PSI was used as control, to compare how ERα protein levels increased when proteasome is inhibited. When SETD7 activity was inhibited by R-PFI, ERα protein increased approximately 3-fold and 7-fold higher in relation to the control, at 24 hours and 48 hours respectively (Figure 16A and 16B). The same was observed when cells were incubated with the proteasome inhibitor because using PSI, proteins tend to accumulate in cell (Figure 16). This is in agreement with the knowledge that the proteasome degrades ERα bound to E_2 once it has completed activation of transcription (188).

In summary, SETD7 inhibition results in an accumulation of ERα overtime, a similar effect as observed with the PSI. Therefore, we propose that SETD7 may induce ERα protein degradation, suggesting that SETD7 might inhibit E_2-induced proliferation through increased ERα protein turn-over. Alternatively, this may be related to inhibition of ERα gene transcription. However, combined SETD7 inhibition -which increases ERα- with E_2 treatment reduce proliferation, this may be related to loss of ERα transcriptional activity and/or reduced H3K4me induced in specific genes by SETD7 inhibition.

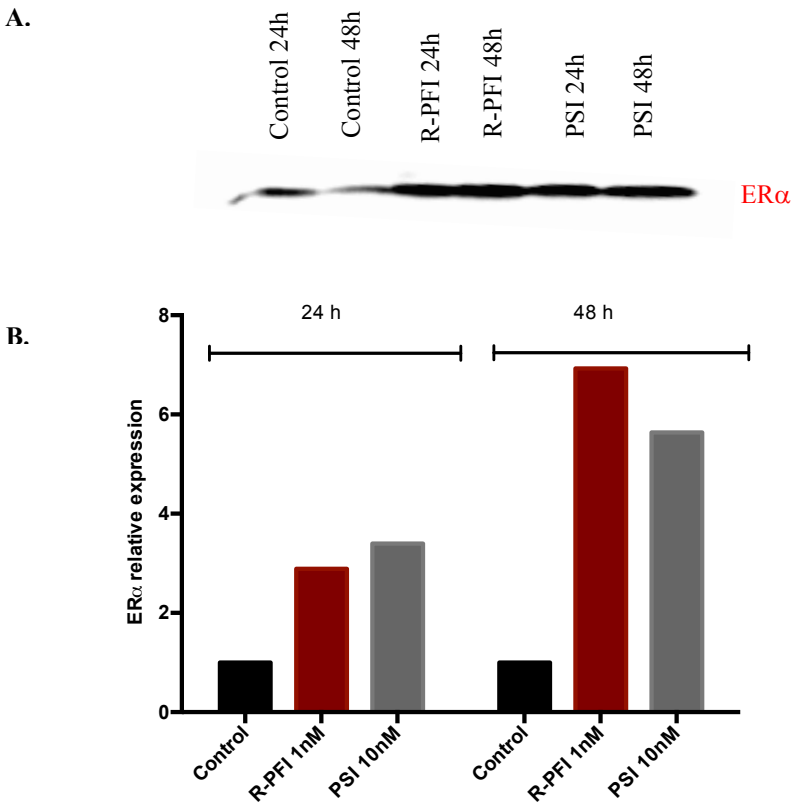

Figure 16: Effect of SETD7 inhibition and proteasome inhibition in T47D after 24 and 48 hours A. Cells were co-treated with R-PFI at 1nM and PSI 10 nM or with the same volume of DMSO in case of control. Twentyμg total protein was separated using a 7,5% SDS-Page. Afterwards, ERα protein levels were analysed by WB. **B.** Relative quantification of the intensity of the bands in the blot in relation to 24h and 48h.

4.6. ERα protein activity is influenced by SETD7

With the aim to study if SETD7 regulates ERα activity, we analysed the protein levels of an ERα target gene by Western blot. We chose nuclear receptor co-repressor nuclear repressor interacting protein 1 (RIP140), a transcriptional downstream target gene of ERα(124–126). We incubated HC11 cells in the presence or absence of SETD7 inhibitor R-PFI, proteasome inhibitor PSI and E_2. HC11 cells were incubated with those treatments for 24h. As expected, when HC11 cells were treated with E_2, RIP140 protein levels increased (Figure 17). When SETD7 was inhibited by R-PFI, RIP140 levels remained similar to the control possibly because of the absence of E_2 (Figure 17). The co-treatment of cells with both R-PFI and E_2, resulted in RIP140 levels similar to E_2 alone which suggests that SETD7 inhibition does not affect ERα transcriptional activity. When we combined PSI and E_2, the levels of RIP140 decreased in relation to E_2. this was expected because transcriptional activity of ERα is dependent on its own degradation in a ligand-dependent manner.

In summary, while SETD7 inhibition blocks E_2/ERα-induced proliferation, this may be related to reduced ERα protein levels but not to its activity.

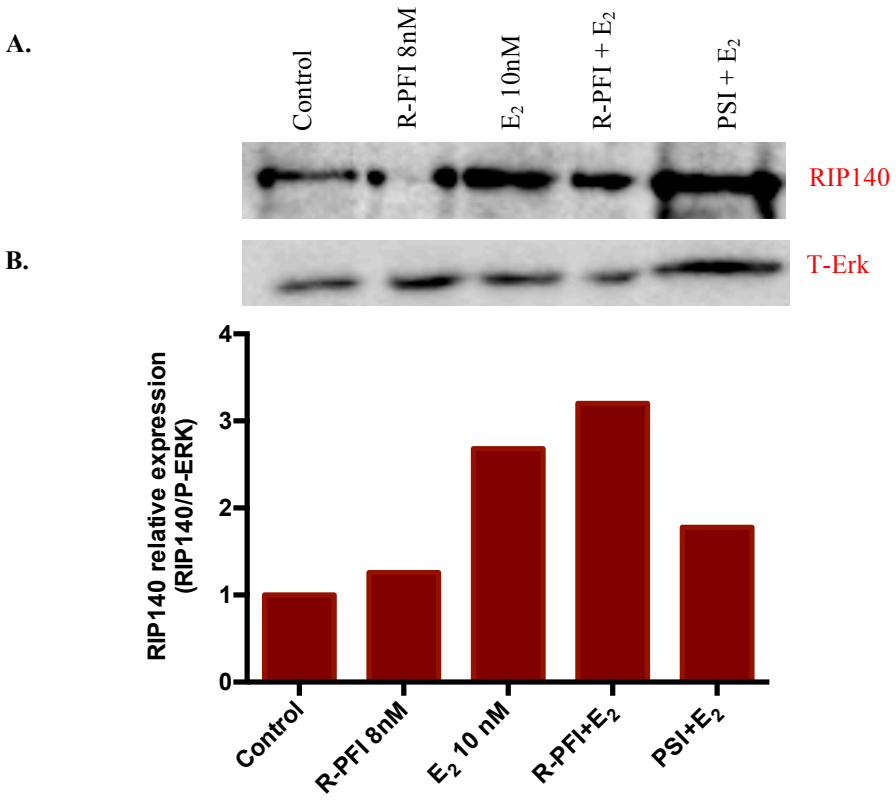

Figure 17: Effect of SETD7 and proteasome inhibition on ERα activity through RIP140 protein levels. A. Cells were co-treated with R-PFI at 8nM, PSI 10 nM and E_2 or with the same volume of DMSO or Ethanol in case of control. Twenty µg total protein was separated using a 12% SDS-Page. Afterwards, RIP140 protein levels and housekeeping T-Erk were analysed by WB. **B**. Relative quantification of the intensity of RIP140/P-EKR protein level in relation to control condition.

5. DISCUSSION

Approximately, 70% of breast cancers express ERα and they initially depend on estrogen signaling for growth. Anti-estrogen therapy, as tamoxifen, is the standard treatment for ERα-positive breast cancers. However, its efficacy is limited by initial or acquired resistance to endocrine therapy (189,190). The key pathways involved in endocrine resistance are: 1) upregulation of growth factors pathways that promote ligand-independent ERα activation, 2) loss of ERα expression and 3) the mutant ERαthat are constitutively active in the absence of estrogen (191–193).Thus, it is important to understand how ERα is regulated in order to achieve a better treatment.

ERα is subject of several post translational modifications, where SETD7 appears to methylate, stabilize ERα protein and enhance its transcriptional activity. For this reason, we decided to study SETD7 expression and function in breast cancer cells, and understand the biological association between SETD7 with ERα in the regulation of cell proliferation.

Our findings suggest that SETD7 methyltransferase protein levels are down-regulated by proliferative signals in cancer, including E_2, and the growth factors EGF and FGF_2 (194–196). Furthermore, a previous study done in our lab showed that SETD7 protein level is inhibited by MAPK activation by EGF. E_2, EGF and FGF_2 reduced SETD7 protein levels, suggesting that SETD7 protein levels must be lowered to allow cell proliferation. Our results are supported by literature where one study reported that SETD7 is preferentially expressed in differentiated and non-proliferative cells (147).

Next, we were interested in understanding the impact of SETD7 inhibition on cell number. Our results show that the cellular number increased when SETD7 was inhibited by R-PFI in T47D, HC11 and MC4L2. In breast cancer cells 1nM lead to a great cell number and in HC11 cells the maximal number was obtained at 8nM R-PFI. We also verified an increase in cell number when cells were co-stimulated with EGF and R-PFI. This result can be explained through Hippo pathway regulation by EGF (Figure 18), which negatively regulates proliferation(64). When cells are stimulated with EGF, EGF activates downstream pathways as MAPK that recruit AJUBA proteins leading to YAP translocation to the nucleus where it functions as transcriptional coactivator of proliferation genes by interacting with transcription factors and cyclin D1(197). Previously, we showed that MAPK inhibition decreased SETD7 levels (177), and others have shown that SETD7 methylates YAP and promotes its sequestering in the cytoplasm (198). Thus, SETD7 inhibition could impair cytosolic retention of YAP, leading to proliferation and concomitant increase of cell number (Figure 18).

In the absence of EGF and presence of R-PFI, the fold of cell increase observed in the stimulation of cell population growth was higher than in cells already stimulated with EGF. This is probably because the proliferative rate in the later, with lower SETD7 levels was already close to the maximum achievable probably by the culture conditions as confluence. It remains unresolved whether the increase in cell proliferation in cells

treated or not with EGF is caused by SETD7 methylation of YAP protein itself or if SETD7 additional substrates are involved. Still, we can associate SETD7 activity to a diminished cell number.

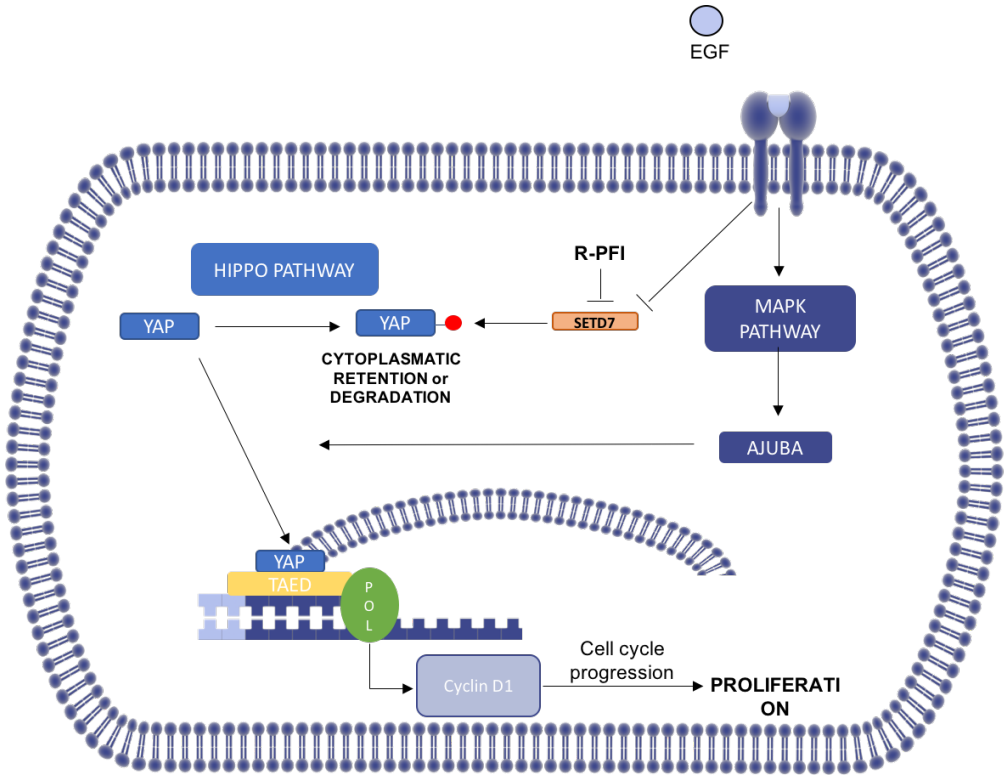

Figure 18: Regulation of cell proliferation YAP translocation. YAP translocation can be regulated by EGF downstream pathways as MAPK. MAPK is involved in regulation of SETD7 levels and recruitment of AJUBA proteins. These processes enhance nuclear translocation to nucleus and increase cell proliferation by cell cycle progression

Next, we were interested if the same effect was verified when cells were stimulated to proliferate by activation of ERα. This question is relevant because others had shown that SETD7 increases ERα protein levels and enhances its transcriptional activity (18,94). Hence, based on our own results with EGF, what would be the effect of SETD7 inhibition in cells treated with E_2? When we combined SETD7 inhibition and E_2 the E_2 effect was blocked or reduced. That result raised another question: why the combined treatments didn´t generate an increase in cell number as did the treatments alone? Our findings reveal that the combined exposure to $E_2$and R-PFI caused an arrest on cell cycle thus blocking cell proliferation.

We also reported that there was an inverse correlation between SETD7 proteins level and ERα proteins level when cells were stimulated to proliferate with EGF. So, when cells were stimulated with EGF, SETD7 levels diminished and the levels of ERα increased. Our results are not supported by literature because several studies reported a reduction on ERα protein and mRNA levels after EGF stimulation(199). But, one study proposes that ERα could interact with EGFR after E_2 activation and, through MAPK pathway, induce the increase of ERα levels. Moreover, several results indicate that there is a requirement of the ERα to drive EGF-

mediated functions(200,201). Other studies suggest that MAPK-mediated phosphorylation at S118 of the receptor protects ERα from degradation via UPS (202). This models could explain the increase on ERα protein levels after EGF stimulation. However, we need to carry out additional studies to establish the interplay between EGF, ERα and SETD7.

We explored the effect of SETD7 activity in regulation of ERα protein levels. Our findings show that when SETD7 activity is compromised, ERα protein levels increase. A similar behaviour was obtained when cells were incubated with proteasome inhibitor PSI. Despite literature reports that SETD7 increases ERα protein stability, our results show that when SETD7 activity was inhibited the ERα protein levels are identical to the levels obtained when proteasome degradation is inhibited. Consequently, it is possible that SETD7 activity negatively regulates ERα turnover. We propose that SETD7 might inhibit E_2-induced proliferation through increased ERα protein degradation or might negatively regulate ERα gene expression (Figure 19). It was reported that N-terminal domain of Rb protein interacts with ERα in breast cancer cells allowing the assembly of ERα-Hsp90-p23 protein complex. This complex protects ERα from degradation by the UPS(122). As Rb is a SETD7 target, we propose that SETD7 could regulate ERα degradation by inhibition of Rb/ERα interaction (Figure 19). But further studies are needed to confirm this hypothesis.

Finally, we evaluated the impact of SETD7 on ERα activity through measurement of the ERα target gene RIP140 protein product. E_2 induces RIP140 transcription (203,204). This was verified by us at the protein level. RIP140 is an ERα transcriptional co-repressor, so that following E_2 activation of ERα, RIP140 cellular levels is high and there is a negative feedback regulation of ERα activity (205,206). For this reason, when cells were incubated with R-PFI, the levels of RIP140 were maintained because even though there was more ERα protein levels, there was no E_2. However, when E_2 and R-PFI were used together RIP140 levels increased, suggesting that SETD7 inhibition did not affect ERα activity. Thus, considering that the amount of ERα is proportional to ERα activity, SETD7 regulates the protein levels of ERα and SETD7 inhibition for 24h does not reduce ERα activity.

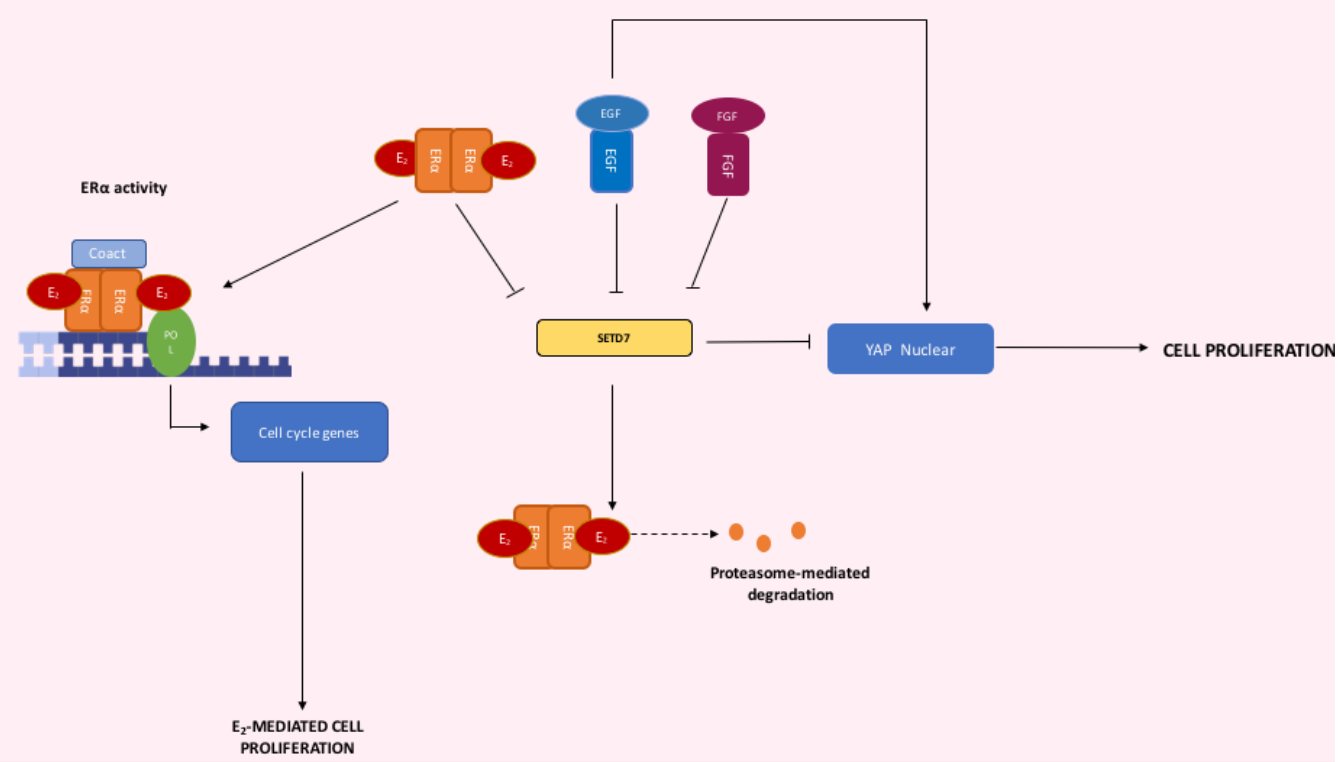

Figure 19: Purposed model of SETD7 regulation in breast cancer cell fate. SETD7 protein levels are upstream controlled by growth factors EGF and FGF as well E_2. SETD7 could negatively control cell proliferation through inhibition of translocation of YAP to nucleus. SETD7 could also be associated to ERα proteasome mediated degradation
"→" indicate possible interactions in agreement with our results; "-->" indicate a purposed interaction to be posteriorly proved. Coact: Coactivator; ERα: Estrogen Receptor α ; E_2: 17ß-estradiol; Pol: RNA Polimerase II

6. CONCLUDING REMARKS

In summary, in this thesis we successfully established SETD7 as a regulator of breast cancer cell proliferation and have gathered some information regarding the role of SETD7 on ERα protein levels and activity. Hence, we can consider SETD7 an important protein regulating multiples signaling pathways relevant to breast cancer proliferation, including estrogen signaling and RTK-mediated signaling.

We linked SETD7 to the regulation of cell number and cell proliferation. When cells are stimulated to proliferate by E_2, EGF or FGF_2, SETD7 levels decreased, suggesting that in a high proliferative state, the cell need to decrease SETD7 levels. Moreover, SETD7 activity was necessary to restrict cell number, in the absence of E_2.

We show that SETD7 activity regulates ERα protein levels, possibly promoting ERα degradation, but it probably dispensable for ERα transcriptional activity in RIP140 target gene, although this hypothesis needs to be confirmed by analysing RIP-140 mRNA levels and ERα binding to the RIP-140 promoter SETD7 is indicated as inhibitor and promotor of degradation of many substrates. The regulation of ERα can be dependent on many factors, other PTMs or even the cell type. Hence, further studies are required to support our findings and to verify if the effects here reported can be observed in different breast cancer cell lines. Thus, the next step to carry on would be analyse if the levels of ERα-ubiquitinated decrease when SETD7 activity is inhibited with the aim of confirm if SETD7 promotes ERα protein degradation.

SETD7 activity contribute to maintain low levels of ERα protein. In that case, SETD7 also play a role as breast cancer tumour suppressor, since overexpression of ERα is frequently observed in early stage of breast cancers (207).

Many of SETD7 targets, including ERα, are involved in carcinogenesis, suggesting that the modulation of SETD7 activity could be a new strategy for breast cancer treatment. Our results indicate that specific agonists of SETD7 activity could be used for ERα negative breast cancer which is more dependent on EGF signaling. In case of ERα-positive breast cancer, SETD7 inhibitors would be preferred.

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8. SUPPLEMENTAL INFORMATION

8.1. FACS analysis of cell cycle

With the aim to check if the cells could be cell cycle arrested it has been realized a setup experiment using FACS analysis. In that experiment T47D were normally grown and cyclate on growth factor-supplemented medium (t_0). When the cells reached 80% of confluence the medium has been changed by a growth factor-free medium (-EGF). In that point, cells were supposed to be arrested on cell cycle (synchronized). Then, 24h later the cells synchronized were stimulated with EGF for 12h (EGF 12h) to restart cell cycle.

By the results obtained, on the figure below it can be observed that at t_0 (Figure 20A; A02 T0 box), most of the cells are distributed by G_0/G_1, S and G_2/M phase. When the cells were synchronized, it can be observed an increase on the cells arrested on $G_/G_1$ of the cell cycle and a decrease on the relative frequency of cells in S phase, in comparison to t_0 (Figure 20A; A03 –EGF box) Finally, once the cells are stimulated with growth factors to proliferate again, there is an increase of cells on S phase, in comparison to –EGF condition (Figure 20A; A04 EGF12hs box) and a reduction of cells in G_2/M. That reduction could be explained by the cells that had complete cell cycle. In conclusion, 24 hours of growth factor-free medium apparently was sufficient to induce an arrest on cell cycle of T47D cells and a stimulation with EGF for 12h was also sufficient to cells restart to recycle. The next step of this experiment will be measure by FACS the impact of R-PFI SETD7-inhibitor, E_2 and PSI proteasome inhibitor on each phase of cell cycle to confirm the results previously presented by BrdU and immunofluorescence technique.

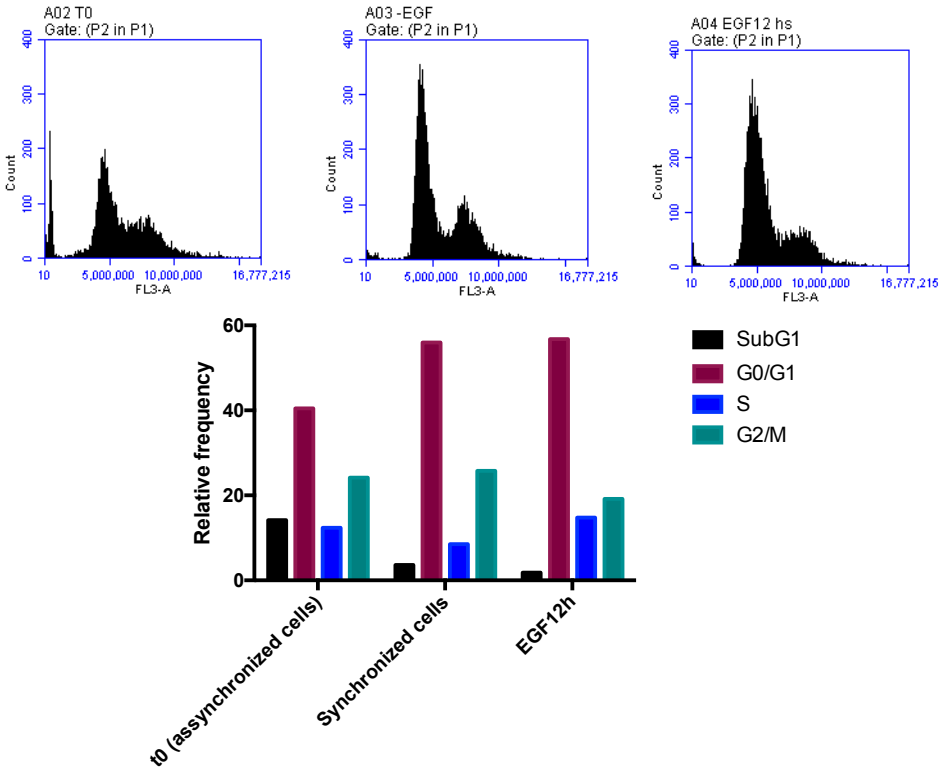

Figure 20: Cell cycle analysis by FACS assay in T47D cells. A. T47D normally cycling at FBS-supplement medium, T47D synchronized by FBS-free medium for 24h and T47D synchronized were incubated with EGF 10ng/mL for 12h **B.** Relative frequency of cell cycle phases on t_0, -EGF and EGF12h.