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POST-TRANSLATIONAL MODIFICATION OF ESTROGEN RECEPTOR ALPHA AND P53 IN BREAST CANCER CELLS

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Post-translational Modification of Estrogen Receptor α and p53 in Breast Cancer Cells

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my parents and my wife!

ABSTRACT

Estrogen receptor α (ERα/ESR1) and tumor protein p53 (p53/TP53) signaling are aberrant and play important roles in breast cancer pathogenesis and evolution. ERα is highly expressed in the majority of breast cancers and is an important contributor to the development of these tumors. p53 aberrances occur as mutations resulting in defective protein or as decreased expression due to genomic mutations or deletions and genetic hyper-methylation. Besides regulation at the genome and transcriptome levels, accumulated evidence shows that post-translational modifications of ERα and p53 also play critical roles in cancer cell proliferation and therapeutic resistance. The overall aim of this thesis was to characterize the protein modification properties of the E3 ubiquitin ligase RNF31 on ERα and p53 signaling and the effect of p21 protein (Cdc42/Rac)-activated kinase 4 (PAK4) phosphorylation on ERα signaling.

In the first study, we find that the E3 ubiquitin ligase ring finger protein 31 (RNF31) is highly expressed in breast cancers, and increases estrogen-stimulated cell proliferation by facilitating estrogen signaling. Furthermore, we show that RNF31 interacts with ERα via its RBR (Ring between ring fingers) domain and induces ERα mono-ubiquitination resulting in increased ERα levels. This modification occurs in the cytoplasm and depends on the ligase activity of RNF31.

In the second study, we investigate the epithelial-mesenchymal transition (EMT) phenomenon in triple-negative breast cancer (TNBC) cells. We provide evidence that AP-1 signaling contributes to EMT in TNBC cells via activation of its target gene ZEB2. We demonstrate that AP-1 binds to two distinct cis-regulatory regions of ZEB2 and regulates its expression by mediating long-range chromatin interactions.

In the third study, we identify that RNF31 is involved in the p53 pathway based on an unbiased approach exploring global gene expression profiling data. We show that RNF31 inhibits p53-dependent cell cycle arrest and cisplatin-induced apoptosis in wild type p53 breast cancer cells. Depletion of RNF31 increases p53 protein levels and its target genes. We demonstrate that RNF31 interacts with the p53/MDM2 complex and facilitates p53 poly-ubiquitination and degradation, possibly by modifying MDM2 stability.

In the fourth study, we investigate the effect of PAK4 phosphorylation on ERα and its association with tamoxifen sensitivity. PAK4 expression is found to correlate with poor tamoxifen response in data from multiple clinical databases. We show a feed-forward regulation between PAK4 and ERα signaling. PAK4, which has been demonstrated to be a direct target gene of ERα, increases ERα stability and phosphorylates ERα on the S305 site. This phosphorylation facilitates activation of ERα signaling.

In conclusion, our data identify the E3 ubiquitin ligase RNF31 as a modulator of both ERα and p53 protein levels, thus facilitating breast cancer cell proliferation in a dual manner. We also identify that PAK4 plays a role in ERα signaling and tamoxifen resistance and that AP-1

regulates ZEB2 and contributes to EMT phenomenon. We suggest that RNF31 and PAK4 might be useful therapeutic targets in ERα-positive breast cancer and add new knowledge about the role of ZEB2 in TNBCs.

LIST OF SCIENTIFIC PAPERS

I. **J Zhu**, C Zhao, A Kharman-Biz, T Zhuang, P Jonsson, N Liang, C Williams, C-Y Lin, Y Qiao, K Zendehdel, S Strömblad, E Treuter, K Dahlman-Wright: The atypical ubiquitin ligase RNF31 stabilizes estrogen receptor α and modulates estrogen-stimulated breast cancer cell proliferation. Oncogene 01/2014; DOI:10.1038/onc.2013.573; 33(4340-4351).

II. Yichun Qiao, Chiou-Nan Shiue, **Jian Zhu**, Ting Zhuang, Philip Jonsson, Anthony P H Wright, Chunyan Zhao, Karin Dahlman-Wright: AP-1-mediated chromatin looping regulates ZEB2 transcription: new insights into TNFalpha-induced epithelial-mesenchymal transition in triple-negative breast cancer. Oncotarget, 2015. 6(10): p. 7804-14.

III. **Jian Zhu**, Chunyan Zhao, Ting Zhuang, Philip Jonsson, Cecilia Williams, Staffan Strömblad, Karin Dahlman-Wright. RING finger protein 31 (RNF31) promotes p53 degradation in breast cancer cells. Oncogene 07/2015; DOI: 10.1038/onc.2015.260.

IV. Ting Zhuang*, **Jian Zhu***, Zhilun Li, Julie Lorent, Karin Dahlman-Wright, Staffan Strömblad. Pharmacological targeting of p21-activated kinase-4 inhibits estrogen receptor alpha signaling and restores tamoxifen-sensitivity in breast cancer cells (Manuscript).

T Zhuang* and J Zhu* contributed equally to the work.

CONTENTS

LIST OF ABBREVIATIONS

ADORA1	Adenosine A1 receptor
AF-1	Activator function 1
AF-2	Activator function 2
AP-1	Activator protein 1
ATM	Ataxia telangiectasia mutated
BAX	BCL2-associated X protein
BMP	Bone morphogenetic protein
BRCA1	Breast cancer 1, early onset
BTG2	BTG family, member 2
ChIP	Chromatin immunoprecipitation
CHX	Cycloheximide
CHK1	Checkpoint kinase 1
E2	17β-estradiol
EdU	Ethynyl-deoxyuridine
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
eNOS	Nitric oxide synthase (Endothelial cell)
ER	Estrogen receptor
ERE	Estrogen response element
ERK	Extracellular signal-regulated kinase
FAS	Fas cell surface death receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPCRs	G-protein-coupled receptors
GREB1	Growth regulation by estrogen in breast cancer 1
HER2	Human epidermal growth factor receptor 2
IGFR	Insulin growth factor receptor
IGFBP3	Insulin-like growth factor binding protein 3
IKKY	Inhibitor of Kappa light polypeptide gene enhancer in B-cells, Kinase Gamma
MAPK	Mitogen-activated protein kinase
MDM2	Murine double minute clone 2
NFKB	Nuclear factor kappa B

NO	Nitric oxide
OS	Overall survival
PAK1	P21 activated-kinase 1
PAK4	P21 activated-kinase 4
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKIB	Protein kinase (cAMP-dependent, catalytic) inhibitor beta
PR	Progesterone receptor
RAS	Rat sarcoma viral oncogene homolog
RBCK1	RanBP-type and C3HC4-type zinc finger containing 1
RBR	RING-In-Between-RINGs
RNF31	RING finger 31
SERMs	Selective estrogen receptor modulators
SRC	SRC proto-oncogene
siRNA	Small interfering RNA
TAM	Tamoxifen
TCGA	The Cancer Genome Atlas
TFF1	Trefoil factor 1
TGFβ	Transforming growth factor beta
TNBC	Triple negative breast cancer
TNF	Tumor necrosis factor
TNM	Tumor, Nodes and Metastases
TP53	Tumor protein 53
Ub	Ubiquitin
ZEB1	Zinc finger E-box-binding homeobox 1
ZEB2	Zinc finger E-box-binding homeobox 2

1 INTRODUCTION

1.1 BREAST CANCER

Breast cancer ranks number one of diagnosed cancers in women worldwide. According to the world epidemiological report of 2012, breast cancer accounts for about 23% of the cancer incidence and 13.7% of cancer-related death in women [1]. Risk factors that are known to contribute to breast cancer include age (over 55), race (white), density of breast (dense), menstrual periods (early menarche and late menopause), previous chest radiation and diethylstilbestrol (DES) exposure [2]. In addition, around 5-10% of breast cancers have a hereditary component, including mutations in genes such as BRCA1, BRCA2, ATM and p53. Among these genes, mutations in BRCA1 and BRCA2 are the most common cause of hereditary breast cancer [3].

Breast cancer can be classified into stages or subtypes according to diverse criteria, such as pathological type, tumor, nodes and metastases (TNM) staging, and molecular subtype classification [4]. These criteria reflect the characteristics of the malignancy. For example, invasive breast cancer can be separated into infiltrating ductal carcinoma, invasive lobular carcinoma, ductal lobular carcinoma, tubular carcinoma, mucinous carcinoma and medullary carcinoma according to the pathological classification [5]. The TNM stage is classified according to the tumor size, lymph node infiltration and distant metastasis, which reflect the relative prognosis in relation to surgery [6, 7]. The most significant advance of the recent 20 years is the receptor status classification based on estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER2) positivity, and the subsequent targeted therapy [8]. According to molecular subtype classification, based on gene expression profiles, breast cancer is divided into the following groups; Luminal A, Luminal B, HER2-enriched, normal-like, and basal-like tumors (Table 1) [9]. The molecular classification is an important reference for prognosis and choice of treatment strategy [10]. The Luminal A breast cancer subtype, which is mostly ER α positive, PR positive and HER2 negative, has the best prognosis [5]. Selective antagonists of ERα such as tamoxifen and/or aromatase inhibitors, such as letrozole, usually achieve good efficacy in Luminal A breast cancer [11]. Aromatase inhibitors correspond to inhibitors of endogenous estrogen synthesis [12]. The Luminal B subtype is mostly ERα and PR positive but is distinguished from the Luminal A subtype by high expression of Ki-67 and HER2 [13]. The Luminal B subtype of breast cancer is more aggressive than the Luminal A subtype [13]. Patients with the Luminal

B subtype can in many cases benefit from ERα antagonists and/or aromatase inhibitors [14], as well as HER2 targeted therapy. For HER2-enriched subtypes, Trastuzumab, which is a specific antibody for HER2, is available as a targeted therapy [15]. Due to a lack of validated drug targets for the basal-like subtype, which are mostly triple-negative breast cancer (TNBC), chemotherapy is the primary treatment for this group [16].

Table 1. Molecular subtype of breast cancer

SUBTYPE	MOLECULAR MARKERS	FIRST TREATMENT	PERCENTAGE
Luminal A	ER+, PR+, HER2-, Ki67 low	Hormonal treatment	40%
Luminal B	ER+, PR+, HER2+ or Ki67 high	Hormonal treatment Trastuzumab	20%
HER2 type	ER-, PR-, HER2+	Trastuzumab Chemotherapy	10-15%
Triple negative/basal-like	ER-, PR-, HER2-	Chemotherapy	15-20%

1.2 ERALPHA SIGNALING IN BREAST CANCER

1.2.1 ERα signaling

The human ERα gene was cloned from MCF-7 cells in 1986 [17]. ERα belongs to the nuclear receptor superfamily of transcription factors, and specifically to the ligand-dependent subfamily of this superfamily. Nuclear receptors have several distinct domains (Figure 1). The Activator Function 1 (AF1) domain at the N-terminal of the ERα protein can transactivate transcription in the absence of ligand binding. The DNA-binding domain

(DBD) binds to estrogen response elements (EREs) in DNA. The AF2 domain is the ligand-dependent transactivation domain. As part of its transactivation function, the AF2 domain also binds to several co-activators and co-repressors of ERα [18].

Unliganded ERα protein is localized to the cell membrane, cytoplasm and the nucleus [19]. Upon estrogen stimulation, the ERα protein can shuttle into the nucleus and form dimers, which subsequently bind to cis-regulatory DNA regions of target genes and transactivate gene expression [20]. A group of nuclear proteins bind to the ERα protein on the DNA and exert effects on ERα signaling. These effects can be functionally separated into activation and repression and the proteins are referred to as co-activators and co-repressors. ERα can also be activated by other means than ligands. For example activation of the tyrosine kinases epidermal growth factor receptor (EGFR) or the insulin growth factor receptor (IGFR) initiates a phosphorylation cascade, which subsequently induces tyrosine and/or serine/threonine phosphorylation of ERα [21, 22]. Such phosphorylation can lead to interaction of ERα with co-activators and subsequent induction of transcription.

The activated ERα protein can also act via a non-genomic function. ERα could interact with several scaffold proteins and pathway molecules including GPCR, SRC, RAS and PI3K, which subsequently activate signaling pathways [23]. This effect leads to several phenotypes in different cells. In endothelial cells, 17β-estradiol (E2) activation of ERα and promotion of the ERα/PI3K interaction induce the expression of nitric oxidase synthase (eNOS). The increased eNOS facilitates the generation of NO and regulates vessel dilation activity [24]. In breast cancer cells, ERα can trans-activate ERK and MAPK pathways and facilitate cell proliferation [25].

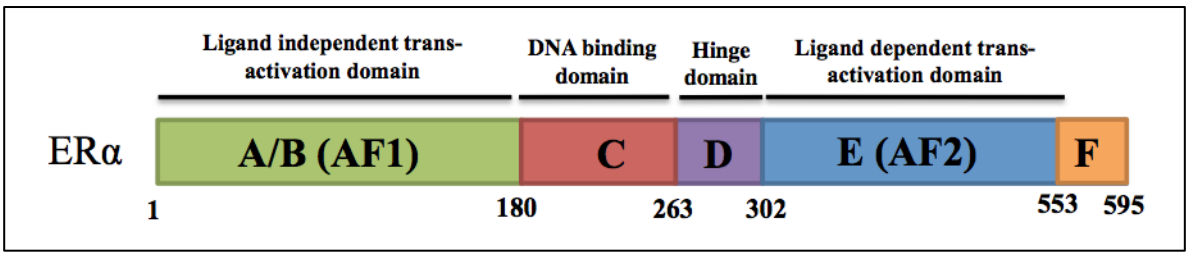

Figure 1. ERα protein domain structure. Numbers represent amino acids from amino to carboxy termini.

1.2.2 ERα and breast cancer

Since 60-70% of breast cancers over-express ERα, endocrine therapy targeting estrogen signaling has been shown to be a successful therapeutic strategy for a large group of breast cancer patients [26]. Hormonal therapy drugs include Selective Estrogen Receptor Modulators (SERMs) and aromatase inhibitors. SERMs display agonistic activities in certain tissues and antagonistic activities in other tissues. For example, a suitable profile of a SERM for the treatment of breast cancer should be antagonistic activity in the breast but agonistic activity on the bone where it is known that estrogens are important to maintain bone mass. Widely used SERMs include tamoxifen and raloxifene. In contrast to raloxifene, tamoxifen is a pro-drug which is metabolized to the active 4-OH-tamoxifen. Both raloxifene and tamoxifen bind to the ERα protein and inhibit the activation of ERα signaling in breast cancer. Aromatase inhibitors, such as anastrozole and letrozole, inhibit aromatase, the enzyme responsible for estrogen synthesis and are clinically used only in post-menopausal patients [27], in which the ovaries have ceased to produce estrogen and estrogen is mainly produced in peripheral tissues. Among all the hormonal drugs, tamoxifen is still the most widely used in ERα positive breast cancer patients. Tamoxifen has a similar structure as E2, however it has an extra chain, which interferes with the conformational change that transforms the ERα protein into its active conformation [28]. Despite the effectiveness of tamoxifen treatment, a significant percentage of ERα expressing tumors develop endocrine resistance.

Many different mechanisms have been shown to account for tamoxifen resistance. Besides rare examples of ERα mutations and amplifications [29, 30], tamoxifen resistance has been shown to be associated with high expression of co-activators, such as SRC [31]. As an ERα co-activator, SRC interacts with ERα protein and promotes E2 stimulated ERα trans-activation and proliferation [32]. Another SRC group member, SRC-3, has been shown to promote the agonistic activity of tamoxifen in the breast thereby conferring tamoxifen resistance [33]. Recently, our group identified RBCK1 (RanBP-Type And C3HC4-Type Zinc Finger Containing 1) as a novel ERα modulator [34]. Another group reported that RBCK1 interacts with ERα as a co-activator which is related to tamoxifen resistance [35].

ERα signaling has been demonstrated to interact with a number of other signaling pathways, such as the HER2, EGFR and NFKB pathways, which could affect cell proliferation and tamoxifen resistance. This crosstalk between ERα and growth factor signaling has several effects. First, ERα exerts its impact on HER2/ERK/MAPK signaling by both genomic and

non-genomic functions. ERα interacts with HER2 protein, resulting in the downstream activation of ERK/MAPK pathway [36]. The interaction between ERα and HER2 reduces tamoxifen induced cell death [37, 38]. Clinically, crosstalk between ERα and HER2 signaling provides one explanation why tamoxifen has a lower efficacy in ER+HER2+ patients [39]. On the genomic level, ERα suppresses the expression of growth factor components, such as EGFR [36]. Thus the estrogen effects on growth factor pathways are complicated, and the net effect may differ in different cell types. Also, many growth factor signaling kinases regulate the phosphorylation of ERα; examples of such kinases are ERK, MAPK, RAS, AKT, PKA and p21-activated kinase 1 (PAK1) [40-42]. Phosphorylation of ERα may enhance ERα stability and/or transactivation activity and/or enhance the activity of co-activators, which might render cells less sensitive to tamoxifen [43].

1.2.3 ERα post-translational modifications

ERα protein activity can be regulated by various post-translational modifications. The known modifications include phosphorylation, ubiquitination, sumoylation, acetylation, methylation and O-linked N-acetylglucosamine (Figures 2 and 3). The many sites of modifications are widely distributed over the ERα protein. Modifications of ERα protein can affect its functions in several ways. For example, acetylation in the hinge domain of ERα changes the ligand sensitivity and subsequent histone de-acetylation effect [44]. For example, p300 is shown to acetylate ERα protein on the DBD, which is shown to enhance ERα activity [45]. Phosphorylation of ERα increases its interaction with ERα co-activators [46]. For example, Tharun *et al.* showed that phosphorylation at Y537 of ERα changed the helix loop conformation and subsequently increased ligand or co-factor binding efficacy [47]. In addition, many ERα protein modifiers could act as co-activators, which co-occupy with ERα on promoter regions, such as p300 and PIAS [48, 49]. Hanstein *et al.* first reported that p300 interacts with ERα as an important co-activator [50]. Several years later, Wang *et al.* reported p300 as an acetylation ligase on ERα and that the acetylation effect enhanced ERα transcriptional activity [45]. Further studies have identified additional ERα modifiers, which also act as co-activators [51-54]. It is explainable that the co-activators exert their impacts on ERα through the post-translational modifications. However, it is still not clarified if the process of modification is required for co-activators to exert their impacts on ERα activity.

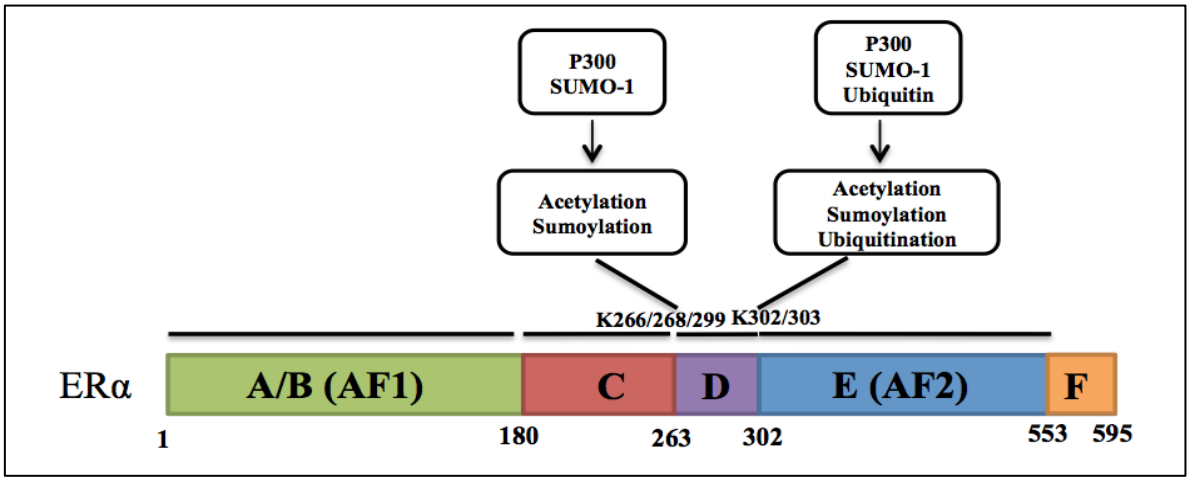

Figure 2. ERα protein acetylation, sumoylation and ubiquitination sites and their corresponding enzymes.

Several studies have shown a critical role of phosphorylation for ERα signaling. Several ERα phosphorylation sites have been reported and many of these have proven to be related to tamoxifen resistance (Figure 3). For example, the MAPK pathway could directly phosphorylate ERα at several sites including S104/S106/S118, causing constitutive activation of ERα through the AF-1 domain potentially leading to tamoxifen resistance [55, 56]. Additionally, MAPK kinases phosphorylate ERα site Y537, which leads to a conformational change of the ERα protein and potentially causes tamoxifen resistance by increasing co-factor binding efficacy [57-59]. Together, these data indicate a crosstalk between the classical MAPK pathway and the ERα pathway and a mutual supportive function in cell survival and proliferation.

Besides the phosphorylation sites referred to above, phosphorylation at S305 of ERα is also reported to relate to endocrine treatment outcome. S305 site can be phosphorylated by PKA and PAK1 [60, 61]. Phosphorylation of S305 has several effects. First, it will lead to a conformational change of the ERα protein leading to increased sensitivity for E2 and decreased effect of tamoxifen inhibition [62]. Additionally, phosphorylation of S305 will lead to increased ERα protein stability possibly by affecting methylation or ubiquitination of K302 [63]. Interestingly, immunohistochemistry results show that S305 phosphorylation is tightly correlated to tamoxifen resistance and related to PAK1 expression levels [41].

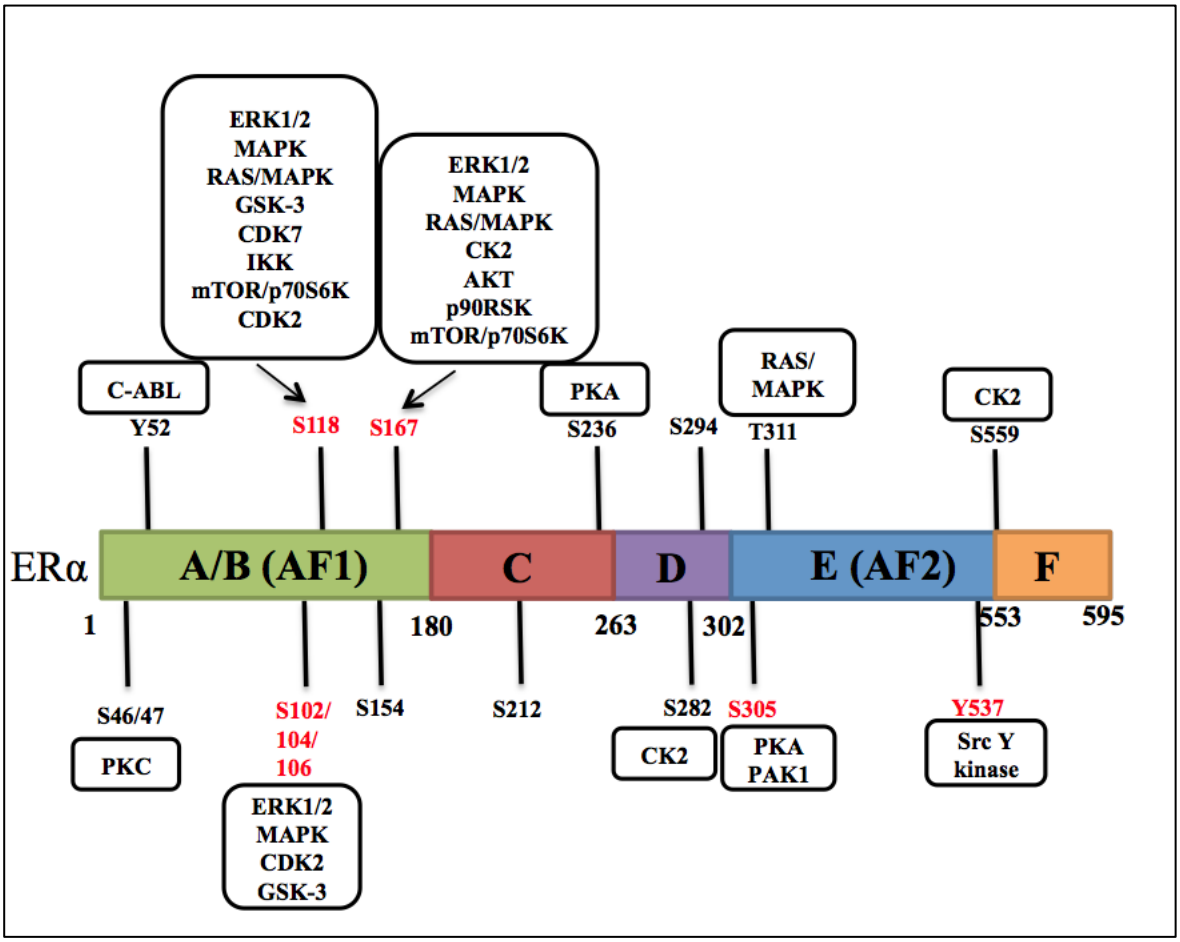

Figure 3. ERα protein phosphorylation sites and their corresponding phosphorylating kinases. Sites related to tamoxifen resistance are marked in red.

1.3 P53 SIGNALING IN BREAST CANCER

1.3.1 p53 signaling and cancer

The p53 protein is encoded by the TP53 gene, which is located on chromosome 17 [64]. Inherited loss of the p53 gene leads to multiple tumors at an early age, a syndrome that is referred to as the Li-Fraumeni syndrome [65]. This observation indicates that the p53 gene is a tumor suppressor gene. Structural and functional analysis reveals that p53 is composed of several functional domains (Figure 4) [66]. The N-terminal part of p53, corresponding to amino acids 1-42, constitutes the trans-activation domain, which is involved in interactions with other proteins including MDM2, JNK and p300 [67-69]. The proline-rich domain, from amino acid 42 to amino acid 100, is proven necessary for p53 dependent apoptosis and cell

cycle arrest, and mutations in this domain lead to decreased apoptotic response [70]. The DBD is rich in arginine and related to transcriptional activity [71]. The protein domain from amino acid 305 to amino acid 322 includes the nuclear localization domain, while the domain from amino acid 340 to amino acid 351 includes the nuclear exclusion domain. In addition, the protein domain from amino acid 326 to amino acid 356 corresponds to the tetramerization domain, which is necessary for p53 function [72]. The C-terminal domain from amino acid 364 to amino acid 393 is required for DNA binding capability and DNA damage response. In addition, the C-terminal domain is thought to be the cell fate determinant in that C-terminal deletions or modifications will significantly change the response to p53 target gene induction, cell cycle arrest and apoptosis [73-75].

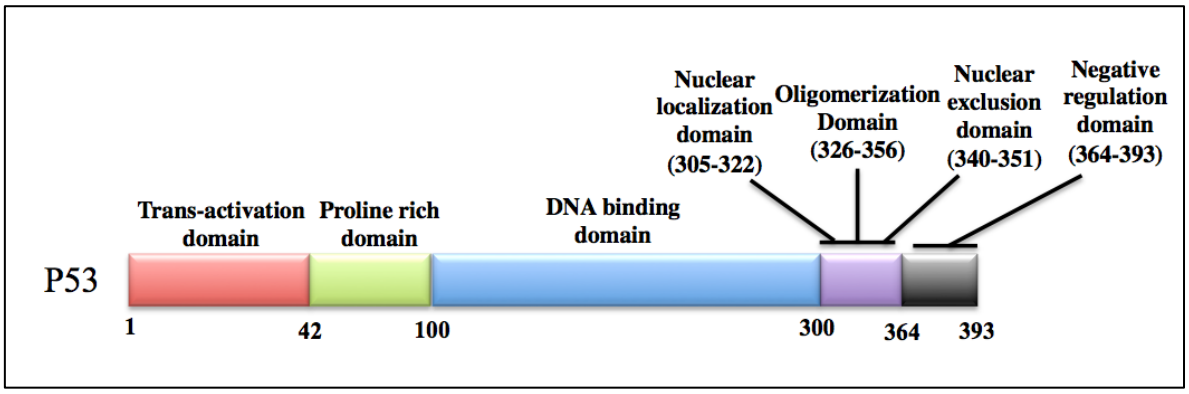

Figure 4. P53 protein domain structure.

The p53 protein is activated by several events, such as DNA damage, oxidative stress and oncogene activation [76]. If activated, the p53 half life will increase leading to enhanced activation of p53 target genes [77]. Several p53 target genes, including p21, are involved in cell cycle arrest [78]. Another group of target genes regulate cell apoptosis, including the BAX and Fas proteins [78]. In addition to its trans-activation function, p53 exerts trans-repression functions on several oncogenes, such as bcl-2 [79]. p53 is also reported to mediate DNA repair via interaction with DNA repair proteins, such as BRCA1 and ATM [80, 81].

The p53 gene is frequently mutated, with a mutation rate varying from 2 to 80% in different cancers. The five tumor types with the highest frequency of p53 mutations are ovarian cancer,

squamous lung cancer, esophageal cancer, colorectal cancer and head and neck cancer [82]. The lowest frequency of p53 mutations is found in thyroid cancer, medulloblastoma, cervical cancer, diffused B-cell lymphoma and renal cell carcinoma (Figure 5) [82]. By analyzing the mutation pattern in tumors, it has been observed that more p53 mutations are found in the DBD than in other domains. However, the mutations are widely distributed over p53 exons and it is difficult to find any specific pattern (Figure 6) [83]. In addition to mutations in the p53 gene, p53 is reported to be hyper-methylated at the promoter regions, presumably leading to reduced expression in a few cancers, including glioma (60-70% of cases) and hepatocellular carcinoma (70-80% of cases) [84, 85].

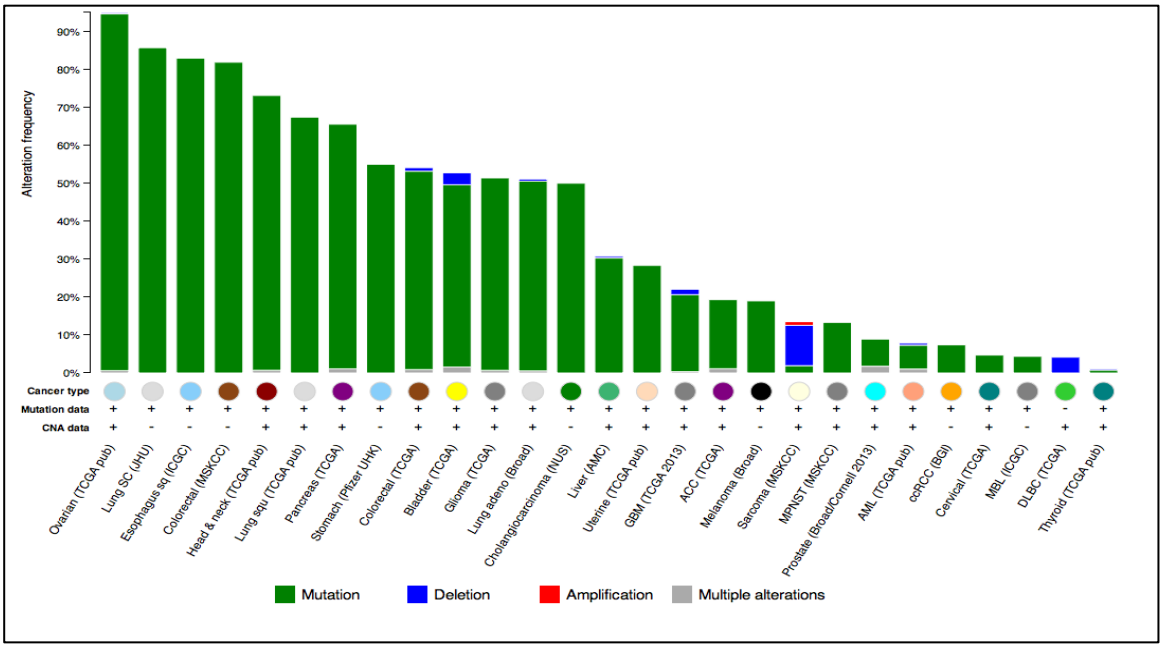

Figure 5. P53 mutation frequency in different tumors (http://www.cbioportal.org/).

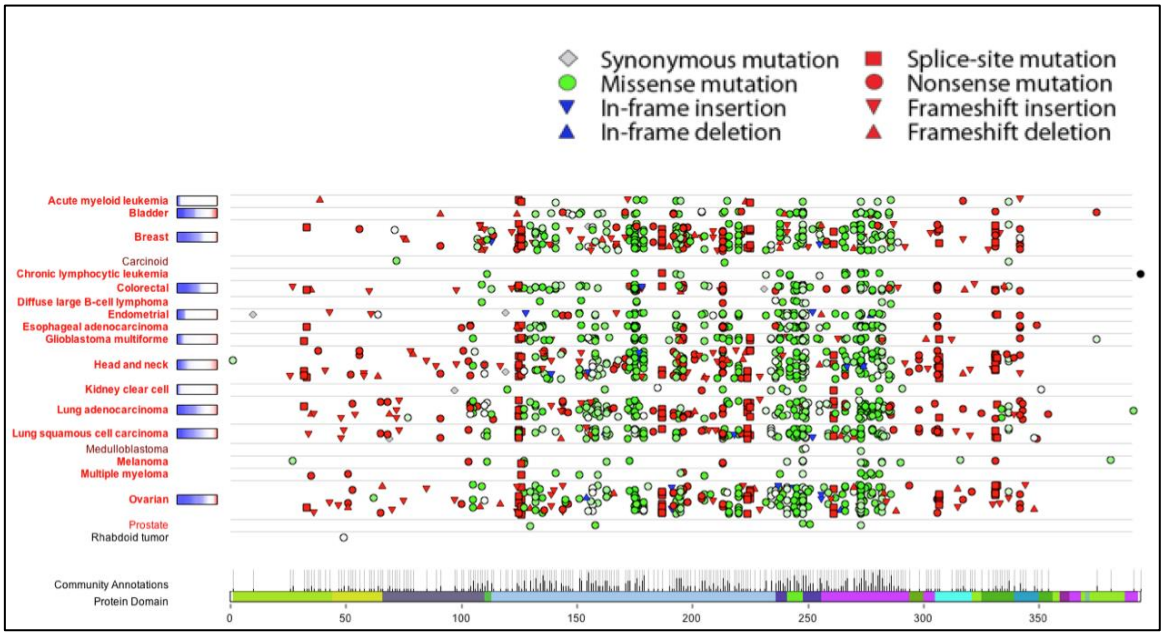

Figure 6. P53 mutation pattern in different tumors (http://www.tumorportal.org/).

Loss of tumor suppression function by mutations in p53, decreased p53 expression by hyper-methylation or post-translational modification in cancers will lead to several changed cellular properties. First, it may lead to enhanced cell proliferation due to failure to induce target genes related to the check point for G1-S and apoptosis, such as p21, BTG2 and BAX [86]. Secondly, it will result in impaired activation of DNA repair and a subsequent genome instability, which facilitates the process of accumulated gene mutations and carcinogenesis.

Besides the loss of p53 protein by nonsense mutations and deletion/insertions that generate out-of-frame translation, p53 point mutations, which lead to the mutant p53 can also gain other functions and perform as an oncogene. Although the detailed mechanisms are not clear, there are several suggested models [87]. Mutant p53 is more stable and accumulates in the nucleus [88]. Mutant p53 also interacts with the DNA repair complex to inhibit its function, which increases genomic instability [89]. Additionally, mutant p53 can interact with tumor suppressor proteins, such as p63 and p73, to inhibit their function [90]. Besides the Missense mutations which results in more stable protein, p53 could also have nonsense mutations, or deletions/insertions that generate out-of-frame translation, reducing mRNA levels and less or no protein [91].

1.3.2 p53 signaling in breast cancer

Similar to other cancers, p53 also plays a significant role in breast cancers. According to the TCGA database, 31% of breast cancers harbor p53 mutations [83]. Interestingly, p53 mutations display a negative correlation with ERα expression, in that about 15% of Luminal A ERα-positive tumors harbor p53 mutations, whereas about 80% of the basal ERα-negative subtype harbor p53 mutations [92]. In some breast cancers, p53 is downregulated either by promoter hyper-methylation or by post-translational modification [93]. It has been shown that wild-type p53 induces ERα expression and this phenomenon can possibly explain why wild-type p53 exists mostly in ERα-positive cancer type [94]. Crosstalk between p53 and ERα signaling at several levels has been demonstrated. The wild-type p53 protein induces ERα gene expression by binding to its promoter region, while ERα has been shown to interact with p53 and suppress p53 target gene expression, such as the expression of p21. Thus, the function of wild-type p53 could maintain ERα expression, which means that tumors with this ERα-p53 loop could be viewed as less aggressive and could be well controlled by ERα antagonists. This ERα-p53 feedback loop in ERα-potitive tumors could be one reason for why ERα-positive and p53-wild type breast tumors are less aggressive than the ERα-negative and p53-mutant breast tumors [95].

1.3.3 p53 post-translational modifications

The p53 protein is subject to several kinds of protein modifications, including phosphorylation, ubiquitination, acetylation and methylation, which are tightly related to the physiological function of p53 (Figure 7) [96]. p53 is subject to several phosphorylation events that are relevant for p53 function [97]. For example, p53 is phosphorylated at S6/S9/S15/S18 by CHK1 under conditions of DNA damage, which subsequently induces p53-dependent cell cycle arrest and the recruitment of DNA repair proteins [98]. Several phosphorylation sites relate to increased p53 protein stability, including S33, S81 and S315, while some phosphorylation sites, such as T150 and T155, promote p53 degradation [99]. Besides phosphorylation, p53 can also be acetylated and methylated [100, 101]. With regard to p53 acetylation, K120 and K164 acetylation has been shown to promote DNA damage-induced cell cycle arrest and apoptosis, while acetylation on K320/K370/K372/K373/K381/382 always leads to enhanced p53 DNA binding activity [102, 103]. Also the effect of methylation on p53 function depends on the specific site of methylation. K372 methylation stabilizes p53 and promotes p53 target gene expression, while K370/K382 methylation functions to suppress p53-mediated gene transcription [104].

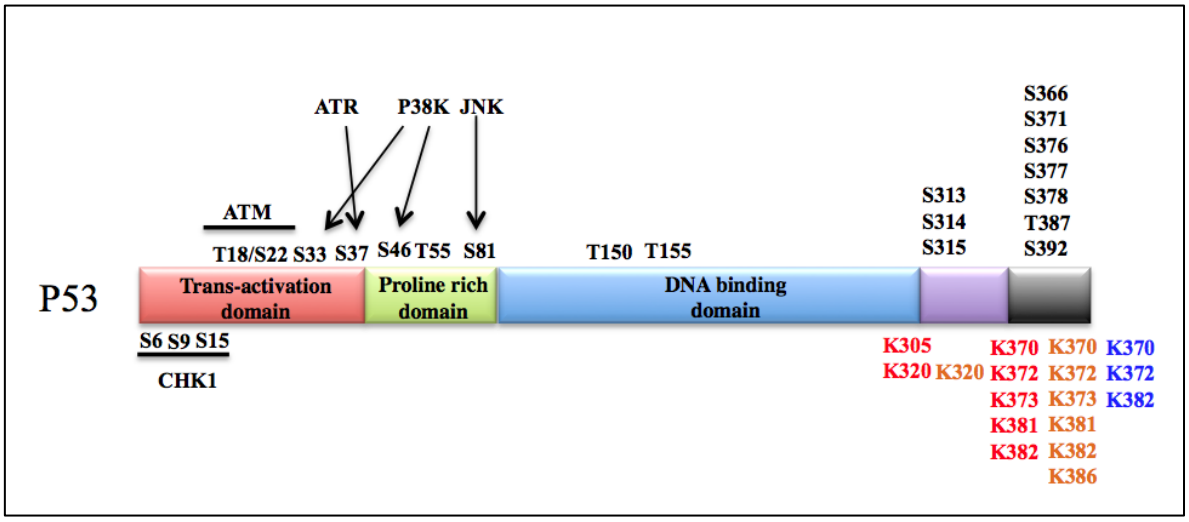

Figure 7. P53 protein phosphorylation sites (black), ubiquitin sites (yellow), methylation sites (blue) and acetylation sites (red).

p53 is under precise control in unstressed conditions. If the p53 pathway is not activated, the p53 half-life is approximately 20 minutes, regulated mainly by ubiquitination and proteasomal degradation [105]. Several ubiquitination sites are found at the C-terminal domain of p53, including K370/K372/K373/K381/K382/386 [106]. Several E3 ubiquitin ligases have been shown to directly poly-ubiquitinate the p53 protein and induce its proteasomal degradation, including MDM2, COP1 and Pirh2 [107]. The most studied of these is the MDM2 protein. MDM2 is a direct target gene of p53 [108]. When p53 is activated, it will induce the expression of MDM2. The MDM2 protein will interact with p53 at the N-terminus and block its transcriptional function [109]. MDM2 also facilitates poly-ubiquitination at several lysine residues in the p53 DBD and C-terminus, which subsequently induces the degradation of p53 [110-112]. This MDM2-p53 negative feedback effectively keeps the cells responding appropriately to certain stimulus [110]. Besides this cross talk between MDM2 and p53, more and more E3 ubiquitin ligases are found to modify the MDM2-p53 complex and indirectly regulate p53 poly-ubiquitination and degradation, including RNF2 and Smurf [113, 114]. E3 ubiquitin ligases that indirectly modify p53 are highly expressed in cancers and thought to be involved in carcinogenesis by suppressing p53 function [115].

1.4 RNF31 IN BREAST CANCER

1.4.1 E3 ubiquitin ligases in cancer

E3 ubiquitin ligases function to catalyze the transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme to attach it to the lysine of a protein substrate. Ubiquitin molecules are attached to lysine residues on substrates via lysine residues on ubiquitin [116]. Different forms of ubiquitination have been identified such as mono-ubiquitination and poly-ubiquitination [117]. Mono-ubiquitination can be viewed as a necessary process for poly-ubiquitination or a separate event [118]. Mono-ubiquitination has been demonstrated to be linked to a change of substrate functions such as signal transduction or protein trafficking in addition to protein degradation [118]. For example, mono-ubiquitination of histone 2A (H2AX) by RNF8 is a necessary step of the DNA repair response [119]. Poly-ubiquitination has different lysine residues on ubiquitin protein as points of ubiquitination, including K63, K48, K27, K29, K33, K11 and linear ubiquitination [120-122]. The K48 and K63 ubiquitination process is related to proteasome dependent degradation [116]. However, the other atypical forms of ubiquitin, such as K27, K11 and linear ubiquitin, are less well understood, while there are accumulating evidences showing that they involve in DNA repair, signal transduction and protein trafficking [116, 123, 124].

Accroding to their functional domains, E3 ubiquitin ligases can be devided into two groups: the HECT (Homologous to the E6-AP Carboxyl Terminus) group and the RING finger group [125]. There are about 30 different HECT E3 ligases in mammals that are involved in protein transfering, immune reaction, and DNA damage response [125]. In general, the HECT family of E3 ligases are composed of two functional domains. The functional domain at the C-terminus is responsible for the interaction with E2 and ubiquitin molecules, while the N-terminal domain is responsible for substrate interaction [125]. One group among the HECT family are the SMURF proteins (Smad ubiquitinylation regulatory factor), which regulate TGFβ and bone morphogenetic protein (BMP) signaling [126]. SMURF proteins interact with Smad proteins and regulate the poly-ubiquitination and degradation via the HECT domain. This process negatively control the protein levels of the Smad proteins and subsequently controls TGFβ pathway output. There are about 700 different RING E3 ligases, most of which are not well studied [127]. According to the current knowledge, the functions of RING E3 ligases cover multiple aspects of cell physiological functions, including cell proliferation, cell migration, DNA damage, and

protein trafficking [124, 127, 128]. Many of the RING E3 ligases are found to be involved in carcinogenesis [129]. BRCA1 is the most thoroughly studied RING E3 ligase in cancer. As a tumor suppressor protein, BRCA1 is shown to regulate gene expression, DNA repair after double stain break and protein ubiquitination [130]. ERα has been suggested as a putative BRCA1 target and BRCA1 inhibits ER function [131]. Defects in BRCA1 ligase functions will lead to loss of the DNA repair response [132]. BRCA1 mutations are found in about 70% of familial breast cancer and ovarian cancer [133]. In addition, recent studies showed that RNF54 (RBCK1) interacts with ERα and facilitates ERα target gene transcription [34]. Analysis of publically available data sets indicates that RBCK1 expression correlates with poor tamoxifen response [35].

1.4.2 RNF31 as an E3 ubiquitin ligase

Ring finger protein 31 (RNF31), also named HOIL-1-interacting protein (HOIP), was first cloned in 2004 from MCF-7 cells [134]. Figure 8 shows the domain structure of the RNF31 protein [135]. The PUB domain (putative ubiquitin binding) at the N-terminal is reported to bind cofactors [136]. The ZNF-RBZ domain (Zinc finger domain in Ran-binding proteins) is related to the ubiquitin binding function [137]. The UBA domain (ubiquitin binding associated) has been shown to bind RBCK1 and mediates linear ubiquitination of IKK γ, which facilitates signal transduction of NFKB [138]. The RING-IBR-RING domain at the C-terminal is thought to be the most important one for its ubiquitin ligase function [139]. The deletion of this domain will lead to loss of function of its substrates, such as IKK γ [135, 140].

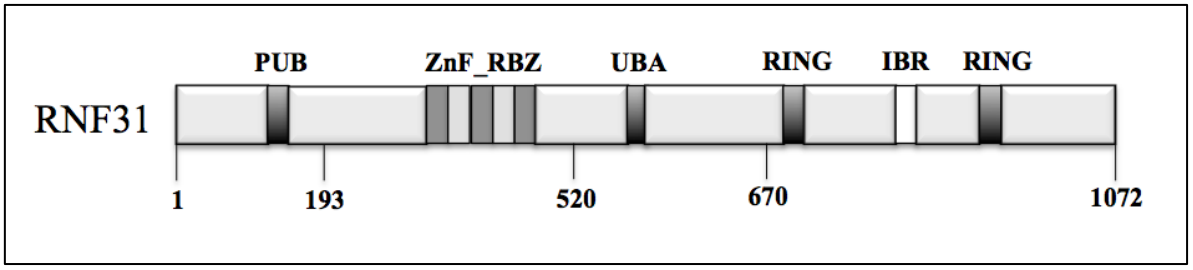

Figure 8. RNF31 protein domain structure. PUB domain - putative ubiquitin binding domain; ZnF_RBZ domain - Zinc finger domain in Ran-binding proteins; UBA domain - ubiquitin binding associated domain; RING-IBR-RING domain - RING-in between RING-RING domain.

RNF31 is highly expressed in muscle, heart, and testis [135]. In cells, RNF31 mainly localizes to the cytoplasm. Whole-body knockout of RNF31 will lead to embryonic lethality through TNFR1-mediated endothelial cell death [141]. The most well studied function of RNF31 is that it together with RBCK1 and SHARPIN, forms the linear ubiquitin assembly complex (LUBAC) which facilitates linear ubiquitination of IKK γ and NFKB signaling transduction as demonstrated in several conditional knockout mice models [140, 142]. For example, conditional deletion of the RBR domain in B cells (B-HOIP $^{\Delta \text{linear}}$) leads to lack of or reduced NFKB and ERK signaling. Phenotypically, lack of development of B cells was observed with deficient thymus-dependent and thymus-independent antigen response [142]. In addition, RNF31 is reported to modify ERK and JNK pathways leading to cisplatin resistance [143].

1.5 PAK4 IN BREAST CANCER

1.5.1 PAK4 structure and function

The PAK family was first identified as a downstream substrate of the Rho GTPases CDC42 and RAC1. The PAK4 protein domain structure is shown in Figure 9. The PBD (p21-binding domain) is close to the N-terminal, while the kinase domain is localized to the C-terminal. There are two important motifs in the kinase domain, the ATP-binding domain and the integrin-binding domain [144]. The PAK4 protein localizes to both the cytoplasm and the nucleus and is involved in several physiological functions, including cell migration, cell proliferation, and apoptosis [145]. PAK4 facilitates polymerization of actin filaments by promoting the phosphorylation of LIMK1 and cofilin [146]. PAK4 has been shown to promote pancreatic cancer cell proliferation through the AKT and NFKB pathways [147]. PAK4 also inhibits cell apoptosis by phosphorylating the pro-apoptotic protein BAD [148].

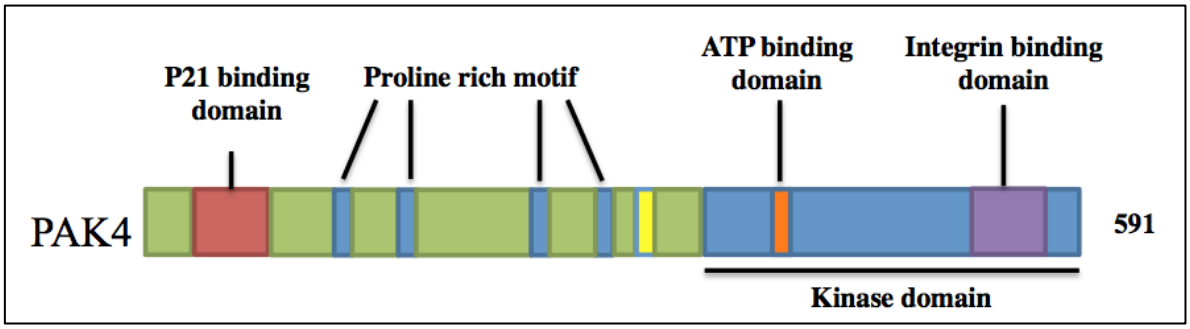

Figure 9. PAK4 protein domain structure.

1.5.2 PAK4 in breast cancer

PAK4 is highly expressed in breast cancer tissues, breast cancer cell lines, and mouse mammary tumors, while it is rarely detectable in the normal mammary gland [149]. PAK4 can be viewed as a general poor-prognosis marker in breast cancer [150]. Several studies have shown that PAK4 expression is higher in breast cancer cell lines and breast cancer tissues than in normal breast tissues [151]. High PAK4 expression is tightly linked to aggressive phenotypes [152, 153]. For example, PAK4 is shown to promote cell migration and anchorage-independent cancer growth [154]. Additionally, PAK4 in cancer cells has been shown to be necessary for multilayered growth in 3D culture and for lost cellular polarity [155]. Analysis of databases of clinical samples reveals that PAK4 expression is related to poor overall survival (OS), poor chemotherapy response and poor relapse-free survival (RFS) after surgery of ovarian cancer [156]. In ERα positive breast cancers, PAK4 expression is also shown to relate to poor OS and poor tamoxifen treatment outcome.

1.6 AP-1 FAMILY AND ZEB2 IN TRIPLE-NEGATIVE BREAST CANCER

The activator protein 1 (AP-1) constitutes a group of transcription factors including seven members, Fra-1 (FOSL1), Fra-2 (FOSL2), c-fos (FOS), fosB (FOSB), c-Jun (JUN), JunB (JUNB) and JunD (JUND). AP-1 controls several cellular phenotypes including proliferation, differentiation, and apoptosis by binding to target gene motifs containing the TRE (TPA-response element) (TGAG/CTCA) [157]. In breast cancer, AP-1 family members are often highly expressed [158, 159]. Increased levels of AP-1 proteins facilitate cancer cell proliferation via regulation of cyclin proteins. AP-1 proteins have also been reported to

mediate tamoxifen resistance in ERα-positive breast cancers [160]. Our group has shown that Fra-1, as one of AP-1 family members, is highly expressed in TNBC compared with ERα-positive breast cancers, and that it promotes cell migration, metastasis, and EMT [161].

ZEB2 (Zinc Finger E-Box Binding Homeobox) belongs to the zinc finger/homeodomain protein family [162]. ZEB2 protein is mainly localized to the nucleus and functions as a DNA-binding transcriptional repressor. A few studies show that ZEB2 expression correlates to cancer cell mobility [163-165]. Further studies indicate that ZEB2 induces EMT by repressing the expression of proteins important for the epithelial state, including E-cadherin (CDH1) [166]. Consequently, high ZEB2 expression levels have been shown to correlate with poor prognosis in cancers, such as colorectal cancer and bladder cancer [167, 168].

2 AIM

Transcriptional factor signaling, including nuclear receptor signaling, is central for the control of cell behavior such as cell proliferation, apoptosis, and metabolism. ERα and p53 signaling are frequently studied in cancers, and proven to be key regulators for breast cancer progression, apoptosis, and drug resistance. Much is known about how nuclear receptors and other transcription factors regulate their target genes. In recent years, there is a growing interest in how transcription factors are modified and how deregulated modification may affect cancer behavior. RNF31 as an E3 ligase discovered decades ago, was rarely studied in cancer until in recent years. The increased expression of RNF31 in breast cancer may indicate an important biological function. Additional oncogenic transcription factors, such as AP-1, are proven to have important roles in carcinogenesis and metastasis including in TNBC. **The general aim of this thesis was to study how modification of ERα and p53 affects their signaling capacities and the efficacy of ERα antagonists.**

The specific aims were:

I. To investigate the role of RNF31 in relation to ERα signaling and proliferation in breast cancer.

II. To investigate the role of RNF31 in relation to p53 signaling and cell proliferation in breast cancer.

III. To investigate the effect of PAK4-mediated ERα phosphorylation on estrogen signaling and tamoxifen resistance in breast cancer.

IV. To investigate the regulatory role of AP-1 for ZEB2 gene expression, which mediates EMT in TNBC.

3 METHODOLOGICAL CONSIDERATIONS

The detailed methods and systems are described in each study, while general considerations and limitations are discussed in this section.

3.1 CELL LINES AND IN VITRO SYSTEMS

The cell lines used in these studies were derived from different breast cancer tumors. These immortalized cell lines are important tools for cancer research, as they can be cultured infinitely and undergo genotypic changes or phenotypic changes. Notably, results derived from the same cell line in different labs have often been found to be contradictory, due sometimes to the passages and culture conditions of the cell lines. It is therefore important to record the cell passage numbers and not let the cells undergo to many passages but to regularly return to the frozen stock. Also the regular comfirmation of the cell lines and free of microplasm contamination need to be done.

The MCF-7s cell line used in the studies reported in this thesis, originates from the work at the Michigan cancer foundation in 1970 [169]. This cell line shares the same origin and has similar biological behavior as the MCF-7 cell line from the American Type Culture Collection (ATCC). However, with respect to E2 response, the MCF-7s cell line maintains higher estrogen response compared with MCF-7 from ATCC.

3.2 SMALL INTERFERING RNA TRANSFECTION

In order to reduce expression of a particular gene, small interfering RNA (siRNA) is transfected into cell lines. Basically, the siRNAs form double-stranded RNAs, which are subsequently degraded in a process catalyzed by Dicer enzyme. The resulting RNA interact with the RISC complex in the cytoplasm, and inhibit target genes through hybridizations with their mRNA and its subsequent degradation. The challenge for this technique is off-target effects, which refers to that siRNAs might unspecificly binds to some RNAs with similar sequences, which induce the subsequent RNA cleavage and gene silence. In order to minimize the false positive results from the off-target effect, it is important to use at least two different target siRNAs.

3.3 WST-1 ASSAY AND FLOW CYTOMETRY

To study cell proliferation, the WST-1 assay, ethynyl-deoxyuridine (EdU) and propidium iodide (PI) staining were used in this thesis. The WST-1 assay is a colorimetric assay measuring cell number by the amount of tetrazolium cleaved into formazan by a mitochondrial enzyme. The EdU assay measures EdU incorporation into DNA, quantifying the percentage of EdU positive cells. PI staining measures the relative content of DNA in each cell, discriminating G1 phase cells from cells in the S or G2-M phase.

The principle of WST-1 assay is to measure the amount of colored dye converted from tetrazolium salt. This reaction is catalyzed by mitochondrial dehydrogenase enzymes. In general, the WST-1 assay is relatively simple by measuring the absorbance at 450nm. WST-1 readings can be affected by different conditions, such as temperature and pH. When comes to the PI staining-based flowcytometry, the merit of this experiment is that cells can be clearly separated into G1, S and G2-M phases. The limitation is that it cannot discriminate cells in the G2 phase from cells in the M phase. The EdU experiment is quite sensitive and it reflects the dynamic proliferation process during a certain time span. Before flowcytometry analysis, cells are grown in EdU containing culture medium and EdU is incorporated into proliferating cells. Thus, the relative density of EdU signaling will reflect the relative proliferation activity. In our research, we used at least two different techniques to determine cell proliferation in each study.

3.4 MICROARRAY ANALYSIS

The Agilent SurePrint 8x60K arrays were applied for global gene expression according to standard protocols. Data is uploaded into the Gene Expression Omnibus (GEO) database (accession number GSE46010). In the initial analysis, several P-values and fold change thresholds were applied (P-values 0.05, 0.01 or 0.005; Fold changes 1.5 or 2.0). A lower threshold cutoff value for fold change and a less stringent p-value will increase the sensitivity and decrease the specificity, while a higher threshold cutoff for fold change and a less stringent p-value will decrease the sensitivity and increase the specificity. However, the groups of enriched pathways were overall insensitive to the filters applied. In order to increase the specificity of the analysis, we applied a filter of P-value less than 0.001 for

significantly modulated gene expression and at least a two-fold difference in the mean expression levels.

4 RESULTS AND DISCUSSION

STUDY I: THE ATYPICAL UBIQUITIN LIGASE RNF31 STABILIZES ERα AND MODULATES ESTROGEN-STIMULATED BREAST CANCER CELL PROLIFERATION

In order to investigate the role of RNF31 in breast cancer cell proliferation, we depleted RNF31 by siRNA in MCF-7 cells. We observed that RNF31 knockdown significantly decreased E2-dependent cell proliferation, mimicking the effect of ERα depletion in this cell line. The effect on proliferation and cell cycle arrest upon RNF31 knockdown could be partially rescued by overexpression of ERα. This indicates that RNF31 can facilitate estrogen-stimulated cell proliferation. Further experiments showed that RNF31 depletion significantly decreased ERα protein level, ERα target gene expression, ERα-regulated reporter gene activity and ERα recruitment to the promoter regions of target genes. Analysis of breast cancer samples reveals that RNF31 is highly expressed in breast tumors compared with adjacent tissues. Analysis of global gene expression in response to RNF31 knockdown in MCF-7 cells showed that the ERα pathway is one of the most inhibited pathways and a group of ERα target genes were decreased, among which 70% showed consistent change in the TGCA database.

In order to decode the regulatory mechanism of ERα by RNF31, immunoprecipitation was carried out, showing that RNF31 interacts with ERα and increases its protein stability, an effect which was observed in both vehicle and E2-treated conditions. Further experiments showed that the RNF31 RBR domain is required for interaction with the ERα protein, stabilization of the ERα protein, and increased ERα-regulated reporter gene activity. Furthermore, our results demonstrate that RNF31 increased mono-ubiquitination of ERα, and that this was dependent on the RBR domain and the E3 ligase activity. This indicates that RNF31 may stabilize ERα protein via mono-ubiquitination. Using an immunofluorescence assay, we found that RNF31 co-localizes with ERα mainly in the cytoplasm.

RNF31 shares functional domains with RBCK1, another member of the same family of E3 ubiquitin ligases. Previous studies have shown that RNF31 together with RBCK1 forms the linear ubiquitin complex and mediates linear ubiquitination of IKKγ, which subsequently facilitates NKFB signaling (Figure 10) [138, 170, 171]. This suggests that ERα may be modified by linear ubiquitination.

Interestingly, our group has shown that RNF31 and RBCK1 both regulate estrogen signaling, but through distinct mechanisms. RBCK1 interacts with ERα mainly in the nucleus, while RNF31 interacts with ERα in the cytoplasm. Additionally, RNF31 mainly exerts its role in ERα signaling by stabilizing ERα through mono-ubiquitination, while RBCK1 acts as a co-activator to ERα regulating ERα signaling [172, 173].

In summary, we have identified RNF31 as a novel modifier of ERα signaling, detailing this mechanism and thereby increased the knowledge of the regulation of estrogen signaling as well as suggesting a potential new target for modulating estrogen signaling in breast cancer (Figure 10). Previous studies have revealed that RNF31 is lower expressed in bones, thus targeting RNF31 may have negligible effects on osteoporosis and be suitable for treatment of post-menopausal breast cancer patients. With regard to the possibility of developing RNF31 inhibitors, it would be interesting to test the blocking efficacy of such compounds on estrogen signaling.

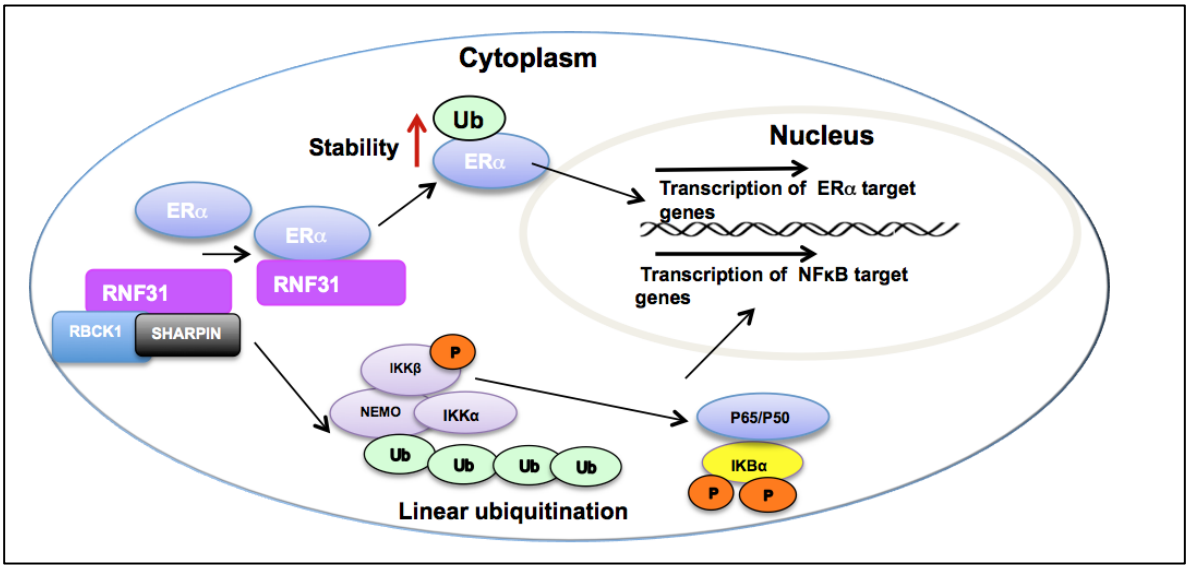

Figure 10. The proposed regulatory effect of RNF31 on ERα.

STUDY II: RNF31 PROMOTES p53 DEGRADATION IN BREAST CANCER CELLS

In analysis of our non-biased global gene expression profiling data in study I, we observed that the p53 pathway is significantly affected upon RNF31 knockdown. Most of the affected p53 target genes were up-regulated by RNF31 knockdown, suggesting that RNF31 down-regulates p53 signaling. The list of thirty up-regulated p53 target genes upon RNF31 knockdown was used to search in TCGA breast tumor database. Among the 30 genes, 50% were observed to be negatively correlated with RNF31 expression. In further experiments we demonstrated that RNF31 depletion increased the p53 protein along with its target genes, including p21, IGFBP3, and BTG2, in three different breast cancer cell lines (MCF-7, MDA-MB-175 and ZR-75-1), representing different breast cancer subtypes. Our results further show that RNF31 depletion decreased the fraction of proliferating cells in the MCF-7 and ZR-75-1 cell lines. Knockdown of p53 in siRNF31 transfected cells resulted in increased fraction of proliferating cells as compared to only siRNF31-treated cells, thus supporting that interaction of RNF31 and p53 regulates cell proliferation. Using dual staining with Annexin V and PI, we found that knockdown of RNF31 facilitated cisplatin-induced apoptosis, while knockdown of p53 in addition to knockdown of RNF31 rescued this effect. This supports that interaction of RNF31 and p53 inhibits apoptosis.

The mRNA levels of p53 showed little change 24 h after knockdown of RNF31, while the p53 protein was significantly increased, suggesting that RNF31 regulates p53 at the protein level. Measurement of p53 half-life revealed that RNF31 mainly regulated p53 stability. An immunoprecipitation assay revealed that RNF31 interacted with the MDM2/p53 complex and increased p53 poly-ubiquitination in an MDM2-dependent manner. This was also supported by treatment with Nutlin-3, a compound that disrupts the p53-MDM2 interaction. Further experiments showed that RNF31 affected MDM2 stability and proteasomal degradation by inhibiting MDM2 poly-ubiquitination. However, it is not clear how RNF31 affect the poly-ubiquitination of MDM2. There are several possible explanations: RNF31 may compete with other E3 ligases and inhibit MDM2 degradation. Another possibility is that RNF31, as atypical E3 ligase, could function to increase MDM2 stability through mono-ubiquitination. More research is needed to elucidate the regulatory function of RNF31 on MDM2.

It is well established that functional p53 is necessary for chemotherapy-induced cell death. One approach, which increases the efficacy of chemotherapy, is to increase p53 protein levels [174]. In this study, we report that RNF31 depletion can arrest the cell cycle and enhance

cisplatin-induced cell death. This study uncovers a potential oncogenic role of RNF31: the suppression of p53 signaling (Figure 11). As such, RNF31 could be a potential target to increase the efficacy of chemotherapy. Further, we provide additional knowledge of the molecular mechanism underlying regulation of p53 signaling in breast cancer cells.

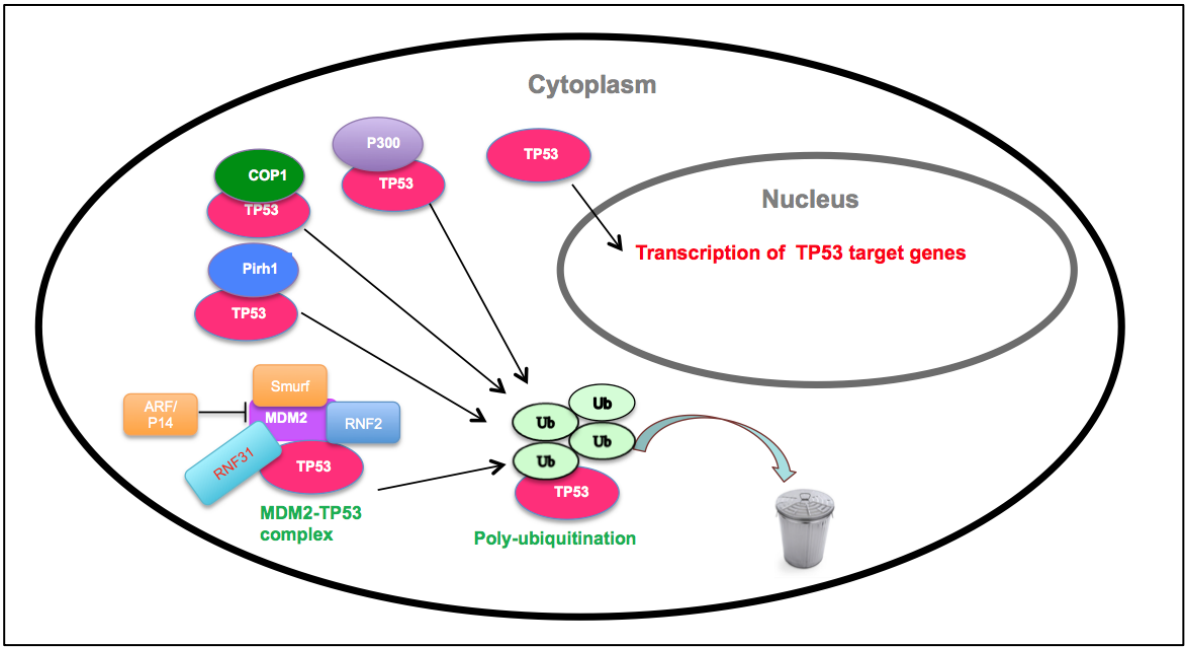

Figure 11. The proposed regulatory effect of RNF31 on P53.

STUDY III: p21-ACTIVATED KINASE GROUP II SMALL COMPOUND INHIBITOR (GNE-2861) INHIBITS ERα SIGNALING AND RESTORES TAMOXIFEN-SENSITIVITY IN BREAST CANCER CELLS

The hypothesis that PAK4 can induce tamoxifen resistance is derived from analysis of the clinical databases METABRIC and KMPLOT. We observed that the PAK4 expression level is correlated with poor tamoxifen response. When expressing PAK4 in MCF-7 cells we observed that they displayed a higher IC50 for tamoxifen compared to the parental cell line. Upon treatment with PAK4 inhibitor (GNE-2861), both MCF-7 cells and tamoxifen-resistant LCC2 cells displayed decreased IC50 for tamoxifen, compared to vehicle-treated cells. This suggests that PAK4 may be involved in tamoxifen resistance.

PAK4 depletion or treatment with a PAK4 inhibitor decreased ERα protein levels, ERα target gene expression, and ERα-regulated reporter gene activity. As assayed by EdU assay, PAK4 knockdown or treatment with a PAK4 inhibitor decreased E2-stimulated cell proliferation in MCF-7 cells. In order to understand how PAK4 regulates ERα, levels of ERα mRNA and protein were determined in PAK4-depleted cells. We found that following PAK4 depletion ERα protein levels were decreased, but its mRNA levels were unchanged. This indicates that PAK4 regulates ERα mainly through post-transcriptional modification. We further showed that the ERα half-life was increased upon PAK4 over-expression. Furthermore, the PAK4 – induced ERα protein levels could be diminished upon MG132 treatment. This indicates that PAK4 increases ERα stability and inhibits proteasomal degradation. Using an *in vitro* protein phosphorylation assay, we found that PAK4 phosphorylated ERα at the S305 site. Consistent with this, a mutant ERα where S305 was replaced by alanine was not phosphorylated at this site and PAK4 expression did not activate ERα-regulated reporter gene.

Additionally, by analyzing ERα ChIP-seq data, we observed that ERα bound to the intron region of PAK4, with the DNA-binding being facilitated by E2 treatment. Consistent with this, E2 treatment was shown to increase PAK4 mRNA and protein levels.

In summary, we showed that ERα bound to the PAK4 gene and promoted its transcription in response to E2 treatment. The increased levels of PAK4 protein resulted in phosphorylation and stabilization of ERα protein, which subsequently enhanced ERα signaling and ERα target gene expression including PAK4. This loop might promote breast cancer proliferation and tamoxifen resistance. Based on the current literature, PAK4 is the only PAK family member, which is found to be a target gene of ERα. Our study suggests that PAK4 has a tight relationship with ERα, and we propose that a forward feed loop between ERα and PAK4 in

ERα-expressing breast cancers influences proliferation and tamoxifen resistance (Figure 12). This suggests that PAK4 inhibition may be a potential strategy to reverse tamoxifen resistance.

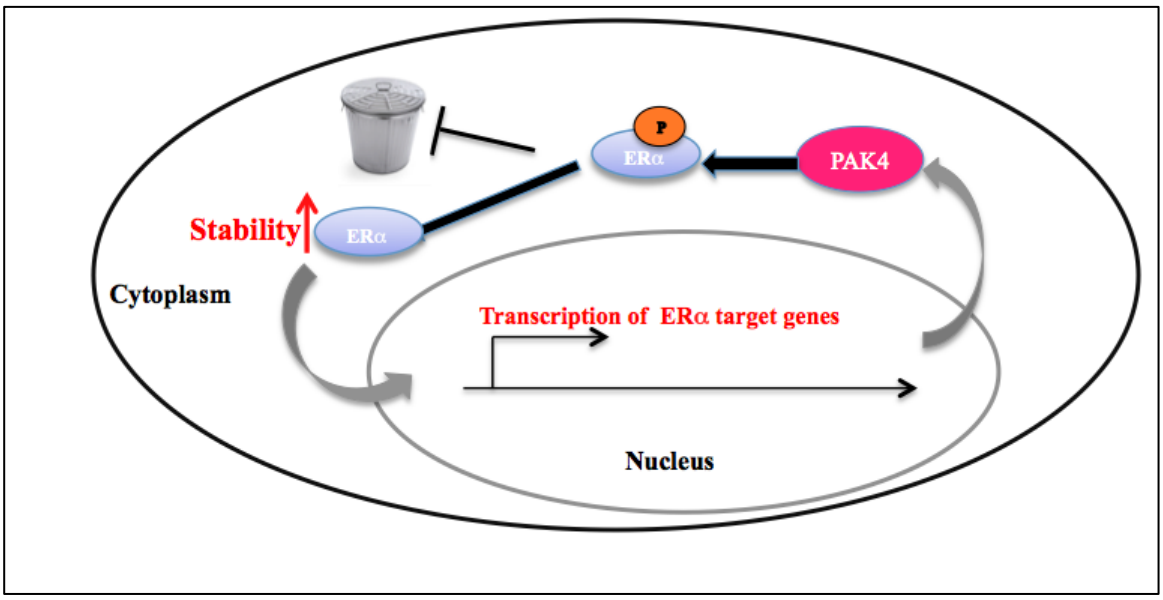

Figure 12. The proposed regulatory effect of PAK4 on ERα.

STUDY IV: AP-1-MEDIATED CHROMATIN LOOPING REGULATES ZEB2 TRANSCRIPTION: NEW INSIGHTS INTO TNFα-INDUCED EMT IN TNBC

The aim of this study was to identify the regulatory role of AP-1 for ZEB2 gene expression, which mediates EMT in TNBC cells. Upon TNFα treatment, triple-negative BT549 and Hs578T cells change morphology into spindle-like shape. Furthermore, TNFα treatment increases mesenchymal makers including N-cadherin and fibronectin and decreases epithelial markers including E-cadherin. After depletion of AP-1 family members Fra-1/c-Jun or ZEB2, we observed similar changes in cell morphology and EMT markers. This indicates that possible link between Fra-1/c-Jun and ZEB2, both of which are involved in EMT in TNBC cells.

By analysis of phosphorylated Fra-1 and c-Jun in TNFα-treated cells, we observed that TNFα can increase their phosphorylation level. Additionally, TNFα treatment can increase ZEB2 mRNA and protein levels in both BT549 and Hs578T cells, an effect which could be compromised by knocking down Fra-1 or c-Jun. ChIP-qPCR showed that Fra-1 and c-Jun

can bind to the promoter region of ZEB2, and that this binding is increased upon TNFα treatment.

In this study, we report a new role of Fra-1/c-Jun in mediating EMT by transcriptional regulation of ZEB2 in TNBC cells.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

ERα and p53 play important roles in carcinogenesis, cancer progression, and drug sensitivity in breast cancer. During the past decades, much research has focused on the transcriptional functions of these transcription factors. However, transcription factors are subject to post-translational modifications that might affect their activity and signaling capabilities. General findings of this thesis are the ubiquitin modification effect of RNF31 on ERα and p53 signaling and the subsequent effect on cancer cell behavior, and the phosphorylation effect of PAK4 on ERα and its related effect on tamoxifen resistance.

Since ERα is regarded as the main player in the development and progression of ER-positive breast tumors, compounds blocking ERα function have been developed and resulted in significantly improved treatment successes. However, treatment with compounds blocking ERα function, including SERMs, often leads to resistance [175]. In addition to bypassing estrogen signaling, the modification of ERα could affect endocrine therapy sensitivity by several mechanisms. A central finding of this thesis is the identification of two different ERα modulators. Although both identified modifications lead to increased ERα stability and enhanced estrogen signaling, they display distinct molecular mechanisms. RNF31 modifies ERα through mono-ubiquitination, while PAK4 modifies ERα via phosphorylation. However, although PAK4 and RNF31 can stabilize ERα and increase estrogen signaling, it is still unclear exactly how the modifications stabilize the protein. There is data showing that phosphorylation of S305 will affect K302/K303 ubiquitination, which subsequently affects ERα stability [176]. More detailed mechanistic studies are needed to characterize the link between S305 phosphorylation, K302/K303 ubiquitination, and ERα stability.

Although the RING finger protein members of E3 ubiquitin ligases share similar functional domains for their E3 ubiquitin ligase activity, they behave differently with respect to cancer biology. For example RNF53 (BRCA1) has been shown to suppress carcinogenesis by facilitating p53 signaling and suppressing ERα signaling [131, 177]. However, RNF2 together with RNF1 suppresses the p53 pathway and promote carcinogenesis [178]. An important finding from our studies is that another RNF protein, RNF31, might play an oncogenic role in breast cancer. In study I, we characterize RNF31, and show that this E3 ligase is highly expressed in breast tumors. Global gene expression profiling analysis reveals that RNF31 promotes many oncogenic pathways including the NFKB, TGFβ and Wnt pathways, while it inhibits the tumor suppressor p53 pathway. Interestingly, a small peptide

targeting RNF31 has been developed and is under clinical trial for lymphoma [179]. We believe that targeting RNF31 by this peptide could be tested, initially in animal models of breast cancer.

The knowledge of RNF31 and its role in breast cancer is still very limited. We propose that RNF31 mono-ubiquitinates ERα. However, other studies propose that RNF31 can mediate linear ubiquitination [139, 170, 180]. Since RNF31 is an atypical ubiquitin ligase, its different ubiquitination patterns to different substrates should be thoroughly investigated. Additionally, since we only characterize the role of RNF31 in supporting estrogen signaling and inhibiting p53 signaling in ERα-positive breast cancer cells, further investigation is required to characterize the role of RNF31 in TNBC cells, which are ERα negative and express mutant p53. Moreover, this thesis is mainly focused on elucidating the regulatory role of RNF31 on ERα and p53 signaling, and PAK4's role in ERα signaling. Future studies should emphasize the related clinical significance.

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