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POST-TRANSLATIONAL MODIFICATIONS IN MAMMARY GLAND DEVELOPMENT AND MAMMARY TUMOR PROGRESSION

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POST-TRANSLATIONAL MODIFICATIONS IN MAMMARY GLAND DEVELOPMENT AND MAMMARY TUMOR PROGRESSION

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To my dearest family

ABSTRACT

Breast cancer is one of the most common cancers in women. Estrogen receptor α (ERα) signaling and p53 signaling have important roles in breast cancer progression. Therefore, post-translational modifications of ERα and p53 play critical roles in breast cancer. The overall aim of this thesis is to characterize the role of RING-finger protein 31 (RNF31) on ERα and p53 signaling and the function of P21-activated kinase 4 (PAK4) on ERα signaling. Moreover, the role of PAK4 in mouse mammary development and mammary tumor progression was also analyzed.

In the first study, RNF31 was shown to active and stabilize ERα, and subsequently to increase estrogen-stimulated cell proliferation in breast cancer cells. In breast cancer clinical databases, the gene expression of RNF31 and ERα target genes were correlated. The suggested mechanism is that RNF31 interacts ERα via the RBR domain and facilitate ERα mono-ubiquitination.

In the second study, RNF31 depletion was shown to increase the gene expression of p53 target genes. RNF31 depletion caused cycle arrest and cisplatin-induced apoptosis in a p53-dependent manner in breast cancer cells. Depletion of RNF31 increased p53 protein levels and the mRNA levels of its downstream target genes. The suggested mechanism is that RNF31 interacts with the p53/MDM2 complex and stabilizes MDM2 and consequently facilitates p53 poly-ubiquitination and degradation.

In the third study, high PAK4 expression level was correlated with poor tamoxifen response in breast cancer patients in clinical databases, based on analysis of available mRNA expression. In MCF-7 cells, PAK4 overexpression promoted tamoxifen resistance, while PAK4 inhibition sensitized tamoxifen-resistant breast cancer cells to tamoxifen. Mechanistically, we identified a regulatory positive feedback loop, where PAK4 acts as a downstream target gene of ERα; while PAK4 can phosphorylate ERα at Ser305, thereby increasing ERα protein stability and activating ERα signaling. In conclusion, PAK4 may be a suitable target for tamoxifen resistance in breast cancer.

In the fourth study, we elucidated the function of PAK4 in mammary development and mammary tumor progression *in vivo*. We observed no difference in mammary gland development between control mice and PAK4 conditional knockout mice. To test the role of PAK4 in mammary tumor development, conditional depletion of PAK4 was introduced in the MMTV-PyMT breast cancer mouse model. Importantly, conditional PAK4 depletion caused an increased tumor latency in MMTV-PyMT mice, indicating a role for PAK4 in early mammary tumor development.

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. 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 07/2014; DOI: 10.1038/onc.2015.260.

III. **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)
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IV. **Ting Zhuang**, Tania Costa, Parisa Rabieifar, Staffan Strömblad. Increased MMTV-PyMT mammary tumor latency by MMTV-Cre-driven conditional gene depletion of p21-activated kinase 4. (Manuscript)

LIST OF ADDITIONAL PUBLICATIONS (NOT INCLUDED IN THE THESIS)

I. 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.

CONTENTS

LIST OF ABBREVIATIONS

2D	Two-dimensional
ACK	Activated Cdc42-associated kinase
ARF	ADP-ribosylation factor
BAD	Bcl-2-associated death promoter
BCL-2	B-cell lymphoma 2
Cdc42	Cell division control protein 42 homolog
CK	Cytokeratin
DBD	DNA-binding domain
DCIS	Ductal carcinoma in situ
DNA	Deoxyribonucleic acid
DUB	Deubiquitinating enzyme
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E2	17β-estradiol
E3	Ubiquitin ligase
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ERα	Estrogen receptor alpha
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FL	Floxed
GAP	GTPase-activating protein
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GEM	Genetically engineered mouse
GTP	Guanosine triphosphate
HDM2	Human double minute clone 2
HECT	Homologous to the E6-AP Carboxyl Terminus
HER2	Human epidermal growth factor receptor 2
HOIP	HOIL-1-interacting protein
IBD	Integrin binding domain

IBR	In-Between-RING
IDC	Invasive ductal carcinoma
IKKγ	IkB kinase subunit gamma
ILC	Invasive lobular carcinoma
JNK	c-Jun N-terminal kinase
LBD	Ligand-binding domain
LUBAC	Linear ubiquitin assembly complex
MDM2	Murine double minute clone 2
MEK	Mitogen-activated protein kinase kinase
MLK	Mixed-lineage kinase
MMTV-LTR	Mouse mammary tumor virus long terminal repeat
MRCK	Myotonic dystrophy kinase-related Cdc42-binding kinase
mRNA	Messenger RNA
MT	Metallothionein
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NES	Nuclear export signal
NLS	Nuclear localization signal
NR	Nuclear receptor
PAK	p21-activated kinase
PBD	p21-GTPase binding domain
PDK	Pyruvate dehydrogenase kinase
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
PUB	Putative ubiquitin binding domain
PyMT	Polyoma virus middle T antigen
Ran	Ras-related nuclear protein
Ras	Rat sarcoma viral oncogene homolog
RBCK1	RBCC protein interacting with PKC 1
RBR	RING-In-Between-RING
REG	Regulatory domain
RING	Really interesting new gene
RNF31	RING-finger protein 31
ROCK	Rho-associated protein kinase

Ser	Serine
SERM	Selective estrogen receptor modulator
SH3	SRC homology 3
SHARPIN	Shank-associated RH domain-interacting protein
TAD	Transactivation domain
TGFβ	Transforming growth factor beta
TET	Tetramerization domain
Tet	Tetracycline
TNBC	Triple negative breast cancer
UBA	Ubiquitin binding associated domain
WAP	Whey acidic protein
ZNF_RBZ	Zinc finger domain in Ran-binding proteins domain

1 INTRODUCTION

1.1 NEOPLASM

The word “Neoplasm” came from Ancient Greek (neo means “new” and plasma means “formation”), which refers to the tissue having abnormal appearance, abnormal proliferation pattern and undergoing some form of mutation. In most cases, neoplasm forming a mass, it is commonly called tumor or solid tumor; but in few cases, neoplasm does not form a mass, such as in leukemia. The word “tumor” is of Latin origin and means “swelling”. It is noteworthy that, “tumor” is not only referring to “neoplasm”, but also referring to general mass. However, the two words are used as synonyms in daily clinical work.

Neoplasia describes the state of neoplasm growth. The type of proliferation in neoplasia is called neoplastic proliferation, as the cells do not die as they should and divide more quickly, forming tissue without normal function. In contrast to this term, non-neoplastic proliferation is more common in response to inflammation, tissue damage and repair, etc. It is usually occurring according to a physiological requirement and the formed tissue is mature and functional. This kind of proliferation is under control and will be stopped when the initial factor is removed. The proliferating cells are a polyclonal population, which comes from different parental cells. Compared to non-neoplastic proliferation, neoplastic proliferation has several unique characteristics: 1. Neoplastic proliferation is not coordinated with the body, and is harmful to the body. 2. Neoplastic proliferation is monoclonal. A cell population originates from one neoplastic transformed parental cell, which is a phenomenon called neoplasia clonality. 3. In neoplastic proliferation, the cell morphology, metabolism, function, and differentiation are abnormal. 4. In neoplastic proliferation, the cells have relative autonomy. The growth is rapid and out of control. Even if the initial factors have been removed, the proliferation effect cannot be eliminated, because of the gain-of-function oncogenes and/or the loss-of-function tumor suppressor genes, which can be passed on to the offspring cells.

There are benign and malignant neoplasms. Benign neoplasms usually grow slower and have no capability to invade into the surrounding tissues or metastasize to other parts of the body. Benign neoplasms are usually not fatal unless vital organs are pressed, such as brainstem compression. In contrast, malignant neoplasms grow faster and have the capability to invade the surrounding tissues and to metastasize to distant organs. Metastasis is the main course of death from malignant neoplasms. Actually, benign and malignant neoplasms are not totally black and white, and sometimes there is a grey zone in between. Pre-malignant neoplasms,

which are non-invasive, have the potential to become malignant neoplasms. In clinic, pre-malignant neoplasms are difficult to diagnose and/or to design treatment strategies against.

The malignant neoplasms, also known as cancers, can be divided into different groups, *e.g.* carcinoma, sarcoma, melanoma, lymphoma, leukemia. Carcinomas are the most commonly diagnosed cancers. They are originated from epithelial cells in breasts, lungs, pancreases, and other organs.

1.2 BREAST CANCER

1.2.1 Epidemiology

Breast cancer is one of the most common cancers and the second most common cause of cancer death in women worldwide [1]. Several risk factors of breast cancer have been discovered (Table 1) [2-4].

Table 1. Established and probable risk factor for breast cancer

Factor	High-risk group
Age	Age ≥ 55-year-old
Race	White
Geographical location	Developed country
Age at menarche	Menarche before age 11
Age at menopause	Menopause after age 45
Age at first full pregnancy	Age of first childbirth ≥ 40-year-old
Family history	Breast cancer in the first-degree relative when young
Previous benign disease	Atypical hyperplasia
Mammographic density	Density ≥ 75% of the mammogram
Cancer in another breast	
Socioeconomic group	Group I and II
Lifestyle	
Diet	High intake of saturated fat
Body weight	Body mass index >35
Alcohol consumption	Excessive intake

Smoking	Initiate smoking before first birth
Exposure to ionizing radiation	Abnormal exposure in young female after age 10
Taking exogenous hormones	
Oral contraceptives	Current use
Hormone replacement therapy	Use for ≥ 10 years
Diethylstilbestrol	Use during pregnancy

1.2.2 Pathophysiology

Breast cancers are usually originated from epithelial cells of mammary glands. The carcinogenesis of breast cancer could be due to different molecular events, such as DNA damage and genetic mutations. Each molecular abnormality may lead to the distinct genomic profiling and a different breast cancer subtype. Some individuals with a family history of breast and/or ovarian cancer inherit defects in DNA, such as mutations in BRCA1/2, TP53, or PTEN. An abnormal estrogen exposure can also lead to mutations, which may contributes to the breast cancer formation [5]. Besides the genetic events, the deficiency of immune system also contributes to the development of breast cancer. High activity of proliferation signaling and/or low activity of cell cycle inhibition signaling may affect several cancer cell behaviors, such as cell proliferation, cell survival, cell apoptosis, cell adhesion, and cell motion.

1.2.3 Breast cancer categories

1.2.3.1 Grade

Breast cancer grade indicates the differentiation level. According to tubule formation, nuclear pleomorphism and mitotic count, breast cancer can be classified as low-grade (well differentiated), intermediate-grade (moderately differentiated), and high-grade (poorly differentiated). Lower-grade tumors usually have a better survival rate and can be treated less aggressively; while higher-grade tumors usually link with worse survival rate and require more aggressive medications.

1.2.3.2 Stage

Breast cancer stage indicates the overall distribution of the cancer cells in the whole body, which is mostly referenced to make the therapeutic decisions. The TNM staging is the most commonly recommended, and is based on the size of the tumor (T), lymph node involvement (N), and whether the cancer has metastases (M) to obtain the overall stage. Breast cancer

stage scales from 0 to IV, spanning from noninvasive breast cancer, early invasive cancer to locally advanced breast cancer and metastatic breast cancer. The 5-year overall survival decreases from 99% in stage 0 to 24% in stage IV [6].

1.2.3.3 Histopathological classification

Histopathology classification is based on light microscopy observation of biopsy specimen. Most breast cancers are carcinomas. Carcinoma is a type of cancer originating from epithelial cells. Adenocarcinoma is the most common pathological subtype in breast cancers, which refers to a carcinoma featuring glandular-related tissue cytology and gland-related molecular products. The three most common histopathological types stand for three-quarters of breast cancers: Invasive (or infiltrating) ductal carcinoma (IDC) (55%), Ductal carcinoma in situ (DCIS) (13%), and Invasive (or infiltrating) lobular carcinoma (ILC) (5%) [7]. DCIS, also called intraductal carcinoma, is non-invasive, as the cells have not invaded through the basal layer of the ducts into the surrounding breast tissue. DCIS is a pre-cancer and up to 30% of DCIS cases will develop an invasive ductal carcinoma within 10 years after the DCIS diagnosis. An accurate way to predict the transformation from DCIS to invasive carcinoma is still missing. IDC is the most common type of breast cancer. It starts from a milk duct, breaks through the basal layer of the duct, and has the capability to metastasize through lymphatics and blood stream. ILC starts in the glands (lobules) of breast, and breaks through the basal layer of lobules. Also, like IDC, ILC can metastasize to other parts of the body.

1.2.3.4 Clinical-pathological classification

Breast cancer clinical-pathological classification is based on the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [8, 9]. Breast cancers are grouped as hormone receptor-positive (positive for estrogen receptor and progesterone receptor), HER2-positive, triple positive (positive for estrogen receptor, progesterone receptor, and HER2), and triple negative breast cancer (TNBC) (negative for estrogen receptor, progesterone receptor, and HER2). The adjuvant therapy strategies for different groups are diverse. Endocrine therapy is the priority treatment for hormone receptor-positive patients. Trastuzumab is the target treatment for HER2-positive patients. Moreover, triple-negative breast cancer (TNBC) patients mainly use chemotherapy and radiotherapy.

1.2.3.5 Molecular subtype

Genome-wide microarray analysis has been used to classify invasive breast cancer into different groups: Luminal A, Luminal B, normal-like, HER2 type, and basal-like (Table 2) [10-12]. There are also some breast cancers that do not fall into any of these groups, and they can be listed as unclassified. Most of the breast cancers belong to the luminal groups. Luminal A tumors tend to be tumor grade 1 or 2. Among all these groups, luminal A has the best prognosis [13]. The women in the luminal B group are usually diagnosed in an earlier age than in the luminal A group [14]. The luminal B group patients have a poorer prognosis than the luminal A group, because they tend to have worse tumor grade, larger tumor size, and more lymph node-metastasis [13, 15, 16]. The HER2 type is not equal to HER2 positive breast cancer. The HER2 type tends to be lymph node-positive and high tumor grade. The prognosis of the HER2 type breast cancer is usually worse than any luminal type. Women with HER2 type tumors are often diagnosed at younger ages than those with luminal A or luminal B tumors. Most of the basal-like breast cancers and triple negative breast cancers (TNBC) overlap. However, there are still some basal-like tumors not belonging to TNBC; and also, some TNBC not belonging to the basal-like group. Most of the BRCA1 mutation associated breast cancers are both basal-like and TNBC [17]. The basal-like tumors tend to be very aggressive and usually have a poor prognosis. Normal-like tumors tend to be small and have a good prognosis. There is a dispute about whether normal-like tumors constitute a specific molecular subtype, or if they are just a group of unclassified tumors.

Table 2. Molecular subtypes of breast cancer

Molecular subtypes	Molecular markers	Prevalence
Luminal A	ER+ and/or PR+, HER2-, low Ki67	40%
Luminal B	ER+ and/or PR+, HER2+ (or HER2- with high Ki67)	20%
HER2 type	ER-, PR-, HER2+	10-15%
Basel-like	ER-, PR-, HER2-, cytokeratin 5/6+ and/or EGFR+ Overexpression of CK15, CK17, vimentin and c-kit	15-20%

Normal-like	ER-, PR-, HER2-, cytokeratin 5/6- and EGFR- Expression of CK8/18	10%

1.2.4 Current treatments and therapeutic challenges

Through a century change, the greatest progress of breast cancer treatment has been seen: the revolution of locoregional surgery; the application of adjuvant chemotherapy; the therapeutic exploration of estrogen receptor; the targeting of the human epidermal growth factor receptor complex; the use of neoadjuvant treatment; and the approaches of biology-driven systemic therapies. Surgery usually is the primary therapy for breast cancer. Adjuvant therapy is given after primary therapy to increase the disease-free survival. Neoadjuvant therapy is given before primary therapy, to shrink the tumor for surgery [18]. Neoadjuvant therapy is given in the same manner as adjuvant therapy, including chemotherapy, endocrine therapy, radiation therapy, target therapy (*e.g.* Trastuzumab), or a combination treatment. A commonly used drug in endocrine therapy is tamoxifen, which blocks estrogen receptor activity. ER positive patients benefit from tamoxifen treatment, however, many patients develop tamoxifen resistance over time. Endocrine therapy resistance is a major challenge in the clinic. Loss of ER expression cannot explain all of the resistance. It is urgent for scientists to characterize the resistance mechanisms. Based on such scientific studies, this challenge may be conquered.

1.3 P21-ACTIVATED KINASE 4

1.3.1 Small GTPases and the p21-activated kinase family

The Ras superfamily consists of various families of small GTPases. Small GTPases are a type of monomeric GTP-binding proteins, which are homologous to the alpha subunit of heterotrimeric G-proteins, and usually in the range of 20-25 kDa. They function as hydrolase enzymes to hydrolyze guanosine triphosphate (GTP). They are "molecular switches" – active when GTP is bound and inactive when GDP is bound. Three classical regulators of GTPases are GEFs (guanine nucleotide exchange factors), GAPs (GTPase-activating proteins), and GDIs (guanine nucleotide dissociation inhibitors). Based on structure, sequence and function similarity, the Ras superfamily can be divided into five main families: Ras (mainly for cell proliferation), Rho (mainly for cell morphology), Ran (mainly for nuclear transport), Rab (mainly for vesicle transport), and Arf (mainly for vesicle transport) family GTPases. Among them, only the Ras and Rho families transmit signals from cell-surface receptors. The Rho family GTPases regulate many aspects of cell morphology. There are three heavily studied

members of this family: Rac1 (mainly affects lamellipodia), Cdc42 (mainly affects filopodia), and RhoA (mainly affects stress fibers). The main effectors of these GTPase are PAK, ACK, MLK, MRCK, and ROCK.

The p21-activated kinase (PAK) family is among the most extensively studied effectors of Rac1 and Cdc42. The PAK family consists of six members and can be divided into two groups based on sequence homology: PAK1-3 in group I and PAK4-6 in group II. PAKs are involved in many cellular functions, such as cell proliferation, cell migration, cell survival and cell death [19]. Moreover, they also play critical roles in tumor progression, such as oncogenic transformation, metastasis and angiogenesis [19]. PAKs, especially PAK1 and PAK4, have often been found overexpressed and/or hyperactivated in many different human cancer forms, such as breast cancer, ovarian cancer, colon cancer and prostate cancer [19]. Among them, breast cancer is the most extensively studied cancer for PAKs. Dominant negative PAK1 leads to a significant reduction of the size of MDA-MB-631 xenograft tumors in mice. Interestingly, the transgenic mice with constitutively active PAK1 develop mammary tumors [20]. These results indicate an essential role of PAK1 in breast cancer.

PAKs regulate several cell signaling pathways controlling cancer cell proliferation, survival, invasion, metastasis, angiogenesis, epithelial-mesenchymal transition, and metabolism. First, for cell proliferation, several PAK members positively regulate key cell cycle signaling pathways such as ERK, AKT and WNT in many cancer cell types. In the ERK pathway, PAK1 can phosphorylate c-RAF at S338 and MEK1 at S298 [21]. In a kinase-independent manner, PAK1 scaffold function may also contribute, as the over-expression of kinase-dead PAK1 can activate ERK in the absence of c-RAF S338 phosphorylation [22]. Moreover, PAK1 scaffold function can also facilitate Akt stimulation by PDK1 and contribute recruitment of Akt to the membrane [23]. The phosphorylation of β-catenin by PAK1 at S663 and S675 stabilizes β-catenin and promotes its nuclear localization, which subsequently upregulates its transcriptional activity [24]. PAK4 was shown to have the similar role [25]. Second, several PAK members have been shown to phosphorylate BAD directly or indirectly indicating a regulatory role in apoptosis [26, 27]. Third, both PAK1 and PAK4 can phosphorylate LIM kinase, which subsequently phosphorylates Cofilin, resulting in polymerization of actin filaments thereby promoting cell motility [28, 29].

1.3.2 PAK4 structure and function

PAK4 is the most extensively studied group II PAK. PAK4 was firstly identified as an effector of Cdc42 to induce actin polymerization and the formation of filopodia [30].

Like other PAKs, PAK4 consists of a conserved C-terminal serine/threonine-kinase domain and an N-terminal regulatory domain. Unlike the auto-inhibition of group I PAKs, the group II PAKs are constitutively phosphorylated. However, a recent study found an autoinhibitory pseudosubstrate in the PAK4 N-terminal region (Figure 1) [31, 32]. In the resulting new model of PAK4 regulation, the binding of an SH3 domain to the newly defined autoinhibitory pseudosubstrate leads to the promotion of PAK4 kinase activity [33].

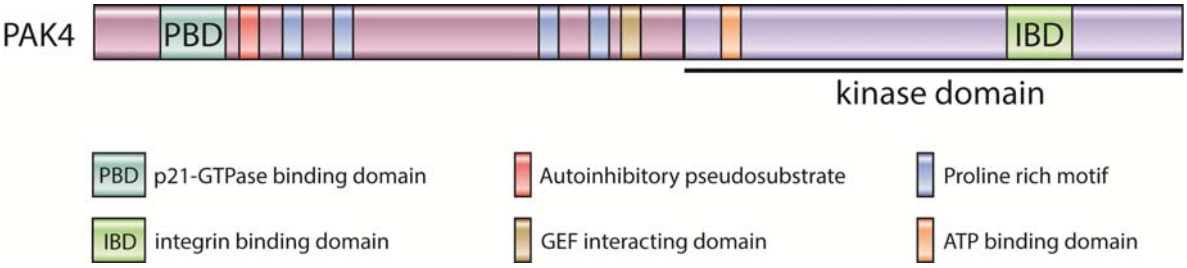

Figure 1. PAK4 protein domain structure.

Besides the Lim kinase, BAD and β-catenin mentioned above, additional PAK4 substrates have been identified, and some of them are shared with other PAKs. For example, PAK4 phosphorylates GEF-H1, consequently reducing RhoA activity [34]. GEF-H1 is also the substrate of PAK1 and PAK2 [35, 36]. Moreover, PAK4 can regulate cell migration by phosphorylating integrin β5 [37].

PAK4 is highly expressed during development [38]. Although the PAK4 expression is universal, it only has relatively high expression levels in limited adult organs, such as prostate, testis, and colon, and in most of the other adult tissues the expression levels are quite low [30]. Moreover, PAK4 may be involved in cancer progression [39]. For example, PAK4 may play a role in cell transformation, since a constitutively active PAK4 mutant transforms mouse embryonic fibroblasts NIH3T3 cells *in vitro*; dominant-negative PAK4 partially inhibits Ras-induced transformation in NIH3T3 cells [40, 41]; and overexpression of PAK4 makes NIH3T3 cells tumorigenic in athymic mice [42]. PAK4 may also be required for anchorage-independent growth of HCT116 human colon carcinoma cells [40, 41]. Moreover, by phosphorylating BAD, PAK4 can also protect HeLa cells from apoptosis [43]. Importantly, PAK4 is up-regulated in most human cancer cell lines [41], and has also been found overexpressed in several human cancer forms, including breast cancer, colon, esophageal, pancreas, and ovarian cancer [42, 44-46]. High PAK4 expression in ovary cancer is linked to poor patient survival and chemotherapy resistance [45]. In breast cancer cells, PAK4 inhibits cell adhesion [40, 47, 48] and promotes cell migration by inducing αvβ5 mediated breast

cancer cell motility [29, 37, 48-50]. However, the potential role of PAK4 in breast cancer remains largely elusive.

1.4 RING-FINGER PROTEIN 31

1.4.1 Ubiquitination

Ubiquitin is a 76-amino-acid polypeptide (8kDa) that can be covalently conjugated to other substrate proteins through a process called ubiquitination. The attachment of ubiquitin to substrates requires three enzymes: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase). As the initial step of ubiquitination, E1 uses ATP hydrolysis energy to attach and activate ubiquitin; then passes this activated ubiquitin to E2. E3 provides platforms for binding of E2 and a selected substrate protein; E3 thereby transfer ubiquitin to the specific substrate. This process may repeat several times to form different types of ubiquitin chains (poly-ubiquitination), and some ubiquitinated proteins can be targeted by the 26S proteasome for degradation [51]. Protein modification with one single ubiquitin is called mono-ubiquitination, and can be a start of a poly-ubiquitination or a separate event [52]. Ubiquitination can be reversed by deubiquitinating enzymes (DUBs) to remove ubiquitin from the substrates.

Humans have approximately 500-1000 different E3 ligases, which can be divided into four families according to the functional domains: HECT, RING-finger, U-box and PHD-finger [53]. Among them, the RING-finger E3 ligase family is the largest. The two most well-known examples in the RING-finger E3 ligase family that have been associated with carcinogenesis are Murine double minute clone 2 (Mdm2) and BRCA1. The human homologue of Mdm2 is also called Hdm2. Mdm2 is found overexpressed in many human cancers. Mdm2 interacts and targets p53 for degradation. Mdm2 has functions in protein ubiquitination, DNA double strain break repair, and gene expression regulation. BRCA1 is a tumor suppressor. BRCA1 mutations are found in around 70% of all familial breast or ovarian cancers [54].

RING-In-Between-RING (RBR) E3 ligase is a subfamily of the RING-finger E3 ligase family. RBR is defined by an RING1-in-between-ring (IBR)-RING2 motif. The RBR family has functions in NF-kB signaling and nuclear receptor (NR) signaling. Some of the RBR family members are critical in human diseases, such as Parkin in Parkinson's disease, Dolfin in familial amyotrophic lateral sclerosis and ARA54 in prostate cancer.

1.4.2 RNF31 structure and function

RING-finger protein 31 (RNF31), also called HOIL-1-interacting protein (HOIP), is encoded by the RNF31 gene that was first cloned in 2004 [55]. It belongs to the RBR family. Figure 2 shows the RNF31 protein domain structure [56]. The PUB domain binds to cofactors. The ZNF_RBZ domain is related to the ubiquitin binding function. The UBA domain can bind RBCK1 and mediates linear ubiquitination of IKKγ. The RING-IBR-RING domain is the main functional domain in an ubiquitin ligase.

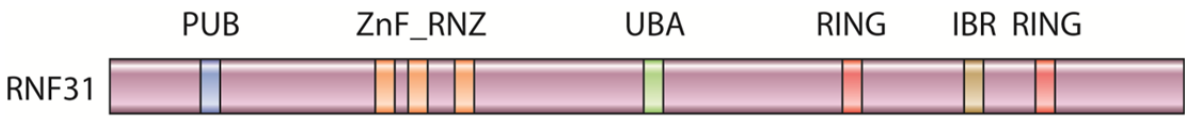

Figure 2. RNF31 protein domain structure. PUB: putative ubiquitin binding domain; ZNF_RBZ: Zinc finger domain in Ran-binding proteins domain; UBA: ubiquitin binding associated domain.

RNF31 is highly expressed in muscle, heart, and testis [56]. RNF31 was originally identified as a muscle-specific tyrosine kinase receptor interacting protein [57]. RNF31 knockout in mice leads to embryonic lethality. In cancer-related studies, RNF31 has been reported to cause cisplatin resistance through ERK and JNK pathways. Moreover, RNF31 can form the linear ubiquitin assembly complex (LUBAC) together with RBCK1 and SHARPIN. This LUBAC can facilitate linear ubiquitination of IKKγ [58].

1.5 ESTROGEN RECEPTOR ALPHA

1.5.1 Estrogen receptor alpha signaling

Estrogen receptor (ER) is a member of the nuclear receptor (NR) superfamily. ER has two main forms, ERα and ERβ, which are encoded by the ESR1 and ESR2 genes, respectively. ERα was firstly reported in the 1960's and cloned from MCF-7 cells in 1985 [59, 60]. ERβ was cloned in 1996 [61], which will not be discussed in detail here.

ERα protein has 595 amino acids, which consist of four main functional domains: a DNA-binding domain (DBD), a ligand-binding domain (LBD) and two transcriptional activation functions (AF-1 and AF-2) (Figure 3).

Figure 3. ERα protein domain structure. A-F: **(A/B)** N-terminal regulatory domain: contains the activation function 1 (AF-1), which is hormone-independent. **(C)** DNA-binding domain (DBD): binds to DNA estrogen response elements. **(D)** Hinge region: contains nuclear localization sequences and interacts with AP-1. **(E)**

Ligand binding domain (LBD): contains the activation function 2 (AF-2), which is hormone-dependent. **(F)** C-terminal domain: the function is not clear.

ERα is activated by estrogen (17β-estradiol, E2). Upon estrogen binding, the ERα protein can shuttle from cytoplasm into the nucleus to form dimers, which subsequently bind to estrogen response elements in the DNA and active downstream target genes [62].

1.5.2 ERα in breast cancer

Around 70% of breast cancers are ERα positive. The risk of breast cancer is higher in breast tissues with high ERα expression [63]. Given that ERα is the target gene of itself, ERα can exert a positive self-regulation. Moreover, high levels of ERα expression in breast cancer cells can lead to increased E2-independent activity of ERα [64]. ER–positive cancers tend to depend on ERα signaling for cell growth, which makes ERα a suitable target for breast cancer therapy.

For ER–positive breast cancer patients, selective estrogen receptor modulators (SERMs) are standard endocrine treatment. The most common used drug in SERMs is tamoxifen. Tamoxifen shares a similar structure with E2, and acts as a competitive inhibitor of E2 binding to ERα. Unlike SERMs, which can be used for all ages of breast cancer patients, aromatase inhibitors are only used for post-menopausal patients [65]. By suppressing aromatase enzyme activity, aromatase inhibitors block the estrogen production from androgens. In pre-menopausal women, ovarian aromatase is responsible for estrogen synthesis; while in post-menopausal women, aromatase in fat and muscle mainly function to produce circulating estrogen [66]. In the hypothalamic/pituitary feedback loop in pre-menopausal women, lower estrogen levels lead to an up-regulation of aromatase enzymes in the ovary. Therefore, aromatase inhibitors are ineffective in pre-menopausal patients.

The largest challenge of tamoxifen treatment is drug resistance. There are many mechanisms that may contribute to tamoxifen resistance. However, the mechanisms of tamoxifen resistance are not entirely clear. It is known that either loss of ERα function or upregulation of ERα function (or loss of control) can lead to tamoxifen resistance. Tamoxifen resistance has been linked to high expression of ERα co-activators, such as SRC, which can promote ERα transactivaty and cell proliferation [67]. Moreover, tamoxifen resistance may also occur due to the cells shifting to depend on other pathways for cell proliferation, such as HER2, EGFR, and NFκB pathways. In this case, blocking of ERα pathway is not efficient. ERα post-translational modifications also have functions in tamoxifen resistance, such as

phosphorylation. Multiple phosphorylation sites in ERα have been discovered by mass spectrometry on phosphopeptides. Some of them have been detected in breast tumor biopsy samples, such as S118 [68-74], S167 [74, 75], S282 [76, 77], S305 [77], and T311 [76]. There are some phosphorylation sites linked to tamoxifen resistance, such as S104/S106, S167, and S305 [78]. Among these, S305 is the only phosphorylation site that has displayed clinical correlation with tamoxifen resistance. ERα-S305 phosphorylation positive breast cancer patients tend to be resistant to adjuvant tamoxifen treatment, while ERα-S305 phosphorylation negative breast cancers have been linked to a better recurrence-free survival with tamoxifen treatment [79, 80]. Consequently, blocking ERα-S305 phosphorylation may become a new therapy strategy.

1.6 P53

The p53 protein was firstly reported in 1979 [81]. TP53, the human gene that encodes p53, was uncovered in 1984 [82]. p53 was initially believed to be an oncogene, because p53 levels were higher in many tumors compared to normal tissue, and higher in transformed cell lines compare to non-transformed [83, 84]. Until the second half of the 1980s, p53 was amended as a tumor suppressor gene, because it was found inactivated in human cancers and loss of p53 promoted cancer [85, 86]. The history of p53 research over the past 30 years proves that p53 is one of the most extensively studied genes and proteins in the cancer area.

Human p53 protein consists of 393 amino acids and can be divided into three fragments (N-terminal, central core, C-terminal), and each of them corresponding to specific functions (Figure 4) [87]. The N-terminal fragment contains the transactivation domain (binds to transcription factors) and a Src homology 3-like (SH3) domain (interacts with SIN3). SIN3 can protect p53 from degradation. The central core is the DNA-binding domain. The C-terminal contains nuclear localization and export signals (NLS and NES), a regulatory domain and the tetramerization domain.

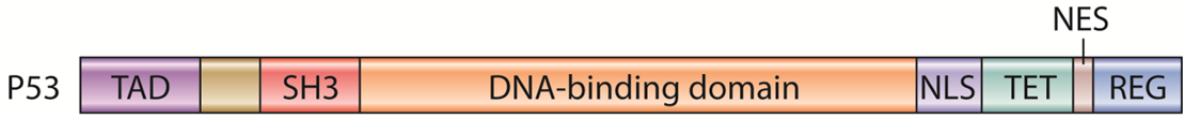

Figure 4. P53 protein domain structure. TAD: Transactivation domain; SH3: Src homology 3-like domain; NLS: Nuclear localization signal; TET: Tetramerization domain; NES: Nuclear export signal; REG: Regulatory domain.

The regulation of p53 is tightly controlled through several mechanisms, such as transcriptional modifications, translational modifications, post-translational modifications,

and subcellular localization. In unstressed cells, p53 has a short half-life with continuous ubiquitylation and subsequent 26S proteasome degradation. The ubiquitylation is mainly due to the interaction of p53 with MDM2 [88]. This interaction can be disrupted in the cells in situations like DNA damage, oxidative stress, or oncogene activation. When p53 ubiquitylation is suppressed and its half-life thereby increased, the stabilized p53 protein accumulates in the nucleus to form homotetrameric complexes and works as a transcriptional regulator. P53 initiates cellular response through transcriptional modifications of distinct target genes that primarily function to prevent the proliferation of damaged cells. Although P53 is mainly a nuclear protein, p53 also has functions in the cytosol by protein-protein interactions. p53 can translocate to the mitochondria, where it interacts with anti-apoptotic proteins (*e.g.* BCL2 and BCL/X_L) to induce cell apoptosis [89].

The importance of p53 in cancer is illustrated by the fact that p53 is one of the most frequently mutated tumor suppressor genes in most of the human cancers [90]. Mutant p53 may lose functions by several different mechanisms. Compared with wild-type p53, mutant p53 proteins generally increase the intensity of phosphorylation and acetylation at the sites, which contribute to the stabilization effect, and subsequently facilitate accumulation of dysfunctional mutant p53 in the nucleus [87]. In the nucleus, the mutant p53 can form tetrameric complexes together with wild-type p53 and hamper the functions of wild-type p53. In addition to abolishing the tumor suppressor function of wild-type p53, mutant p53 often act as an oncogene with new activities, termed “gain-of-function”, that can contribute to apoptosis resistance, genomic instability, aberrant cell cycle, invasion, and inflammation [91, 92].

As in other cancers, p53 plays important roles in breast cancer. Approximately 31% of breast cancer patients carries p53 mutations [93]. In Luminal A, Luminal B, HER2 type, and basal-like breast cancers, the percentage of mutant p53 contains are 15%, 30%, 75%, and 80%, respectively [16]. This illustrates that breast cancers with lower ERα and worse prognosis tend to more frequently carry p53 mutations.

2 AIMS

The general aim of this thesis is to contribute to the knowledge of breast cancer. The specific aims for each paper are described as following:

I. To elucidate the role of RNF31 in ERα signaling in breast cancer.
II. To identify the role of RNF31 in p53 signaling in breast cancer.
III. To determine the role of PAK4 in ERα signaling in breast cancer.
IV. To investigate the role of PAK4 in mouse mammary gland development and mammary tumor progression.

3 MATERIALS AND METHODS

Breast cancer is a group of diseases that have different pathology patterns, genomic features, and outcomes. No single model could mimic all aspects of breast cancer. However, it is still necessary to develop new models to contribute to our understanding and therapeutic targeting of breast cancer. There are different breast cancer models, such as cell lines, xenografts and genetically engineered animals. In this section, the advantages and disadvantages of these breast cancer models will be discussed, although some of them were not used in the studies of this thesis.

3.1 CELLS

Cells are the most commonly used models for breast cancer studies, because there are relatively economical, and easily to propagate and culture. For breast cancer research, the commonly used cells are cell lines and primary cells. There are many differences between human breast cancer cell lines and patient primary breast cancer cells [94, 95], such as genomic alterations, suggesting that during the establishment or after a period of growth and several passages, cell characteristics can change and may become quite different from the initial cells. Unlike primary cells, which are usually isolated from primary tumor lesions, most cancer cell lines are isolated from metastasized cells, which are more aggressive. Noteworthy, most cell culture were performed on traditional two-dimensional (2D) plastic. Compared to 2D culture, three-dimensional (3D) cultures are much better for dynamic interactions between cells and extracellular matrix (ECM) [96]. The ECM has also been shown to be an important regulator of cancer cell morphology and behavior [97]. Moreover, normal cell culture is homotypic and lacks many features of tissues, such as blood vessel, and other cell types communication, which is remarkably different from the breast cancer microenvironment. Heterotypic cultures, which culture the tumor cells together with stromal cells, allow more focus on tumor-stromal cells interaction, such as fibroblasts and macrophages.

3.2 XENOGRAFTS

Many cancer cell lines can be cultured as xenografts, which allow us to analyze the tumor formation, progression and metastasis in a lifelike biological system. However, the xenografts are usually performed in immunocompromised mice, with defect immune systems, which are important in tumor pathophysiology. Moreover, for xenografts, cells are usually subcutaneous injected into the flank of the mouse, which is different from mammary gland

microenvironment. In comparison, orthotopic transplantation into the mammary fat pad is more favorable. Also, from non-invasion to invasion tumors, the cancer cells break through the basement membrane. However, the xenograft tumors do not have this histological structure and do not have the restriction of the basement membrane. For metastasis, the xenografts metastasis mostly occur in the lung; while in human, breast cancer can metastasize to the lung, lymph nodes, bone, liver, and brain.

Clinical isolates can also grow as xenografts, which is a preclinical model and a distinct way to expand patient-derived breast cancer tissue [98]. However, because of the difficulty of clinical samples access, the uncertain transplantation efficiency, and the treatment predicting limitation, this technology has not yet been commonly adopted.

3.3 GENETICALLY ENGINEERED MICE

Among genetically engineered breast cancer research models, genetically engineered mouse (GEM) is the most common used model. Genetic modifications in breast cancer GEM models include the loss of tumor suppressor genes (such as Trp53, Brca1, or Pten) or gain of oncogenes (such as Erbb2, Myc, or PyMT). For the tissue specificity of oncogene targeting, special promoters are used, such as mouse mammary tumor virus long terminal repeat (MMTV-LTR), whey acidic protein (WAP), metallothionin (MT), and cytokeratin 14. Ideally, these promoters should be mammary-specific, but most of them are also expressed in other tissues [99]. Another negative aspect of many of these models is that some promoters, like MMTV and WAP, are hormonally regulated. The expression of these promoters increases during pregnancy and peaks at lactation which may affect tumor etiology [100]. Because most of the breast cancers originate in the mammary gland epithelial cells, the MMTV promoter, which is mainly expressed in mammary gland epithelial cells, is widely used in breast cancer GEM models [101]. This is also the reason why this promoter is used in paper IV study. Advanced genetic modification strategies are used in conditional and inducible GEM, such as Cre/loxP recombinase-mediated gene deletion GEM and tetracycline (Tet)-regulatable transgenes (Tet-Off and Tet-On) GEM. Comparing breast cancer mouse models with human breast cancers, both of them have similar breast cancer oncogenes, multiple genetic mutations, and analogous tumor pathological progression. For sure they also have many differences. Similar with the xenografts, metastasis of breast cancer GEM is also more commonly occurring in the lung; while in human, breast cancer can metastasize to the lung, lymph nodes, bone, liver, and brain. Moreover, mouse mammary tumors have less fibrosis and inflammation as compared to human breast cancers. It is also worth mentioning

that because of the diversity of human breast cancers, no individual GEM can represent this disease perfectly.

In paper IV study of this thesis, we used one of the most extensively studied breast cancer GEM, MMTV-Polyoma virus middle T antigen (MMTV-PyMT), since it shares many properties with human breast tumors. For example, during tumor progression, there is a gradual loss of steroid hormone receptors; and the tumor progression stages (hyperplasia, adenoma, early and late carcinoma) are similar to human breast cancers [102]. Moreover, this mouse model has short latency, high penetrance and a metastatic potential independent of pregnancy. One obvious drawbacks of this model is that PyMT is not expressed in human breast cancer. However, several critical pathways contributing to carcinogenesis in MMTV-PyMT mice are also altered in human breast cancers. The deletion of c-Src results in a significant inhibition of PyMT tumor initiation, which shows the essential role of the Src kinase in the PyMT mouse model [103]. Another kinase that plays a critical role is focal adhesion kinase (FAK); it has been shown that specific depletion of FAK can reduce tumor metastasis in PyMT mice [104]. The evidence for the importance of TGFβ is that blockade of TGFβ inhibits mammary tumor metastasis in PyMT mice [105]. Besides the signal pathway analysis, gene expression profiling has indicated that the tumor generated in MMTV-PyMT mice shares features with the luminal subtype of human breast cancer [106].

4 RESULTS AND DISCUSSION

4.1 PAPER I

The atypical ubiquitin ligase RNF31 stabilizes estrogen receptor alpha and modulates estrogen-stimulated breast cancer cell proliferation

Estrogen receptor α (ERα) is a clinically important mediator of proliferation in ER positive breast cancer. Therefore, insight into the molecular mechanisms that control ERα expression and stability are of outmost importance for the understanding of breast cancer.

Upon knocking-down of RNF31 in MCF-7 cells, cell proliferation decreased dramatically in an E2-dependent manner, which mimicked ERα depletion. Further experiments showed that the depletion of RNF31 reduced ERα protein levels, activity and target gene expression levels. This means that RNF31 contributes to ERα pathways.

In patient specimen, RNF31 was expressed at higher levels in breast tumors compared to adjacent breast tissues. Both in RNF31-depletion microarray data of MCF-7 cells and in the TCGA breast cancer patient database, the expression of RNF31 was correlated with ERα-regulated genes.

Further, RNF31 was found to interact with ERα via the RBR domain and facilitate ERα mono-ubiquitination. Immunofluorescence staining showed that the interaction occurred mainly in the cytosol.

In previous studies, RNF31 was shown to form the linear ubiquitin assembly complex (LUBAC) together with RBCK1 and SHARPIN. LUBAC conjugated linear poly-ubiquitin chains to substrates such as IKKγ, which subsequently facilitates NFκB pathway signaling. Here, we have presented another type of ubiquitin induced by RNF31, which is the mono-ubiquitination on ERα (Figure 5).

There is a large medical need to derive novel therapeutic strategies and targets for breast cancer including novel strategies that modulate estrogen signaling. In this study, we identified such a novel mediator of estrogen signaling that we believe it should be further explored for its potential as a target in breast cancer. This study identified for the first time the E3 ubiquitin ligase RNF31 as a modulator of ERα signaling in human breast cancer cells by a non-transcriptional mechanism, correlating with association and mono-ubiquitination of ERα and enhanced ERα protein stability. Importantly, RNF31 depletion caused the inhibition of

estrogen-dependent cell proliferation, suggesting inhibition of RNF31 as a potential therapeutic strategy in breast cancer.

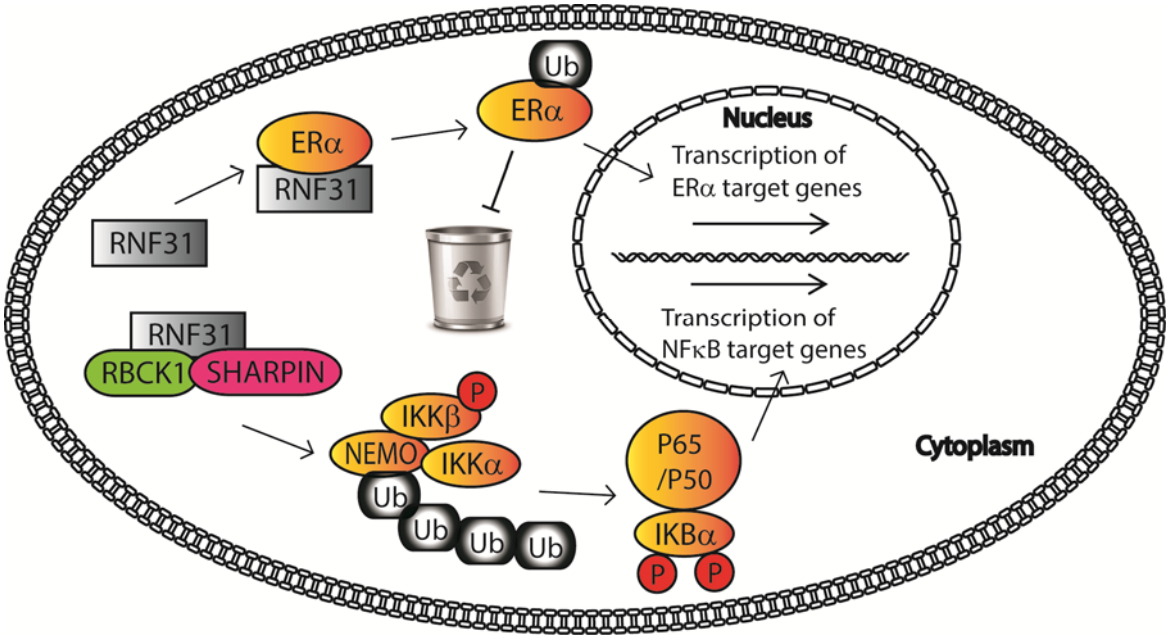

Figure 5. Hypothetical model for the functional interplay of RNF31 with ERα signaling in breast cancer cells.

4.2 PAPER II

RING finger protein 31 promotes p53 degradation in breast cancer cells

P53 is an important tumor suppressor protein. Wild type p53 function often correlates with good chemotherapy response and good prognosis in cancers. Therefore, insights into the molecular mechanisms that control p53 levels are important for the improvement of breast cancer therapeutics. In this study, we identified such a novel mediator of p53 signaling, which deserves further exploration for its potential as a target in breast cancer.

In the microarray analysis performed in paper I, we found that knocking-down RNF31 in MCF-7 cells significantly upregulated many P53-activated genes. We then put this gene list into the TGCA clinical sample database and found that 50% of the genes also display a negative correlation in clinical data. These data showed that RNF31 might be a suppressor for P53 signaling.

Further experiments showed that RNF31 knockdown in MCF-7 cells promotes p53 protein stability, p53 downstream target genes activity, and p53-dependent G1 cell cycle arrest.

To analyze if RNF31 may function in cell death, we switched to the ZR751 cell line, which also express wild type p53, as MCF-7 cells don't express caspase 3 and are resistant to

cisplatin. In cisplatin treatment, RNF31 depletion induced cell death in a p53-dependent manner.

By depletion of RNF31 in MCF-7 cells, we observed a remarkable change of p53 protein levels without any change on p53 mRNA. This indicates that the regulation of p53 may be caused by a post-translational modification. Later experiments showed that RNF31 associated with the p53/MDM2 complex, and induced p53 poly-ubiquitination in MDM2-dependent manner.

We then studied how RNF31 regulate p53 through MDM2. Through co-overexpression of RNF31 with MDM2 in HEK293 cells, we found that MDM2 was stabilized by RNF31. By an immunoprecipitation ubiquitin assay, we found that RNF31 could reduce MDM2 poly-ubiquitination. This effect was also observed in MCF-7 cells.

In summary, this study identified for the first time the E3 ubiquitin ligase RNF31 as a modulator of p53 signaling. p53 protein could be degraded by a few E3 ligases such as Pirh1, COP1 and P300, and in particular by MDM2. RNF31 protein interacted with the MDM2/p53 complex (Figure 6). In this process, RNF31 stabilized MDM2 by prohibiting its poly-ubiquitination. This amplified the MDM2 effect on p53 and facilitied p53 degradation. This is how RNF31 suppresses p53 pathways in breast cancer cell. Importantly, RNF31 depletion in breast cancer cells caused cell cycle arrest and induced apoptosis in a p53-dependent manner suggesting inhibition of RNF31 as a novel potential therapeutic strategy in breast cancer.

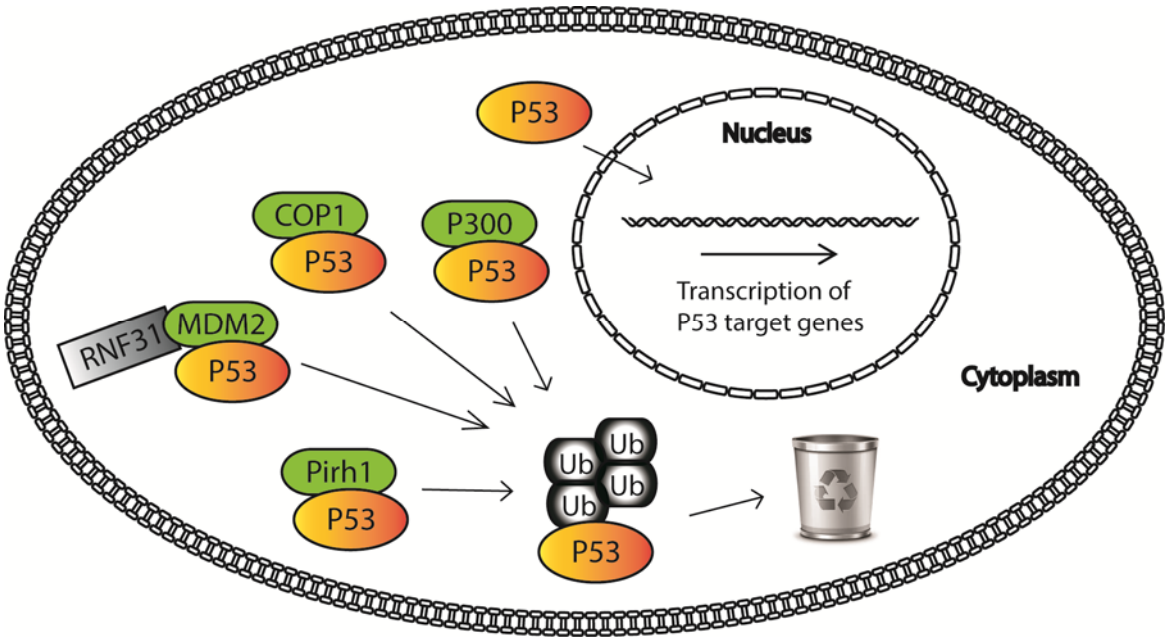

Figure 6. Hypothetical model for the functional interplay of RNF31 with p53 signaling in breast cancer cells.

4.3 PAPER III

P21-activated kinase group II small compound inhibitor GNE-2861 perturbs estrogen receptor alpha signaling and restores tamoxifen-sensitivity in breast cancer cells

Resistance to endocrine therapy remains an important clinical issue in breast cancer treatment. This study describes a potential mechanism in tamoxifen resistance, and a potential marker for endocrine therapy response prediction.

PAK4 expression levels were consistently correlated with poor tamoxifen response in both METABRIC and KMPLOT databases. According to this, we hypothesized that PAK4 might contribute to tamoxifen resistance. Then we tested this hypothesis in human breast cancer cell lines. In MCF-7 cells, PAK4 overexpression promoted tamoxifen resistance. Consistently, the exposure of tamoxifen-resistant MCF-7/LCC2 breast cancer cells to a group II PAK (PAK4, 5, 6) inhibitor, GNE-2861, sensitized these cells to tamoxifen. This indicates that PAK4 may be involved in tamoxifen resistance.

To explore how PAK4 may cause tamoxifen resistance in breast cancer cells, it is necessary to unravel the role of PAK4 in estrogen receptor alpha (ERα) signaling. Interestingly, PAK4 depletion or GNE-2861treatment decreased ERα protein levels, ERα target gene expression levels and ERα regulated reporter gene activity in MCF-7 cells. PAK4 depletion or GNE-2861treatment also decreased E2 stimulated cell proliferation in MCF-7 cells.

Futher, ERα protein levels were decreased, but with no changes in ERα mRNA levels upon PAK4 depletion. We further found that PAK4 increased ERα stability. Using an *in vitro* protein phosphorylation assay, we found that PAK4 could phosphorylate ERα at Serine 305. Previous experimental and clinical data suggested that ERα Ser305 phosphorylation may contribute to tamoxifen resistance in breast cancer [79, 80, 107-110]. One possible mechanism is that an altered orientation between ERα and its coactivator SRC-1 elevates the ERα transcription activity in the presence of tamoxifen [107, 108]. Also, phosphorylation of ERα Ser305 by PAK1 could trigger a secondary phosphorylation on Ser118, which may also contribute to tamoxifen resistance [110].

Interestingly, we found a positive feed-forward loop between PAK4 and ERα (Figure 7). PAK4 is a novel ERα target gene, and PAK4 in turn stabilized ERα protein and activated ERα pathway signaling. The stabilization and PAK4-mediated activation of ERα-dependent

transcription seems to occur via PAK4-mediated phosphorylation of ERα-Ser305. These results suggest that PAK4 may offer a novel drug target for breast cancer patients with tamoxifen resistance, and GNE-2861 may act as a candidate.

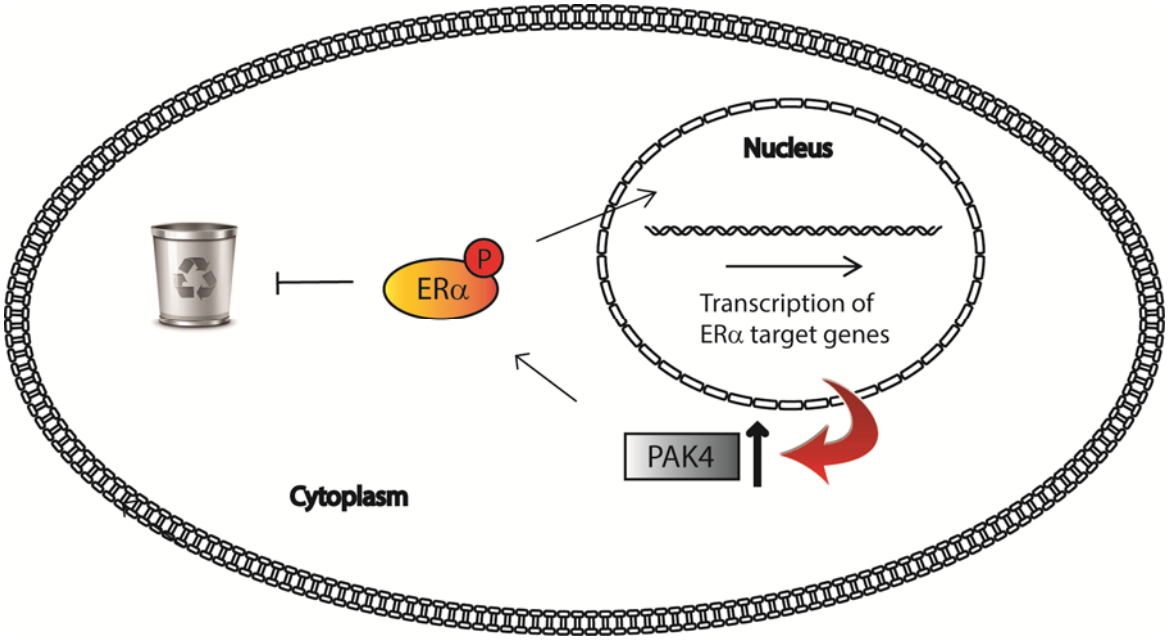

Figure 7. Hypothetical model for the functional interplay of PAK4 with ERα signaling in breast cancer cells.

4.4 PAPER IV

Increased MMTV-PyMT mammary tumor latency by MMTV-Cre-driven conditional gene depletion of p21-activated kinase 4

Given the increased PAK4 expression levels in breast cancer, it is important to elucidate the function of PAK4 in mammary cancer progression in vivo. However, the *in vivo* cancer-related functional evidence is so far limited to xenograft models. In this study, transgenic mice have been used to study endogenous mammary tumor development.

Before a transgenic model with PAK4 depletion can be utilized to study cancer, it is critical to assessing role of PAK4 in the development of the normal mammary gland. We first identified the levels of PAK4 gene expression in the mouse mammary glands. PAK4 mRNA is expressed at relatively low level in mammary glands from virgin to pregnancy, but increased during lactation.

Because of the embryonic lethality upon complete PAK4 gene depletion in mice, we have setup a mouse model lacking PAK4 in the mammary epithelium (MMTV-Cre; $PAK4^{fl/fl}$) using the Cre/loxP system. To test the efficiency of the PAK4 gene depletion in MMTV-Cre;

PAK4$^{fl/fl}$ mice, the PAK4 mRNA was isolated from lactation mice. MMTV-Cre; PAK4$^{fl/fl}$ mice displayed a strong reduction of PAK4 mRNA levels compared to the control mice.

To observe the ductal growth in juvenile and in adult virgin mice as well as the development during pregnancy and lactation, we examined mammary gland whole mounts and hematoxylin and eosin (HE) stainings of the mouse mammary gland tissues. However, we did not find any difference in mammary gland development between control mice and PAK4 conditional knockout mice. To study the function of the mamamry gland, progeny nursed by MMTV-Cre; PAK4$^{fl/fl}$ female were found to have the same weight as in the control group during weaning. As Cre is expressed from postnatal day 22 in MMTV-Cre line D mice [111, 112], we conclude that loss of PAK4 from this stage does not cause defects in mammary gland development from juvenile to lactation. The analysis of mammary gland involution after lactation is in progress.

In MMTV-PyMT mice, PAK4 was found highly expressed in tumors compared to the surrounding normal mammary tissue. To test if genetic depletion of PAK4 may affect mammary tumor development and progression, conditional depletion of PAK4 was introduced into transgenic MMTV-PyMT mice, in which the tumors are induced by the polyoma middle T oncoprotein (PyMT). PAK4 depletion efficiency was tested by immunoblot of the mouse samples. By tumor palpation, conditional depletion of PAK4 was found to be associated with increased tumor latency ($P<0.01$). Mouse mammary gland whole mount stainings also showed that MMTV-Cre; PAK4$^{fl/fl}$; MMTV-PyMT mice have less lesions compared to the control mice at 12 weeks of age. These results indicate a role for PAK4 in early tumorigenesis. Previous studies have shown that PAK4 may contribute to cell transformation, because in NIH3T3 cells, constitutively actived PAK4 caused cell transformation, while dominant-negative PAK4 partially inhibited Ras-induced cell transformation [40, 41]. Moreover, recent findings by Costa et al in our laboratory indicated a role for PAK4 in cancer cells to stay out of cellular senescence, a process acting as a barrier in early cancer development.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

In paper I and II, we reported novel roles for RNF31 in breast cancer. RNF31 increased ERα protein stability, ERα signaling activity and estrogen-dependent cell proliferation in breast cancer cells. Importantly, we also observed a positive correlation of gene expression between RNF31 and ERα downstream target genes in breast cancer patient databases. The possible mechanism involves mono-ubiquitination modification of RNF31 on the ERα protein. Moreover, we extended our microarray data analysis to explore the negative regulation of RNF31 on p53 signaling. As an atypical E3 ubiquitin ligase, RNF31 increased MDM2 protein stability, and consequently contributed to p53 protein poly-ubiquitination and degradation. A negative correlation in gene expression levels between RNF31 and p53 targets genes was observed in breast cancer patient databases. Functionally, RNF31 depletion increased cell cycle arrest effect and cisplatin-induced apoptosis in a p53-dependent manner in breast cancer cells. These studies together suggest that, RNF31 may constitute a potential therapeutic target for breast cancer.

In paper III and IV, we focused on the role of PAK4 in breast cancer. PAK4 expression was shown to correlate with tamoxifen resistance in two breast cancer clinical databases and to functionally promote tamoxifen resistance in human breast cancer cell lines. Further experiments showed that PAK4 could phosphorylate ERα at Ser305 thereby increasing ERα protein stability and signaling activity. To study the *in vivo* function of PAK4 in breast cancer, we generated PAK4 mammary gland conditional knockout mice. While we did not find any effect of this PAK4 depletion in mammary gland development, the depletion of PAK4 caused the prolonged tumor latency in MMTV-PyMT mice. Together, this may indicate that also PAK4 may be a potential target for breast cancer therapy.

In papers I and II, we showed that RNF31 facilitates ERα mono-ubiquitination and p53 poly-ubiquitination. Other studies also showed that RNF31 can form the linear ubiquitin assembly complex (LUBAC) together with RBCK1 and SHARPIN, which facilitate signal transduction of the NFKB pathway. It would be very interesting to elucidate the roles of RNF31 for different substrates in a variety of ubiquitin modifications.

In papers I and II, we found that RNF31 plays important roles in breast cancer cells *in vitro*. However, the knowledge of RNF31 function in breast cancer is still limited. There is a lack of *in vivo* RNF31 breast cancer studies. As RNF31 knockout in mice leads to embryonic lethality, RNF31 conditional knockout mice will be a suitable model to analyze the roles of RNF31 in mammary development and tumorigenesis.

In paper I, II and III, the roles of RNF31 and PAK4 were analyzed in ERα–positive cell lines. Endocrine treatment is specific for ERα–positive breast cancer patients, while Trastuzumab is the target treatment for HER2-positive patients. However, there is at present no specific targeted treatment for triple negative breast cancer (TNBC). Also, it may therefore be interesting to examine the potential roles of RNF31 and PAK4 in triple negative breast cancer (TNBC).

In paper III, ERα was found to be a substrate of PAK4. Interestingly, ERα is also a subtrate of PAK1. PAK1 and PAK4 are the most extensively studied members among the PAK group I and II, respectively. There is also other substrates shared between PAK1 and PAK4, such as Lim kinase, GEF-H1, BAD, Paxillin, Raf-1, and β-catenin. It will be interesting to elucidate if there are more overlapping substrates between different PAK kinases.

In paper III, a group II PAK inhibitor, GNE-2861, has been used. Inhibitors of both RNF31 and PAK4 are under development. However, none of these has yet been successfully passed any clinical trial. One PAK4 inhibitor (PF-3758309, Pfizer) has been tested in Phase I clinical trials, but was withdrawn by the reason remains undisclosed. An RNF31 inhibitor has been used in a pre-clinical study of diffuse large B-cell lymphoma (DLBCL) [113]. This inhibitor could also be tested in breast cancer in future studies. The exploitation of RNF31 and PAK4 specific inhibitors may offer us another choice to cancer therapeutics.

In paper IV, the role of PAK4 in breast cancer metastasis has not been analyzed, because no lung metastasis has been found in this study. This may be because of the strain specificity or because the endpoint time we set is relatively early. As PAK4 has been shown have important functions in cell adhesion and motility, it would be interesting to use an alternative model to study metastasis *in vivo*.

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