Novel insight into the regulation of steroid hormone receptor signaling by protein arginine methyltransferase 6 and regulation of TRIM22 expression in breast cancer

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**Novel insight into the regulation of steroid hormone receptor
signaling by protein arginine methyltransferase 6 and
regulation of TRIM22 expression in breast cancer**

SUN YANG

SCHOOL OF BIOLOGICAL SCIENCES

2012

**Novel insight into the regulation of steroid hormone receptor
signaling by protein arginine methyltransferase 6 and
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School of Biological Sciences

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LIST OF ABBREVIATIONS

4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid	HEPES
40 S ribosomal S6 kinase 1	S6K1
5-aza-2'deoxyctidine	5-aza-dC
90kDa ribosomal S6 kinase	RSK
activation domain 2	AD2
activation function 1	AF1
activation function 2	AF2
activator protein 1	AP-1
acute promyelocytic leukemia	APL
Adenosine, periodate oxidised	AdOx
ammonium persulfate	APS
androgen receptor	AR
base pair	bp
BCL-2 antagonist of cell death	BAD
bovine serum albumin	BSA
Brahma-related gene 1	BRG1
breast cancer type 1 susceptibility protein	BRCA1
B-upstream segment	BUS
cAMP response element-binding	CREB
camptothecin	CPT
CCAAT/enhancer binding protein β	C/EBP β
Chromatin immunoprecipitation	ChIP
coactivator associated arginine methyltransferase	CARM1
Co-immunoprecipitation	Co-IP
CREB binding protein	CBP
cyclic AMP	cAMP
cyclin dependent kinase 7	CDK7
cyclin-dependent kinase 2	CDK2
cyclin-dependent kinase inhibitor 1C	CDKN1C
cycloheximide	CHX
cytomegalovirus	CMV
deoxyribose phosphate	dRP
diethylpyrocarbonate	DEPC
DNA methyltransferase	DMNT
DNA-binding domain	DBD
encephalomyocarditis virus	EMCV
endothelial nitric oxide synthase	eNOS
enhancer of polycomb 1	Epc1
Enzyme-Linked Immunosobent Assay	ELISA
epidermal growth factor	EGF
Epstein-Barr virus	EBV
estradiol	E2
estriol	E3

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estrogen receptor	ER
estrogen related orphan receptor	ERR
estrone	E1
etoposide	VP16
Ewing sarcoma	EWS
extended interferon-stimulating response element	eISRE
fetal bovine serum	FBS
fibroblast growth factor receptor 1	FGFR1
Fluorescence-activated cell sorting	FACS
focal adhesion kinase	FAK
Forkhead box A1	FOXA1
Forkhead box O	FOXO
GATA-binding protein 4	GATA4
glucocorticoid receptor	GR
G-protein coupled receptors	GPCR
green fluorescence protein	GFP
growth regulation by estrogen in breast cancer 1	GREB1
guanine nucleotide exchange factor	GEF
guanosine triphosphatase	GTPase
heat shock protein	hsp
heterogeneous nuclear ribonuclear protein	hnRNP
histone acetyltransferase	HAT
histone deacetylase	HDAC
hormone response element	HRE
human double minute -2	HDM-2
human immunodeficiency virus-1	HIV-1
Human Papillomavirus 16	HPV16
Hydroxyurea	HU
I-kappa-B kinase ϵ	IKK ϵ
inhibitory function	IF
insulin-like growth factor	IGF
Interferon	IFN
interferon regulator factor 3	IRF-3
interferon regulatory factor-1	IRF-1
interleukin-6	IL-6
Kaposi's sarcoma	KS
KCNQ1 opposite strand/antisense transcript 1	KCNQ1OT1
Latency-associated nuclear antigen	LANA
Latent membrane protein 1	LMP-1
ligand binding domain	LBD
liquid chromatography-mass spectrometry	LC-MS
Loss of heterozygosity	LOH
loss of imprinting	LOI
mammalian target of rapamycin	mTOR
MAP kinase kinase 6	MKK6

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Midline 1	MID1
mineralocorticoid receptor	MR
mitogen activated protein kinase	MAPK
mitomycin C	MC
mixed lineage leukemia	MLL
monocyte-derived macrophages	MAC
mouse embryonic fibroblast	MEF
murine double minutes 2	MDM2
nitric oxide	NO
N-tropic murine leukemia virus	N-MLV
nuclear bodies	NBs
nuclear factor κ B	NF- κ B
Nuclear hormone receptor corepressor	NCoR
nuclear receptor	NR
nucleocapsid	NC
Receptor interacting protein 140	RIP140
phenylmethylsulphonylfluoride	PMSF
phorbol-12-myristate-13-acetate	PMA
phosphatidylinositol-3-OH kinase	PI3K
phospholipase C	PLC
polymerase β	Pol β
post-translational modification	PTM
progesterone receptor	PR
Progesterone receptor response element	PRE
Promyelocytic leukaemia	PML
protein arginine methyltransferase	PRMT
protein inhibitor of activated STAT 1	PIAS1
protein kinase A	PKA
protein kinase C	PKC
protein lysine methyltransferase	PKMT
proximity ligation assay	PLA
Reactivation of p53 and induction of tumor cell apoptosis	RITA
replication and transcription activator	RTA
ret finger protein	RFP
retinoblastoma	Rb
retinoic acid receptor α	RAR α
Retinoic-acid-inducible gene I	RIG-I
S-adenosyl-L-methionine	SAM
selective estrogen receptor modulator	SERM
serum response factor	SRF
signal transducer and activator of transcription 1	STAT1
silencing mediator of retinoid and thyroid hormone receptors	SMRT
single-nucleotide polymorphism	SNP
small ubiquitin-like modifier	SUMO
SplA and ryanodine receptor	SPRY

Index

Src homology 2	SH2
steroid hormone receptor	SHR
Steroid receptor coactivator-1	SRC-1
Stimulated Trans-Acting Factor of 50kDa	Staf-50
stimulating protein 1	Sp1
Suppressor of cytokine signaling 1	SOCS1
Su(var)3-9, enhancer of zeste, trithorax	SET
Tat transactivation region	TAR
TATA-binding protein	TBP
TBP associated factor 15	TAF15
Tetramethylethylenediamine	TEMED
Thrombospondin-1	TSP-1
transcriptional intermediary factor 1	TIF1
transcriptional intermediary factor 2	TIF2
translocation associated membrane protein 1	TRAM1
trichostatin A	TSA
Tripartite motif containing	TRIM
tumor necrosis factor α	TNF α
variable region	VR
very low-risk Wilms tumors	VLRWT

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ABSTRACT

Estrogen and progesterone are critically involved in the development of breast cancer and their effects are mediated by the estrogen receptor (ER) and progesterone receptor (PR) respectively. The activities of ER and PR are regulated at multiple steps of steroid hormone receptor (SHR) signaling pathway. Post-translational modifications (PTMs) play pivotal roles in regulating SHR signaling, enable quick responses of these receptors to stimuli without the need for new protein synthesis. Initial mass spectrometry analysis of PTMs on ER identified multiple methylated arginines, suggesting that arginine methylation is an important player regulating ER activity. The objectives of the first part of the thesis were to identify protein arginine methyltransferases (PRMTs) that methylate ER and PR and to determine the functional relevance of the methylation. This study showed that PRMT6 binds to ER, PR and androgen receptor (AR), and methylates them independent of ligand. The site of the interaction and mode of regulation by PRMT6 seems to vary among the receptors but the role of PRMT6 in regulating ER activity has been further defined. PRMT6 binds to ER at the C-terminus domains (LBD-region F) and proximity ligation assays indicated that PRMT6 interacts with ER exclusively in the nucleus. PRMT6 overexpression enhances ER ligand-independent and ligand-induced activity via different mechanisms. PRMT6 significantly enhances the ligand-independent activity of ER without the need for its methyltransferase activity. We present evidences suggesting that the ligand-independent activation of ER by PRMT6 is mediated through its binding to ER which may lead to dissociation of chaperone heat shock protein 70 (Hsp70) and Hsp90. On the other hand, enhancement of ligand-induced ER activity requires enzymatically active PRMT6 and possible arginine residues targeted by PRMT6

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have been identified. In light of the recent report that PRMT6 dysfunction is associated with worse prognosis in ER-positive breast cancers, our results suggest that PRMT6 may promote the mitogenic effect of ER both in the presence and absence of estrogen. The increase in ligand-independent ER signaling with high PRMT6 expression level may result in hormone-independent tumor growth which is resistant to the inhibitory effect of tamoxifen.

In contrast to the mitogenic role of ER in breast cancer, PR can exert inhibitory effect on breast cancer growth. Progesterone-induced growth inhibition in PR-transfected MDA-MB-231 cells was associated with a remarkable upregulation of tripartite motif-containing 22 (TRIM22), a p53 and interferon (IFN) target gene. The objective of the second part of the thesis work was to understand the regulation of TRIM22 gene expression and its clinical significance. TRIM22 protein level is consistently downregulated in breast cancer cell lines and breast tumor tissues as compared to non-malignant mammary epithelial cells and normal breast tissues, making it a potential marker for breast malignancy. TRIM22 expression also correlates with p53 expression in normal breast tissue and it can be induced upon p53 activation in non-malignant cell. However, in breast cancer cells, TRIM22 expression is not inducible in response to p53 activation by genotoxic drugs, suggesting that regulation of TRIM22 by p53 is specifically disrupted during tumorigenesis. In addition, TRIM22 is also a regulator of PR activity. It upregulates both the expression level and ligand induced activity of full length PR but inhibits ligand-independent activity of LBD truncation PR. Together with the reported growth inhibitory role of TRIM22 for leukemic cells, this study presents evidence that TRIM22 may inhibit

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the growth of breast cancer cells via p53 and PR signaling pathway. Further correlation of TRIM22 expression in clinical breast cancer samples with clinicopathological parameters will shed light on the prognostic value of TRIM22 in breast cancer.

Introduction

CHAPTER 1

INTRODUCTION

Introduction

1.1 Steroid hormone receptors

Steroid hormones are important regulators of cellular processes such as reproduction, development, metabolism and homeostasis. There are five groups of steroid hormones, namely estrogens, progestogens, androgens, glucocorticoids and mineralocorticoids. They are synthesized in the adrenal cortex (adrenal androgen, glucocorticoids and mineralocorticoids), the testes (testicular androgen, estrogens), the ovary and placenta (estrogens and progestogens). Steroid hormones are all derived from cholesterol and are lipophilic in nature. Upon release from site of synthesis, they travel in the blood stream with carrier proteins and enter the target cells via passive diffusion. Cellular effects of steroid hormones are transduced by their cognate receptors, the steroid hormone receptors (SHRs).

1.1.1 Classification and domain structure of steroid hormone receptors

SHRs are transcriptional factors belonging to the nuclear receptor superfamily. They are classified as the nuclear receptor subfamily 3 (NR3). This subfamily comprises of the estrogen receptor (ER, NR3A), glucocorticoid receptor (GR, NR3C1), mineralocorticoid receptor (MR, NR3C2), progesterone receptor (PR, NR3C3) and androgen receptor (AR, NR3C4). In addition, the estrogen related orphan receptors (ERR, NR3B) which were cloned by sequence homology to other steroid hormone receptors but have no known ligand identified are also included in this subfamily (Benoit et al., 2006; Dahlman-Wright et al., 2006; Lu et al., 2006).

SHRs share a common domain organization, consisting of the N-terminal variable region (VR, region A/B), a central DNA-binding domain (DBD, region C), a hinge region

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(region D) linking DBD and the ligand binding domain (LBD, region E). An additional C-terminal region F is also present in some cases.

Variable region

The VR of SHR contains the transactivation domain, activation function 1 (AF1), which acts in a constitutive manner (Simental et al., 1991). Although AF1 of the various SHRs differ in size and location, their secondary structures are all mainly random coils in nature in the absence of interacting proteins. Presence of interacting partner stimulates the proper folding of AF1 to facilitate protein binding (Kumar and Thompson, 2003). AF1 can activate transcription independent of ligand; it also synergizes the ligand dependent transcriptional activity of the receptors, possible by direct interaction between the N- and C-terminal domains of the receptors (Metivier et al., 2001).

DNA binding domain

The DBD of SHRs is the most conserved region between the family members. The core structure of DBD comprises of two adjacent zinc fingers made up by about 70 amino acids, involved in making protein-DNA and protein-protein contacts (Luisi et al., 1991; Schwabe et al., 1993a). Three amino acids located at the C-terminal base of the first zinc finger (P box) are critical for the recognition of specific DNA sequence for receptor binding. They are Glu, Gly and Ala in estrogen receptor and Gly, Ser and Val in the other four receptors. Difference in P box residues distinguishes the DNA binding site for estrogen receptor from the other steroid hormone receptors (Aranda and Pascual, 2001; Fairall et al., 1993; Mader et al., 1989). Residues at the N-terminal base of the second zinc finger (D box) are also shown to be important for the dimerization of receptors. D box

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forms the dimerization interface and mutation in these residues affect receptor binding to target gene promoters (Kaspar et al., 1993; Schwabe et al., 1993b; Takimoto et al., 1992).

Hinge region

The hinge region is a flexible region linking the DBD and LBD. Its sequence is not well conserved between the SHRs. It often harbors the nuclear localization signal and may be involved in interaction with corepressors (Aranda and Pascual, 2001).

Ligand binding domain

LBD is the second most conserved region of the SHRs. LBD consists of 12 α -helices which folds into a three layered anti-parallel α -helical sandwich. This forms the hydrophobic pocket for hormone binding. Subtle differences in the conformation of ligand binding pocket are responsible for the ligand selectivity of SHRs (Bledsoe et al., 2002). LBD also contains the ligand-dependent activation function 2 (AF2), this AF2 function is mainly facilitated by helix 12 (Danielian et al., 1992). Upon ligand binding, the position of helix 12 undergoes changes, favoring AF2 interaction with coactivators (Brzozowski et al., 1997). In addition, LBD is also found to be involved in receptor dimerization (Bledsoe et al., 2002).

Region F

ER and some other nuclear receptors also contain a C-terminus region F which is involved in the regulation of ligand binding and interaction with coregulators, modulating the activity of the receptor (Koide et al., 2007; Skafar and Zhao, 2008).

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1.1.2 Mechanism of action

As transcriptional factors, it has long been recognized that SHRs mediate the effect of steroid hormones mainly by direct binding to specific DNA sequences in the nucleus to regulate gene expression. Subsequent research on the SHR signaling reveals that a much more complex network of coregulatory factors and pathways is involved; action of SHRs can be dependent or independent of ligands and may or may not involve direct DNA binding.

Genomic signaling

Unliganded SHRs are associated with chaperone protein complex consisting of heat shock protein 66, 70 and 90 (hsp66, hsp70, hsp90), p23 and many other molecules. Interaction with the chaperone proteins not only facilitates proper folding of the receptors, keeping it in a conformation that favors ligand binding and receptor activation; it is also essential for dimerization of the receptors. Inhibition of hsp90 ATPase activity is associated with receptor degradation (Gougelet et al., 2005; Kimmins and MacRae, 2000; Powell et al., 2010; Pratt and Toft, 1997b, 2003b).

Under the classical signaling pathway for SHRs, upon ligand binding, SHRs undergo conformational changes and dissociate from the chaperone complex. Ligand binding induces dimerization of the receptors, enabling them to bind to specific regulatory DNA sequences, the hormone response elements (HREs), located at the promoter of target genes. HRE is made up by two inverted repeats of 6 nucleotides, separated by 3 unspecified nucleotides. GR, PR, AR and MR can bind to the same set of HREs (AGAACA_{nnn}TGTTCT), whereas ER and ERR recognize a distinct set of HREs

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(AGGTCAnnnTGACCT) (Truss et al., 1992; Truss et al., 1990). SHRs bind to palindromic HREs in the form of dimers to regulate gene expression (Aumais et al., 1996).

Transcriptional activity of DNA-bound SHRs is mediated by N-terminus AF1 and C-terminus AF2. AF1 is constitutively active whereas activation of AF2 is ligand dependent (Danielian et al., 1992; Tora et al., 1989). Synergy between AF1 and AF2 is required for full transcriptional activity of SHRs (Benecke et al., 2000). AF1 and AF2 regulate gene expression by recruiting and interacting with general transcriptional factors and intermediary coregulatory proteins, which are proteins needed for chromatin remodeling and transcription initiation.

Pioneer transcription factors have been demonstrated to be important for the initiation of transcription by SHRs. They prime transcription initiation by prior binding to specific regulatory DNA sequences such as the enhancer elements; they also bind to compact chromatin such as nucleosomes and help to open up the chromatin structure or enable the binding of other transcriptional factors (Carroll et al., 2005; Cirillo et al., 2002; Cirillo and Zaret, 1999). Forkhead box A1 (FOXA1) is one of the main pioneer factors involving in gene expression induced by SHRs. Forkhead motif is enriched in 56% of ER bound chromatin. FOXA1 recognizes and binds to these forkhead motifs, opening the chromatin structure even in the absence of hormone, making these sites accessible for ER binding upon hormone stimulation (Carroll et al., 2005). Similar observation is made by studying the cisrom of other SHRs (Belikov et al., 2009; Wang et al., 2009). Pioneer factors are important determinants for the DNA binding profile and cell type specific action of SHRs.

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In osteosarcoma cell line U2OS where FOXA1 is absent, the binding profile of exogenous ER is determined by another pioneer factor GATA-binding protein 4 (GATA4). In this case, the region bound by ER and the set of genes transcribed is different from that in breast cancer cells (Krum et al., 2008; Miranda-Carboni et al., 2011). However, overexpression of FOXA1 in U2OS cells can recruit ER to region that it only recognizes in breast cancer cells but not in U2OS cells, altering the gene expression profile induced by estrogen (Hurtado et al., 2010). In addition, FOXA1 is recruited to chromatin in a cell type specific manner, contributing to the lineage specific cistroms for SHRs (Eeckhoutte et al., 2006; Lupien and Brown, 2009).

Coregulators of SHRs can be divided into two groups depending on their actions – coactivators and corepressors. The most well studied coactivators are the p160 family proteins. Steroid receptor coactivator-1 (SRC-1) is the first nuclear receptor coactivator identified (Onate et al., 1995). Subsequently, more members of this p160 family were cloned, such as transcriptional intermediary factor 2 (TIF2) and translocation associated membrane protein 1 (TRAM1) (Takeshita et al., 1997; Voegel et al., 1996). These coactivators contain the basic helix-loop-helix motif for DNA binding and the signature nuclear receptor box (LXXLL, L denotes leucine; and X is any amino acid) for interacting with receptors (Aranda and Pascual, 2001; Savkur and Burris, 2004). SRC-1 binds to SHRs directly and coactivates the transcriptional activity directed by AF1 and AF2. It also promotes the interaction between AF1 and AF2 to facilitate transcriptional synergy of these two activation functions. SRC-1 is not only involved in the recruitment and assembly of various transcriptional factors, it also possesses intrinsic histone

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acetyltransferase (HAT) activity and helps in the unpacking of chromatin, making it more accessible to the transcriptional factors (Onate et al., 1998; Spencer et al., 1997). The transcriptional repression function of SHRs is mediated by various corepressor molecules. Nuclear hormone receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptors (SMRT) are two examples of receptor corepressors. NCoR and SMRT complex with histone deacetylase (HDAC) to inhibit gene expression when they are recruited to the target gene promoters via interaction with LBD region of receptors (Lazar, 2003). Furthermore, actions of coregulators are also cell type or target specific; coactivators can function as corepressors in specific context and *vice versa* (Rogatsky et al., 2001).

Besides direct binding to canonical HREs, ligand activated SHRs can also bind to target gene promoters indirectly by interacting with DNA-bound transcriptional factors such as activator protein 1 (AP-1) and stimulating protein 1 (Sp1) to regulate transcription of genes that do not contain canonical HREs (Gottlicher et al., 1998). For example, upon estrogen treatment, ER interacts with Sp1, enhances Sp1 binding to Sp1 sites on the promoter of insulin-like growth factor (IGF) and upregulates expression of IGF (Maor et al., 2006; Porter et al., 1997). Glucocorticoid induced interaction between GR and AP-1 represses the expression of AP-1 target genes such as keratin K5 and K17 in the skin (Radoja et al., 2000).

Non-genomic signaling

Steroid hormones can induce rapid cellular changes within seconds through plasma membrane receptors without the need for direct transcriptional activation or *de novo*

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protein synthesis. The identities of these membrane bound receptors are still under debate, but at least a certain proportion of this activity is caused by cytoplasmic localized SHRs. Membrane-bound SHRs have been reported for all the SHRs and the presence of nine amino acids membrane localization sequence has been identified in the LBD of ER, PR and AR. It mediates palmitoylation of the receptors, facilitates association with caveolin-1 and the subsequent membrane localization (Chaudhri et al., 2012; Pedram et al., 2007; Strehl et al., 2011; Tasker et al., 2006). Membrane initiated non-genomic signaling usually involves the activation of cytoplasmic signaling cascades such as mitogen activated protein kinase (MAPK) or phosphatidylinositol-3-OH kinase (PI3K) signaling via interaction of SHRs with membrane associated receptors such as G-protein coupled receptors (GPCR) (Watson, 1999). ER interacts with G protein in osteoblast to activate phospholipase C (PLC), lead to increase in intracellular Ca^{2+} concentration by mobilization of Ca^{2+} from endoplasmic reticulum (Le Mellay et al., 1997). Estrogen induced acute activation of endothelial nitric oxide synthase (eNOS), increased nitric oxide (NO) production and vasodilation was found to associate with the activation of MAPK signaling via direct interaction between $\text{ER}\alpha$ and $\text{G}\alpha\text{i}$ subunit of GPCR in endothelial cells (Chen et al., 1999c; Wyckoff et al., 2001). Another study by Simoncini *et al* also suggested that ligand bound ER can increase NO production by interacting with the $\text{p}85\alpha$ regulatory subunit of PI3K in the cytoplasm, upregulates PI3K kinase activity and activates protein kinase B/Akt (Simoncini et al., 2000). Similar protective effect on cardiovascular system has been observed for GR by the same mechanism (Hafezi-Moghadam et al., 2002). Glucocorticoid also inhibits the activation of high-voltage-activated Ca^{2+} channel and Ca^{2+} influx in dorsal root ganglion by interacting with GPCR

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and activating protein kinase C (PKC) downstream (He et al., 2003). PR binds to SH3 domains of many cytoplasmic signaling molecules via its N-terminus proline rich region. It associates with c-Src tyrosine kinase to activate c-Src and the downstream MAPK directly, upregulates cell cycle genes such as cyclin D1, which is responsible for progesterone induced proliferation in breast cancer (Boonyaratanakornkit et al., 2001; Skildum et al., 2005).

Ligand-independent signaling

Although activation of SHRs was initially reported to be dependent on direct hormone binding, it has become more apparent that SHR can also be stimulated by other agents such as growth factors. These hormone-independent transcriptional activities mediate the cross-talk between SHR and other cellular signaling pathways and it is responsible for many important biological functions of SHRs (Aguirre et al., 2007; Eickelberg et al., 1999; Hardy et al., 2008; Jenster, 2000; Kim and Lee, 2009; Ueda et al., 2002; Zhang et al., 2010a). Ligand independent activation of SHRs is achieved mainly by direct phosphorylation on SHRs or their coregulators by kinases (Carascossa et al., 2010; Galliher-Beckley et al., 2011; Pierson-Mullany and Lange, 2004a; Rowan et al., 2000).

External stress conditions such as glucose starvation or oxidative stress stimulate the production of glucocorticoids by adrenal glands to activate GR and restore homeostatic balance (Chrousos, 2009). These stress conditions also induce the direct phosphorylation of GR at serine residue 134 (S134) by p38 MAPK, upregulates the binding of GR to zeta isoform of the 14-3-3 signaling proteins (14-3-3 ζ) at the promoter region of GR target genes and alters gene expression patterns in response to glucocorticoid (Galliher-Beckley

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et al., 2011). ER α can be activated by growth factors in a AF1 dependent and AF2 independent manner (Bunone et al., 1996). The requirement of ER α in epidermal growth factor (EGF) induced uterine growth is demonstrated by the failure of EGF to induce DNA synthesis in the uterus of ER knockout mice as compare to wild type mice (Curtis et al., 1996). ER α knock-in mice with mutant ER α that is defective in response to estrogen show normal increased uterine epithelial proliferation with growth factor treatment (Sinkevicius et al., 2008). EGF activates ER α through the phosphorylation of both serine 118 (S118) by MAPK and phosphorylation of S167 by 90kDa ribosomal S6 kinase (RSK) (Bunone et al., 1996; Joel et al., 1998). Elevated level of cyclic AMP (cAMP) activates protein kinase A (PKA), which can phosphorylate coactivator associated arginine methyltransferase (CARM1) at serine 448 (S448), enable it to bind to ER α LBD directly and enhance ER α transcriptional activity (Carascossa et al., 2010). Activation of AR by interleukin-6 (IL-6), EGF, IGF and Her2/Neu is associated with progression of androgen-independent prostate cancer (Craft et al., 1999; Hobisch et al., 1998; Jenster, 2000). IL-6 activates MAPK which phosphorylates AR and SRC-1 to activate AR (Ueda et al., 2002). EGF induces proliferation and invasion of prostate cancer by activating PI3K/Akt which phosphorylates and activates AR (Bonaccorsi et al., 2004). EGF and IGF also upregulates the expression of Rho guanosine triphosphatase (GTPase) guanine nucleotide exchange factor (GEF) Vav3, which promotes nuclear localization and activation of AR (Lyons et al., 2008). PR can also be activated by cyclin-dependent kinase 2 (CDK2) phosphorylation of serine residue 400 when stimulated by growth factors (Pierson-Mullany and Lange, 2004b).

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1.2 Estrogen receptor

1.2.1 Estrogens

Naturally occurring estrogens exist in three forms – estrone (E1), estradiol (E2) and estriol (E3). They act at different stages in life. Estradiol is the predominant form in non-pregnant woman before menopause; estrone is produced during menopause and estriol is important during pregnancy. All three forms bind to ER with high affinity, but estrone and estriol are weaker agonists on ER than estradiol (Gruber and Huber, 1999; Kuiper et al., 1997). Estrogen is essential for the development of reproductive tract, mammary gland, skeletal and central nervous system (Couse and Korach, 1999). Estrogen also reduces bone resorption, the decrease in estrogen level after menopause is the key reason for osteoporosis in women (Krum and Brown, 2008). Furthermore, estrogen is involved in many other processes such as regulation of metabolism.

1.2.2 Isoforms and structures of estrogen receptor

ER is well conserved during evolution; orthologs of ER have been identified in invertebrates with ER-like functionality (Thornton et al., 2003). There are two isoforms of ER (ER α and ER β) encoded by two separate genes, ESR1 and ESR2 on chromosome six and chromosome fourteen respectively (Green et al., 1986; Greene et al., 1986; Kuiper et al., 1996). ESR1 encodes for 595 amino acids whereas ESR2 encodes for 530 amino acids. There is about 97% sequence homology between ER α and ER β at the DBD region, lesser at the LBD region (about 56%) and only 24% at the N-terminal variable region (Dahlman-Wright et al., 2006). Differences at the N terminus AF1 region account for their ability to interact with different subsets of coregulatory proteins.

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The tissue expression pattern is different for these two isoforms. ER α mRNA is predominantly expressed in the uterus, mammary gland, testis, pituitary, liver, kidney, heart and skeletal muscle, whereas ER β transcripts are expressed higher in the ovary and prostate (Couse et al., 1997). ER α and ER β also have distinct biological functions. ER α promotes breast cell proliferation whereas ER β has been found to be antiproliferative (Treeck et al., 2009). Distinctive defects were observed in ER α and ER β knockout mice. ER α knockout mice are viable but infertile for both male and female. Impaired spermatogenesis with low sperm count and decreased sperm motility is observed in ER α knockout male mice (Couse et al., 2001). Initial development and differentiation of the female reproductive tracts are normal but sexual maturation is disrupted, results in a rudimentary mammary gland, hypoplastic uteri and hyperemic ovaries. Uteri of ER α knockout mice are insensitive to E2, no increase in uterine wet weight was observed after E2 treatment in these knockout mice (Couse and Korach, 1999; Lubahn et al., 1993). On the other hand, ER β knockout female mice develop normal mammary gland and uterus but they are subfertile as follicle maturation is disrupted, resulting in fewer and smaller litters than wild type (Dupont et al., 2000; Emmen et al., 2005; Krege et al., 1998). In contrast, ER β knockout male mice are fertile and show no abnormalities in sexual behavior (Arpino et al., 2008). In addition to the effects on reproductive system, ER α and ER β also affect bone metabolism and homeostasis. ER α knockout mice develop shorter femurs, female mice have smaller bone diameters while male mice have lower bone density (Fagan and Yee, 2008; Gaben et al., 2012). ER β knockout female mice show an increase in bone mineral density content (Gaben et al., 2012). For this study, we focus

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mainly on ER α as it is the predominant receptor responsible for the mitogenic effect of estrogen in breast tissue. ER will refer only to ER α hereafter.

1.2.3 Post-translational modifications of ER

Various types of post-translational modifications (PTMs) such as phosphorylation, acetylation and methylation have been identified in ER and shown to be important for the receptor's activity.

Phosphorylation

ER is a phosphoprotein and its transcriptional activity can be regulated by the direct phosphorylation at multiple sites of the receptor. ER serine 118 (S118) phosphorylation was the first site identified as it reduces gel mobility for ER, making it easier to be detected and studied (Joel et al., 1995). E2 induced phosphorylation at S118 can be carried out by cyclin dependent kinase 7 (CDK7) and it involves interaction with transcriptional factor IIH (Chen et al., 2000c). S118 phosphorylation is important for the regulation of ER target gene expression and ER dimerization (Sheeler et al., 2003). It is necessary for ER recognition by splice factor; it also increases recruitment of p68 RNA helicase (Endoh et al., 1999; Masuhiro et al., 2005). In addition, S118 phosphorylation is critical for the ligand-independent activation of ER by MAPK upon EGF treatment (Bunone et al., 1996). The induction of S118 phosphorylation results in the recruitment of peptidyl prolyl cis/trans isomerase Pin1 which isomerizes the serine118-proline119 bond from a cis to trans isomer, hence upregulates AF1 transcriptional activity (Rajbhandari et al., 2011).

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Phosphorylation on serine residues 167 (S167) is reported to be the main ligand induced phosphorylation site by only one study but not supported by others (Arnold et al., 1994; Yamnik and Holz, 2010). S167 to alanine mutation at this site leads to 70% decrease in E2 induced transcriptional activity and 10 fold decrease in DNA binding affinity of ER (Arnold et al., 1994; Castano et al., 1997). S167 can be phosphorylated by PI3K/Akt2, 90 kDa ribosomal S6 kinase (RSK) and I-kappa-B kinase ϵ (IKK ϵ) both in the presence and absence of E2, which contributes to E2 independent growth and tamoxifen resistance for breast cancer cells (Guo et al., 2009; Joel et al., 1998; Sun et al., 2001). A recent study presented new evidence suggesting that Akt and RSK do not act on S167 directly; these kinases upregulate S167 phosphorylation by activating 40 S ribosomal S6 kinase 1 (S6K1). S6K1 is the kinase responsible for S167 phosphorylation *in vivo* (Yamnik et al., 2009). S167 phosphorylation has also been found to be associated with better response to endocrine treatment and longer survival in breast cancer patients (Jiang et al., 2007; Skliris et al., 2010; Yamashita et al., 2005).

S236 phosphorylation by PKA is another example of ligand independent phosphorylation. Mutating S236 to glutamic acid generates phosphomimic that blocks ER dimerization and DNA binding, whereas mutation to alanine has little effect on dimerization and DNA binding (Chen et al., 1999b). Furthermore, ER phosphorylated at S236 by PKA is protected from proteasomal degradation. Knocking down of PKA activity leads to reduce in ER stability (Tsai et al., 2004). S305 phosphorylation by PKA also enhances binding to target gene promoter. Phospho mimic S305E mutation shows increased ligand-independent DNA binding and transcriptional activity (Tharakan et al., 2008). Absence of

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this phosphorylation correlates with favorable outcome with tamoxifen treatment in breast cancer patients (Kok et al., 2011).

Tyrosine 537 (Y537) phosphorylation in ER facilitate homodimerization and ERE binding (Arnold et al., 1995). Mutation of Y537 to alanine or serine gives rise to constitutively active ER mutants that interact with SRC-1 even in the absence of E2 (Weis et al., 1996). This phosphorylation also regulates the direct interaction between ER and SH2 domain of kinases. Phospho Y537 peptide can inhibit the interaction between ER and Src tyrosine kinase, blocking the E2 induced G1/S transition in MCF7 cells. It also inhibits the growth of mammary cancer cell xenografts. Action of this phospho Y537 peptide appears to be specific to the ER expressing cells and targets ER specifically, making it a good antagonist for therapeutic targeting of ER (Auricchio et al., 2008).

Acetylation

HAT p300/ cAMP response element-binding (CREB) binding protein (CBP) coactivates transcriptional activity of ER by acetylating histone, enabling unpacking of chromatin. p300 also regulates ER's activity by direct acetylation on five lysine residues. Acetylation at lysine 266 and 268 is ligand dependent and it enhances ER binding to DNA (Pang et al., 2006). Acetylation at lysine 299, 302 and 303 is constitutive and it suppresses ER's sensitivity to hormone (Wang et al., 2001a).

Methylation

There are two methylation sites reported for ER, one is arginine 260 and the other is lysine 302 (K302). Methylation at arginine 260 (R260) by protein arginine methyltransferase 1 (PRMT1) occurs in the cytoplasm and it affects mainly the non-

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genomic activity of ER. R260 methylation facilitates the recruitment of Src, p85 and focal adhesion kinase (FAK) to the ER complex which in turn activates Akt and regulates cell cycle progression (Le Romancer et al., 2008). K302 methylation by SET7 methyltransferase stabilizes ER; facilitates the recruitment of ER to its target genes (Subramanian et al., 2008).

Cross-talk between different types of PTM

It has been found that PTM at one residue may affect other modifications at nearby sites or different modifications on the same site. S305 phosphorylation negatively regulates K303 acetylation. Mutation of S305 to phosphorylated mimic (Aspartic acid) blocks K303 acetylation, give rise to ER mutant with enhanced transcriptional activity and hypersensitive to low dose of estrogen, resemble the phenotype of naturally occurring K303R mutant observed in one third of premalignant breast hyperplasia (Cui et al., 2004). K302 can be either methylated or acetylation, resulting in stabilization or inhibition of ER activity respectively. Acetylation on K302 exhibits inhibitory effect for methylation on the same site. This may present a mechanism of destabilizing ER to facilitate the downregulation of ER activity with K302 acetylation (Subramanian et al., 2008).

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1.3 Progesterone receptor

1.3.1 Progesterone

Progesterone is also known as P4 (pregn-4-ene-3,20-dione). It is mainly synthesized in the ovary or placenta (during pregnancy) from cholesterol. Progesterone is essential for female fertility. Its level undergoes regular cyclical changes during menstrual cycle and it plays important roles in various stages throughout the pregnancy (Robker et al., 2000). It is essential for normal breast development during puberty and during preparation for lactation. Progesterone induces ductal branching and alveologenesis in the mammary gland (Atwood et al., 2000). Progesterone can also be synthesized in both central and peripheral nervous system, exerts neuroprotective effect and promotes myelination (Schumacher et al., 2004). In addition, progesterone is involved in many other functions such as bone formation and glucose metabolism (Azizi et al., 2003; Picard et al., 2002).

1.3.2 Isoforms and structures of progesterone receptor

PR gene has been mapped to chromosome 11q22. Its transcription can be initiated from two different promoters, giving rise to two main isoforms of PR, form A and B, differ only by the presence of additional 164 amino acids at the N-terminus of PRB. Although they have identical DNA-binding and ligand-binding domain, PRA and PRB have overlapping as well as distinct functions from each other (Kastner et al., 1990).

Similar to other steroid hormone receptors, PR is made up by the N-terminus variable region, DNA binding domain, hinge region and ligand binding domain. The extra 164 amino acid in PRB is named as B-upstream segment (BUS). BUS contains the third

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activation function (AF3) of PRB and it can activate transcription autonomously. BUS facilitates intramolecular interaction between the N- and C-terminus of PRB, enhancing the interaction of PRB with coactivators such as SRC-1 (Tung et al., 2006). It synergizes the transcriptional activity of AF2 and AF1 in both a ligand dependent and a ligand independent manner (Sartorius et al., 1994). Motifs ⁵⁵LXXLL⁵⁹, ¹¹⁵LXXLL¹¹⁹ and the tryptophan 140 in BUS have been shown to be critical for this AF3 activity. Mutation of any of these three sites reduces the AF3 activity. The variable region also contains an inhibitory function (IF) between AF1 and AF3; it is responsible for recruitment of transcriptional inhibitory corepressor proteins. IF downregulates the transcriptional activity of PRA but not PRB. Most probably its inhibitory effect is masked by the presence of BUS upstream in PRB. Deletion of IF drastically increases the transcriptional activity of PRA. IF also makes PRA a trans-dominant repressor of PRB, blocking PRB activity when a high level of PRA is present (Hovland et al., 1998; Huse et al., 1998). Different abnormalities in the reproductive system are observed in PRA and PRB knockout mice, suggesting that they may have different tissue specific functions. PRA knock out leads to impaired superovulation and uterus implantation; whereas defect in mammary gland morphogenesis and alveolar differentiation is observed in PRB knockout mice (Conneely et al., 2001; Mulac-Jericevic et al., 2003). We focus on the activity of PRB in this study as it is a much stronger transactivator than PRA for mediating the effect of progesterone. PR will refer only to the PRB isoform hereafter unless specified otherwise.

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1.3.3 Post-translational modifications of PR

Phosphorylation

Action of PR is closely regulated by PTM on specific amino acids of PR. The most well studied modification on PR is phosphorylation. There are 14 reported phosphorylation sites on human PR, include both basal/constitutive and hormone induced phosphorylation sites. 13 of these phosphorylation sites are located at the N-terminal half of PR, 6 of them are in the unique BUS region, suggesting the importance of phosphorylation in regulating AF1 and AF3 function in PR (Hagan et al., 2011a; Lange, 2004).

PR phosphorylation at S294 by MAPK is induced by hormone treatment and it regulates the turnover of PR protein. S294 phosphorylated PR is targeted for degradation via the 26S proteasomal pathway. Mutation at this site stabilizes PR by blocking the ubiquitination on PR (Lange et al., 2000). EGF can also induce transient phosphorylation on S294. Inhibition of S294 phosphorylation by mutating it to alanine abolishes EGF induced PR nuclear translocation and DNA binding. EGF pretreatment to induced S294 phosphorylation prior to progesterone treatment enhances ligand induced transcriptional activity (Daniel et al., 2007b).

S345 is rapidly phosphorylated by MAPK after progesterone treatment. This phosphorylation results in the interaction of PR with transcriptional factor Sp1, regulating the expression of cell cycle genes p21 and other growth promoting genes to stimulate proliferation (Faivre et al., 2008).

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CDK2 phosphorylates PR at multiple sites both dependent and independent of ligand. It phosphorylates PR at S81 when treated with ligand or when the cells enter G1/S phase of cell cycle. Phosphorylation at S81 has been shown to be important for the binding of PR to PRE site and activation of specific target genes (Hagan et al., 2011b). S400 is a basal phosphorylation site and the level of phospho S400 can be further increased by progesterone or mitogen treatment with corresponding increase in CDK2 level. Expression of constitutively active CDK2 alone can induce PR nuclear localization via S400 phosphorylation, as demonstrated by the absence of nuclear localization when S400 is mutated. When CDK2 level is upregulated by cyclin E during G1/S phase, increase in S400 phosphorylation is also observed, this acts as a mechanism regulating PR activity during cell cycle stages (Pierson-Mullany and Lange, 2004b).

Acetylation

PR's activity can also be regulated by acetylation. Acetylation has been found in the KKKK consensus sequence in the hinge region (amino acid 638-641). Blocking the acetylation at this site leads to delayed nuclear entry in response to hormone treatment and delay in target gene induction, demonstrating that acetylation in the hinge is important in nuclear-cytoplasmic shuttling and transcriptional response kinetic of PR to hormone treatment (Daniel et al., 2010).

Sumoylation

PR lysine residue 388 (K388) can be sumoylated by small ubiquitin-like modifier-1 (SUMO-1). Initial study showed overexpression of SUMO-1 upregulates transcriptional activity of PR not through direct sumoylation on PR but by sumoylation on coactivator SRC-1. Progesterone induced SRC-1 sumoylation is associated with enhanced interaction

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between PR and SRC-1 and increased SRC-1 nuclear retention (Chauchereau et al., 2003). Conversely, K388 sumoylation downregulates transcriptional activity of PR, represses PR sensitivity to ligand and anchorage independent growth for breast cancer cells. Decrease in rate of degradation for PR is also observed with K388 sumoylation. Daniel *et al* showed that stabilization of PR protein with sumoylation is coupled with dephosphorylation at S294. S294A mutant is heavily sumoylated, double mutation on S294 and K388 rescues the turnover rate of PR upon hormone treatment. Whereas Abdel-Hafiz et al suggested that although sumoylation on Lys 388 slows down PR degradation, there is no coupling of PR sumoylation with phosphorylation (Abdel-Hafiz et al., 2009; Daniel et al., 2007a).

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1.4 Complex role of ER and PR in breast cancer

Breast cancer is the most frequent cancer diagnosed in women, and ranked second as a cause of cancer death in women in the United States of America (American Cancer Society) (2012). In Singapore, breast cancer is the most common type of cancer in female population, 7458 new cases of breast cancer were diagnosed from year 2005 to 2009, 29.4% of the total incidence of cancer reported in this period of time. Breast cancer is also the leading cause of cancer death in Singapore female, responsible for 1769 cases of cancer death (17.8%) from year 2005 to 2009 (Singapore Cancer Registry report) (2011). Beside known risk factors such as gender, age, inherited genetic mutations (BRAC1, BRAC2), prolonged exposure to estrogen (early menarche or late menopause), more than 50% of breast cancer has no identifiable risk factors (Lynch et al., 2008).

ER and PR play important roles in the development, prognosis and treatment of breast cancer. Although both ER α and ER β are mediators of estrogen signaling, they display distinct expression patterns and functional roles in human breast. ER α expression is low in normal breast and it is mainly found in luminal cells. However, ER α overexpression has been found in 80% of atypical ductal hyperplasia and 60-70% of invasive breast cancers (Giri et al., 1989; Pallis et al., 1992; Shoker et al., 1999). 55-90% of early breast cancers analyzed are ER α positive. High ER α expression level at early stage may sensitize the cancer cells to the growth stimulatory effect of estrogen. Breast cancer cells tend to lose ER α expression at later stage and proliferate independent of hormone (Althuis et al., 2004 Schmitt, 1995). Due to the mitogenic activity of estrogen and ER α in breast cancer, anti-estrogens are commonly used in breast cancer treatment with ER α positive breast cancer.

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ER α antagonist tamoxifen has shown 25% reduction in recurrence and 17% reduction in mortality in the treatment of ER positive breast cancer (1992). Aromatase inhibitor anastrozole which inhibits the production of estrogen has also been shown to be effective in breast cancer treatment (Baum et al., 2003).

ER β expression is high in normal breast and benign breast tumor but its level is downregulated in breast cancer tissues (Park et al., 2003). It has been found that ER β promoter is unmethylated in normal breast epithelial cells but hypermethylated in breast cancer cells. Inhibition of DNA methylation can reactivate ER β expression, making DNA methylation a possible mechanism responsible for the downregulation of ER β expression in breast cancer (Zhao et al., 2003). This diminishment of ER β expression in breast cancer may be associated with breast tumorigenesis. When ER β is expressed alone, it inhibits proliferation of invasive breast cancer cells in a hormone-independent manner; increase in ER β expression also inhibits hormone-dependent proliferation in T47D breast cancer cell line (Lazennec et al., 2001; Strom et al., 2004). Studies carried out using murine epithelial cell line HC11 also demonstrated that ER α and ER β exert opposite effect on growth upon estrogen treatment; while ER α induces cell proliferation in response to estrogen treatment, ER β promotes apoptosis in an estrogen-dependent manner, knock down of ER β expression also favors HC11 transformation (Helguero et al., 2005). Microarray study for T47D breast cancer cells showed that ER β suppresses ER α upregulated genes involving in proliferation, steroid metabolism and ion transport (Williams et al., 2008). This possible antiproliferative activity of ER β in breast cancer makes it an attractive therapeutic target for breast cancer treatment.

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PR status is also an important marker in predicting clinical outcomes for breast cancer treatments. It often correlates positively with ER status and indicates presence of functional ER. More favorable outcome for endocrine therapy is usually achieved with ER/PR double positive breast cancer (Bardou et al., 2003). The higher PR expression level in ER-positive breast cancer correlates with increase probability of response to tamoxifen (Ravdin et al., 1992). Absence of PR in ER positive breast cancer made it more aggressive and less responsive to selective estrogen receptor modulators (SERMs) such as tamoxifen. Recurrent rate increases from 7.6% in ER-positive/PR-positive tumors to 14.8% in ER-positive/PR-negative tumors for early breast cancer treated with tamoxifen (Baum et al., 2003). So far, direct function of progesterone receptor in breast cancer remains controversial. Progesterones in oral contraceptives were first reported to stimulate proliferation of breast cancer cell (Jeng et al., 1992). The million women study shows that combination of estrogen plus progesterone in hormone replacement therapy is associated with increased risk of incidence and fatal breast cancer (Beral, 2003). Studies using breast cancer cell lines showed that progesterone induces transient proliferation followed by long-term growth inhibition (Chen et al., 2011; Groshong et al., 1997; Leo et al., 2004; Skildum et al., 2005). In the absence of hormone, PR itself can inhibit the growth of breast cancer cell lines (Jacobsen et al., 2005).

Introduction

1.5 Protein arginine methyltransferase (PRMT)

Evidence is emerging that protein methylation plays an important role in fine-tuning cellular response to external stimuli. Protein methylation mainly occurs at lysine and arginine residues, mediated by protein lysine methyltransferases (PKMTs) and protein arginine methyltransferases (PRMTs) respectively. PKMT can be divided into Su(var)3–9, enhancer of zeste, trithorax (SET)-domain containing and non-SET domain containing PKMTs. There are about 50 human SET-domain containing PKMTs but Dot1L is the only non-SET domain containing PKMT found so far (Dillon et al., 2005; Min et al., 2003; Schapira, 2011). In contrast to high number of PKMTs known, until now, there are only 11 PRMTs identified, all of them contain conserved catalytic domain (Wolf, 2009). Both PKMT and PRMT enzymes transfer methyl group from the donor molecule S-adenosyl-L-methionine (SAM) to lysine or arginine residues of their target proteins. Lysine methylation occurs only at the ϵ -NH₂ group whereas arginine methylation can occur at either or both of the two terminal ω nitrogen atoms of the guanidine side chain (Wu and Zhang, 2009). This study focuses on protein arginine methylation by PRMTs.

1.5.1 Classification and structure of PRMT

PRMT homologs have been found in various species including yeast, *Drosophila melanogaster* and zebrafish, suggesting that this class of enzymes is evolutionally conserved in eukaryotes (Bachand, 2007; Boulanger et al., 2004; Hung and Li, 2004). Of the 11 PRMTs identified in human so far, they can be divided into three groups. Type I PRMT includes PRMT1, 2, 3, 4, 6 and 8. They catalyze the formation of monomethylarginine (ω -N^G-monomethylarginine) and asymmetric dimethylarginine (ω -N^G,N^G-dimethylarginine). PRMT5 and 9 belong to type II PRMT, catalyzing the

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formation of monomethylarginine (ω -N^G-monomethylarginine) and symmetric dimethylarginine (ω -N^G,N^{'G}-dimethylarginine). Contradictory results have been reported for PRMT7. It was first classified as type II PRMT, but recent study on the activity of PRMT7 presented new findings suggesting that PRMT7 may be a type III PRMT, only capable of catalyzing the formation of ω -N^G-monomethylarginine. Enzymatic activity of PRMT10 and 11 has yet to be determined. In addition, there is fourth type of PRMT methylating the internal δ nitrogen. This type IV PRMT has only be described in yeast so far (Cook et al., 2006; Lakowski and Frankel, 2009; Lee et al., 2005b; Wolf, 2009; Zurita-Lopez et al., 2012).

PRMT family members share a conserved methyltransferase catalytic core domain of about 310 amino acids. This catalytic domain contains four short motifs that are involved in the interaction with SAM to facilitate methyltransfer to occur. The signature motif I has highly conserved amino acid sequence of VLD/ExGxGxG (residue varies at position x), mutation of these residues lead to loss of enzymatic activity. The other three motifs are post I (V/IxG/AxD/E), motif II (F/I/VDI/L/K) and III (LR/KxxG). There is also a conserved THW loop at the C-terminus of all the PRMT proteins. Motif I, post I and THW loop form part of the SAM binding pocket (Bedford, 2007; Kagan and Clarke, 1994; Katz et al., 2003). In addition, each PRMT has unique N- and C-terminus sequence and motifs. For example, PRMT3 has a zinc finger, PRMT4 has a Plekstrin Homology (PH) domain and PRMT8 has a myristoylation motif at the N-terminus; PRMT9 has a zinc finger at the N-terminus and an F box at the C-terminus. These different motifs may determine the substrate specificity for the respective PRMTs (Wolf, 2009; Zhang and

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Cheng, 2003; Zhang et al., 2000). PRMTs exist as homo- or hetero-oligomers *in vivo*; interaction between different PRMTs has been shown to have synergistic effect on their enzymatic activity (Lee et al., 2005a; Pak et al., 2011; Rho et al., 2001).

1.5.2 PRMT family members

Methylation by PRMTs affects a broad range of cellular processes. PRMT1 is the first mammalian arginine methyltransferase identified. It is the most abundant PRMT and it is responsible for about 85% of protein arginine methylation activity in mammalian cells (Lin et al., 1996; Tang et al., 2000). PRMT2 has been found to be able to coactivate ER and AR (Meyer et al., 2007; Qi et al., 2002). PRMT3 methylates ribosomal proteins to regulate their cellular levels (Bachand and Silver, 2004; Swiercz et al., 2005). PRMT4 activates gene expression by methylating histone H3, it also coactivates steroid hormone receptors (Bauer et al., 2002; Chen et al., 1999a). PRMT5 is the main methyltransferase responsible for generating symmetrical dimethylarginines (Branscombe et al., 2001). It mediates assembly of spliceosome by direct methylation of Sm protein (Meister et al., 2001). PRMT6 downregulates gene expression by methylating histone H3 arginine 2 (Hyllus et al., 2007). It also possesses antiviral activity through direct methylation of viral structure protein (Invernizzi et al., 2007). PRMT7's enzymatic activity is still under investigation and no direct target has been identified. PRMT8 is membrane bound and it is expressed specifically in the brain (Lee et al., 2005a; Taneda et al., 2007). The only possible target of PRMT8 identified so far is the Ewing sarcoma (EWS) protein (Kim et al., 2008). No direct target has been reported for human PRMT9, 10 and 11. Activities of PRMT1 and PRMT4 have been better characterized and will be discuss in detail below.

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PRMT1

PRMT1 has been mapped to 19q13.3 and it gives rise to three transcript variants (v1, 2, 3) by alternative splicing (Scorilas et al., 2000). In addition to that, study by Goulet *et al* showed that PRMT1 can have up to seven transcript variants, differing by their N-terminal sequences. Unlike the first three isoforms whose tissue expression is ubiquitous, expression of these additional isoforms v4 to v7 are only detectable in specific tissues. For instance, v4 is only found in heart, v5 in pancreas and v7 in heart and skeletal muscle. V6 expression is not detected in any of the tissues tested. All variants are enzymatically active except v7 (Goulet et al., 2007). Studies on the first three isoforms revealed that low expression of v1 correlates significantly with longer disease free survival in breast cancer patients but not v2 or v3 (Mathioudaki et al., 2011). High expression of v1 also associates with highly malignant colon tumors and advance stage colon tumors, suggesting that high PRMT1-v1 expression may serve as a prognostic marker for cancer with unfavorable outcomes (Mathioudaki et al., 2008).

PRMT1 methylates histone H4 arginine (R) 3, stimulates the acetylation of lysine (K) 8 and 12 on H4 tail by p300 and transcriptional activation. PRMT1 knock down leads to loss of histone acetylation on both H3 and H4, as well as an increase in H3K9 and H3K27 methylation, indicating that methylation by PRMT1 on H4R3 is a pre-requisite for histone acetylation and chromatin activation. In contrast, histone acetylation also regulates its own methylation. H4R3 methylation is inhibited on acetylated H4 (Huang et al., 2005; Wang et al., 2001b). PRMT1 methylates a wide range of non-histone substrates. It preferentially recognizes arginine in glycine and arginine rich region (RG or RGG repeats), such as

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RNA binding proteins fibrillarin (GGRGRGG), heterogeneous nuclear ribonuclear protein (hnRNP) (GGRGGS) and TATA-binding protein (TBP) associated factor 15 (TAF15) (DRGGGYGG). PRMT1 methylation on TAF15 keeps it in the nucleus for gene activation (Jobert et al., 2009; Kim et al., 1997). PRMT1 targets the cell survival regulator Forkhead box O (FOXO) at R248 and 250 within its Akt recognition motif. This methylation blocks FOXO phosphorylation by Akt and prevents the nuclear export and degradation of FOXO (Yamagata et al., 2008). PRMT1 interacts and methylates R378 of Axin, stabilize Axin protein by decreasing Axin ubiquitination, in turn it downregulates the level of β -catenin and Wnt signaling (Cha et al., 2011). PRMT1 is also involved in ER signaling. E2 induces ER methylation at residue R260 by PRMT1 specifically. This facilitates the interaction of ER with PI3K and Src tyrosine kinase, activates Akt and downstream cellular signaling (Le Romancer et al., 2008).

PRMT4/CARM1

PRMT4 is also called coactivator-associated arginine methyltransferase 1 (CARM1) as it was first identified as a coactivator of nuclear receptor, enhances transcriptional activity of nuclear receptors by interacting with the p160 coactivators (Chen et al., 1999a). There is only one transcript of 2968 base pairs (bp) found, encodes for 608 amino acids. PRMT4 tissue expression is ubiquitous and PRMT4 knockout mice appear smaller during embryonic development and die just after birth (Yadav et al., 2003). PRMT4 is overexpressed in colorectal cancer and breast cancer (El Messaoudi et al., 2006; Kim et al., 2010). PRMT4 methylation of histone H3R17 and H3R26 have been associated with gene activation. Specific antibody raised against methylated H3R17 demonstrated that PRMT4 methylates H3R17 *in vivo*. Binding of PRMT4 to target gene promoter region results in

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increase H3R17 methylation and activation of genes such as pS2 (ER downstream target) (Bauer et al., 2002). PRMT4 can also upregulates cyclin E1 expression in response to growth stimulatory signal via H3R17 and H3R26 methylation (El Messaoudi et al., 2006). PRMT4 works closely with the histone acetylation. Prior acetylation of H3K18 and K23 by CBP acetylase tethers PRMT4 to histone H3 tail for methylation to occur (Daujat et al., 2002). It can repress CBP activation by methylating KIX domain of CBP and blocks CREB binding (Xu et al., 2001).

PRMT4 is important in SHR signaling and it is necessary for estrogen-dependent transcription. ER and PRMT4 recruitment to enhancer region upon E2 treatment is associated with binding of other coactivators, increase histone modifications and chromatin opening. No such activity is observed in regions without PRMT4 occupancy (Lupien et al., 2009). ER reporter gene activity is lost in mouse embryonic fibroblast (MEF) with PRMT4 knock out (Yadav et al., 2003). PRMT4 regulates both the initiation and termination of ER signaling. It upregulates ER target gene expression by histone methylation at the target gene promoter, such as pS2 and E2F1 (Bauer et al., 2002; Fietze et al., 2008). Conversely, it also regulates the disassembly of coactivator complex by methylating SRC-3 and p300/CBP (Feng et al., 2006; Xu et al., 2001). In addition, PRMT4 interacts directly with ER and methylates ER at LBD when it is phosphorylated by PKA at S448, facilitates ligand-independent activation of ER (Carascossa et al., 2010).

PRMT4 is also involved in many other cellular processes. For example, it regulates alternative splicing by directly methylating splicing factors (Cheng et al., 2007). It also

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coactivates nuclear factor κ B (NF- κ B) transcriptional activity upon tumor necrosis factor α (TNF α) or phorbol-12-myristate-13-acetate (PMA) stimulation independent of its enzymatic activity (Jayne et al., 2009).

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1.6 PRMT6

1.6.1 Identification of PRMT6

PRMT6 is the focus of this study. It was identified from the human genome by sequence homology with other family members. There is only one PRMT6 transcript of 2665 base pairs identified, encoding 375 amino acids. PRMT6 expression appears to be tissue specific. Preliminary study showed that its expression is high in kidney and testis, but low in muscle and heart. Using green fluorescence protein (GFP), PRMT6 was found to localize in the nucleus. PRMT6 displays automethylation activity, capable of generating monomethyl- and asymmetrical dimethylarginine (Frankel et al., 2002).

1.6.2 Histone targets of PRMT6

PRMT6 methylates histones at multiples sites to regulate gene expression. It is the primary methyltransferase responsible for asymmetrical dimethylation of histone H3R2 (H3R2me2a). H3R2 methylation by PRMT6 antagonized H3K4 trimethylation, which is associated with activated promoter regions. These two methylations can only coexist *in vitro* but appear to be mutually exclusive *in vivo*. Dimethylated H3R2 inhibits H3K4 trimethylation by blocking the recruitment of mixed lineage leukemia (MLL) methyltransferase complex to H3, resulting in transcriptional repression. Conversely, PRMT6 recruitment and H3R2 methylation is downregulated at promoter sites with H3K4 di- and tri-methylation. Overexpression of PRMT6 leads to a decrease in transcription of H3K4 trimethylation target genes, such as HOX genes and myc-dependent genes (Guccione et al., 2007; Hyllus et al., 2007; Iberg et al., 2008). PRMT6 is also found to methylate histone H2AR29 both *in vitro* and *in vivo*. Methylated H2AR29 is enriched in

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genes repressed by PRMT6 and on inaccessible chromatin, suggesting that PRMT6 downregulated gene expression through specific methylation on H2AR29 (Waldmann et al., 2011).

1.6.3 Non-histone targets of PRMT6

PRMT6 as antiviral factor

PRMT6 may act as an antiviral factor against HIV infection. It has been found to methylate three components of HIV – Tat, Rev and nucleocapsid (NC). PRMT6 methylates HIV transactivator protein Tat at R52 and 53, reduces the interaction between Tat and the Tat transactivation region (TAR) of viral RNA and the formation of Tat-TAR-cyclin T1 tertiary complex, diminishes cyclin T1 dependent Tat transactivation activity. Overexpression of PRMT6 decreases Tat transactivation of HIV-1 long terminal repeat whereas PRMT6 knock down increases HIV production (Boulanger et al., 2005; Xie et al., 2007). PRMT6 methylation of HIV Rev protein at R38 leads to reduced Rev-mediated viral RNA export. It is also able to downregulate Rev expression independent of its enzymatic activity (Invernizzi et al., 2006). In addition, PRMT6 methylates HIV NC at R10 and 32, makes it less able to promote viral RNA annealing and reverse transcription (Invernizzi et al., 2007).

PRMT6 in cell migration and DNA repair

Thrombospondin-1 (TSP-1) is a natural inhibitor of endothelial cell migration and angiogenesis (Dawson et al., 1997; Dawson et al., 1999). PRMT6 is recruited to the promoter region of TSP-1 and it represses the transcription of TSP-1 by dimethylating H3R2 at its promoter region. PRMT6 knock down in osteosarcoma U2OS cells lead to a loss of H3R2me2a at the TSP-1 promoter and increase in H3K4me3, resulting in

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upregulation of TSP-1 expression and inhibition of cell migration (Michaud-Levesque and Richard, 2009).

PRMT6 directly interacts with DNA polymerase β (Pol β) N-terminus deoxyribose phosphate (dRP)-lyase domain, methylating arginine residues 83 and 152 of Pol β . Pol β methylation by PRMT6 increases in response to DNA damage. It enhances Pol β binding to DNA and processivity, which may increase the efficiency of base excision repair (El-Andaloussi et al., 2006).

PRMT6 and steroid hormone signaling

PRMT6 coactivates hormone induced transcriptional activity for ER, PR and GR in the luciferase reporter gene assay. Methyltransferase activity is essential for this coactivator function. Chromatin immunoprecipitation (ChIP) showed that PRMT6 is recruited to the promoter region of ER target genes upon E2 treatment. It cooperates with PRMT4 and SRC-1 to enhance E2 induced transcriptional activation. PRMT6 knock down decreases E2 induced gene expression such as growth regulation by estrogen in breast cancer 1 (GREB1) and PR (Harrison et al., 2010).

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1.7 Tripartite motif containing (TRIM) family of proteins

1.7.1 Domain structure of TRIM family proteins

Tripartite motif containing (TRIM) protein family has 74 members identified in human so far, with more yet to be discovered (Hatakeyama, 2011). They are characterized by the presence of RBCC motif, consisting of a **R**ING domain, **B**-boxes and a **C**oiled-coil region arranged in conserved order. The RING domain at the N-terminus is a zinc finger of 40-60 residues containing regular arrangement of cysteine and histidine, and is able to bind two zinc atoms (Saurin et al., 1996). The RING domain is mainly involved in protein-protein interaction. It has been shown to be responsible for the ubiquitin E3 ligase activity in many TRIM proteins by promoting E2-dependent ubiquitin conjugation to specific substrate, including TRIM protein themselves (Freemont, 2000).

The B-box is a unique structural feature and main determinant of TRIM proteins. There are two different B-box domains, B-box 1 and 2, both are CHC3H2 zinc-finger motifs of about 40 residues, mainly differ by the presence of cysteine in the second coordination residue in B-box 1 but histidine in B-box 2 (Reddy et al., 1992; Reymond et al., 2001). Metal binding stoichiometry experiment using *Xenopus* TRIM protein Xnf7 demonstrated that each B-box can bind one atom of zinc (Borden et al., 1993). One or both B-boxes may be present in one TRIM protein, either in the form of B-box 1 followed by B-box 2 or B-box 2 alone (Reymond et al., 2001). The specific function of B-boxes is still unclear. Mutation in B-boxes in TRIM18/Midline 1 (MID1) has been shown to contribute to X-linked Opitz G/BBB syndrome, causing the malformations of the ventral midline

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(Ferrentino et al., 2007). It may also facilitates the recognition of viral capsid by the C-terminal SplA and ryanodine receptor (SPRY) domain of TRIM5 α (Li et al., 2007).

The coiled-coil region is formed by supercoil of α -helices. The primary sequence of coiled-coil is not conserved except for the presence of hydrophobic residues, often leucine or isoleucine, that allows the tight packing of the different helices (Lupas, 1996). Coiled-coil mediates protein oligomerization, which is important for the localization and function of TRIM proteins (Reymond et al., 2001). For instance, this region is essential for the nuclear body formation and the growth suppressor activity of TRIM19/Promyelocytic leukaemia (PML) (Fagioli et al., 1998).

The C-terminal region of TRIM proteins is less conserved. Currently there are ten different C-terminal domains found in TRIM protein, give rise to greater functional diversity of TRIM family proteins (Ozato et al., 2008). The most common C-terminal domain found is SPRY/B30.2 domain. This domain has been suggested to be necessary for the trimerization and antiviral activity of TRIM5 α (Javanbakht et al., 2006).

1.7.2 Function of TRIM proteins

TRIM proteins have been implicated in various cellular processes, including cell proliferation and differentiation, apoptosis, oncogenesis and antiviral defense. These effects are closely associated with their E3 ubiquitin ligase and E3 SUMO ligase activity.

TRIM proteins and innate immunity

The most studied function of TRIM proteins is their ability to restrict retroviral infection and multiple TRIM proteins possess this activity. A comprehensive screening of 55 TRIM

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proteins (36 human, 19 mice) showed that 20 of them exhibit antiretroviral activity. Overexpression of human TRIM5, 11, 26 and 31 inhibit human immunodeficiency virus-1 (HIV-1) entry, overexpression of human TRIM1, 5, 25, 26 and 62 inhibit N-tropic murine leukemia virus (N-MLV) entry. TRIM proteins also affect late stage of retroviral replication. Human TRIM15, 26 and 32 inhibit HIV release from cell, human TRIM 8, 15, 19, 25, 26, 28 and 35 interfere with the release of MLV whereas human TRIM1, 11, 13, 14, 21, 27, 31, 32 and 62 inhibit viral gene expression (Uchil et al., 2008).

TRIM5 α is the longest isoform of TRIM5. It inhibits HIV infection in Rhesus monkey and to a lesser extent in human. TRIM5 α trimerizes via its coiled-coil and B30.2 C-terminus domain, binds to HIV capsid protein to displace and destabilize the capsid lattice, causing it to dissociate into individual capsid proteins (Javanbakht et al., 2006; Sebastian and Luban, 2005). Furthermore, the E3 ubiquitin ligase activity of TRIM5 α is also important in HIV restriction. Mutation of its amino acid residues in the E2 binding region of RING domain causes the loss of both its ubiquitin ligase activity and the ability to inhibit HIV infection (Lienlaf et al., 2011).

It has been found that TRIM proteins' involvement in innate immunity is more than antiviral response. They also play a part in the defense against other pathogens. Interferon (IFN) production is stimulated when immune system is activated by pathogen and expression of many TRIM proteins can be upregulated by both type I and type II IFN (Ozato et al., 2008). Suppressor of cytokine signaling 1 (SOCS1) is a negative regulator of IFN stimulated gene expression. TRIM8 which has been shown to be induced by IFN γ

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can interact with SOCS1 to promote its degradation, hence blocking its inhibitory effect on IFN signaling (Toniato et al., 2002). Retinoic-acid-inducible gene 1 (RIG-I) RNA helicase recognizes viral RNAs and activates interferon regulator factor 3 (IRF-3) and NF- κ B (Yoneyama and Fujita, 2007). TRIM25 interacts with the caspase-recruitment domain (CARD) of RIG-I through its B30.2 domain and exerts two opposing effects, it can negatively regulate RIG-I expression by ubiquitination or positively regulate it by conjugating the ubiquitin-like ISG15 molecule to RIG-I (Gack et al., 2007).

TRIM proteins and cancer

TRIM family genes are involved directly in tumorigenesis via chromosomal translocation. TRIM19/PML t(15;17) translocation is found in acute promyelocytic leukemia (APL). This translocation gives rise to PML-retinoic acid receptor α (RAR α) fusion protein which abolishes the formation of PML nuclear bodies (PML-NB) and interferes with normal function of both PML and RAR (de The et al., 1991; Gallagher et al., 1997; Kakizuka et al., 1991; Melnick and Licht, 1999). Fusion of TRIM24/ transcriptional intermediary factor 1 (TIF1) with fibroblast growth factor receptor 1 (FGFR1) via t(7;8)(q34;p11) rearrangement leads to the formation of TIF1-FGFR1 fusion protein which turns on the receptor tyrosine kinase activity of FGFR1 constitutively. This oncogenic fusion protein is associated with 8p11 myeloproliferative syndrome (Belloni et al., 2005). In addition, TRIM24 fusion with RET tyrosine kinase domain is found in thyroid carcinoma and TRIM24 fusion with B-raf forms oncogenic protein T18 (Klugbauer and Rabes, 1999; Le Douarin et al., 1995). TRIM27/ret finger protein (RFP) can fuse with the 5' truncated half of ret receptor tyrosine kinase to produce the ret transforming gene (Takahashi et al., 1988).

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TRIM proteins also play important roles in the p53 pathways. TRIM13/ret finger protein 2 (RFP2) ubiquitylates murine double minutes 2 (MDM2). Its expression increases upon induction of apoptosis by ionizing radiation and helps to stabilize p53 (Joo et al., 2011). TRIM19/PML is usually associated with highly acetylated chromatin and may regulate transcription by modulating the availability or activity of transcription factors (Bernardi and Pandolfi, 2007). It regulates apoptotic pathway by recruiting both p53 and p300 to the PML-NBs, facilitates p53 acetylation and activation. It also helps to stabilize p53 by interfering with the ubiquitination of p53 by MDM2 (Bernardi et al., 2008). TRIM24 interacts directly with p53. It mediates the ubiquitination of p53 and downregulates p53 protein level. Knock down of TRIM24 leads to p53-dependent apoptosis (Allton et al., 2009). TRIM27/RFP also associates with SUMO E2 conjugase Ubc9 and Mdm2. Sumoylation of Mdm2 by TRIM27/RFP stabilizes Mdm2 by inhibiting ubiquitination of Mdm2 (Chu and Yang, 2011). TRIM28 promotes p53 ubiquitination and degradation via direct interaction with MDM2 (Wang et al., 2005). It also recruits HDAC1 to p53-MDM2 complex and inhibits the acetylation and activation of p53 (Yang et al., 2007).

Expression level of TRIM proteins is associated with various types of cancer. Overexpression of TRIM24, 25 and 27 is found in breast cancer. Expression of TRIM27 correlates with invasive breast carcinoma and ErbB2 expression; positive expression of TRIM27 is also a predictive marker for poor clinical outcome in endometrial cancer (Tezel et al., 2009; Tsukamoto et al., 2009).

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TRIM proteins in growth and differentiation

TRIM proteins affect cellular growth and differentiation in various types of tissue/cells. TRIM27/RFP is highly expressed in neonatal skeletal muscle. It interacts with enhancer of polycomb 1 (Epc1), interfering the interaction between Epc1 and serum response factor (SRF), blocking myoblast differentiation. Enhanced myotube formation and muscle fiber differentiation is observed in TRIM27/RFP knockout mice (Kee et al., 2012). TRIM32 determines neural cell fate in the mice neocortex. During progenitor cell division, TRIM32 is polarized and concentrated in one of the daughter cell. Overexpression of TRIM32 lead to neural differentiation whereas cells containing low TRIM32 level retain progenitor cell fate (Schwamborn et al., 2009). TRIM19/PML is a mediator of various apoptosis pathways. Functional PML is essential for the activation of caspase 1 and caspase 3. PML knockout mice became resistant to various apoptotic stimuli such as γ -irradiation and anti-Fas antibody. Loss of functional PML due to PML- RAR α protects the APL progenitor cells from Fas-, TNF- and IFN-induced apoptosis (Wang et al., 1998).

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1.8 Tripartite motif containing 22 (TRIM22)

1.8.1 Identification of TRIM22

TRIM22 (also known as Stimulated Trans-Acting Factor of 50kDa, Staf-50) was first identified as an IFN inducible gene by Tissot *et al* when screening a cDNA library prepared from IFN treated Daudi cells. The basal expression level of TRIM22 varies between different types of tissues, with higher level detected in peripheral blood leukocytes, lymphoid tissues and ovary. The first TRIM22 transcript cloned was 2811 bp which encode for 442 amino acids. Later, a longer transcript of 3111 bp was identified and shown to be the predominant TRIM22 mRNA species expressed. It contains eight exons and an open reading frame of 1497 bp which encodes for 498 amino acids (Gongora et al., 2000; Tissot and Mechti, 1995).

TRIM22 has been mapped to 11p15 by *in situ* hybridization (Tissot et al., 1996), located in a cluster of closely related TRIM genes (TRIM6/34/5/22), each has 40-60% homology to TRIM22. Comparison of this cluster in human, cow and dog genomes revealed that while TRIM6 and 34 remain more static and are preserved as single gene orthologs, the cow genome contains an expanded cluster of eight TRIM5 genes but has lost TRIM22. In contrast, the dog genome has TRIM22 but does not encode any functional TRIM5. Both TRIM5 and TRIM22 have evolved under positive selection in primates, possibly caused by their antiviral activities and the direct interaction with viral proteins. However, this positive selection acting on TRIM5 and TRIM22 is mutually exclusive, with only one being positively selected in any given primate lineage (Sawyer et al., 2007).

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1.8.2 Domain structure and cellular localization of TRIM22

TRIM22 contains the typical RBCC motif (with only one B-box 2) and a SPRY domain at the C-terminal (Nisole et al., 2005). It localizes mainly in the nucleus in the form of distinct nuclear bodies (NBs). Deletion of N-terminus RING domain or the nuclear localization signal of TRIM22 (residue 257-275) abolishes NBs formation completely. However, mutation of cysteine 15 and 18, two residues reported to be important for the E3 ubiquitin ligase activity of TRIM22, does not affect NB formation. Valine 493 in the SPRY domain is also found to be important for proper NB formation. Mutation of this residue to alanine changed the NB to a hollow ring structure. Mutation of both residue 493 and 494 to alanine causes decrease in number of NB formed in the nucleus. Nuclear localization of TRIM22 is not affected significantly by the truncation of RING domain or B box, but much higher cytoplasmic localization is observed when the whole RBCC motif is removed. In contrast, TRIM22 protein is predominantly cytoplasmic once the C-terminus SPRY domain is truncated (Sivaramakrishnan et al., 2009; Tissot and Mechti, 1995).

1.8.3 Regulation of TRIM22 expression

TRIM22 induction by p53 activation

TRIM22 works downstream of the tumor suppressor p53; its expression is upregulated upon p53 activation in U-937-4, K562 and MCF7 cells. This induction is rapid and independent of *de novo* protein synthesis, demonstrating that TRIM22 is a direct target of p53 (Obad et al., 2004). Three independent studies have identified the p53 response element in intron 1 of the TRIM22 gene (Kaneshiro et al., 2007; Obad et al., 2004; Wei et al., 2006). The binding of p53 to this response element was demonstrated by mobility

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retardation in gel shift assay. This p53 response element was also shown to be able to enhance the SV40-driven Luciferase expression in a p53 dependent manner (Obad et al., 2004).

TRIM22 induction by interferon

TRIM22 can be induced by both type I and type II IFNs, suggesting their possible involvement in IFN regulated antiviral activity. Induction of the shorter transcript variant of TRIM22 by IFN- α/β is first shown to be independent of *de novo* protein synthesis in HeLa cells (Tissot and Mechti, 1995). However, a recent study demonstrated that induction of the longer transcript variant of TRIM22 by IFN- γ treatment in HepG2 cells is abolished in the presence of cycloheximide, indicating that *de novo* protein synthesis is needed for TRIM22 induction by IFN- γ . A further study in the mechanism of induction reveals that upon IFN- γ treatment, interferon regulatory factor-1 (IRF-1) binds to an extended interferon-stimulating response element (eISRE) - an ISRE-like motif with a 6 bp sequence (AATTTA) upstream of it, at the 5' end of TRIM22. The access of IRF-1 to the TRIM22 promoter site is facilitated by chromatin remodeling enzyme Brahma-related gene 1 (BRG1) and this binding is critical for activating the transcription of TRIM22. Overexpression of IRF-1 upregulates TRIM22 mRNA level whereas silencing of IRF-1 downregulates TRIM22 protein expression induced by IFN- γ (Gao et al., 2010a; Wang et al., 2011).

TRIM22 induction by progesterone

Work done in our lab has shown that TRIM22 expression level can be upregulated drastically after progesterone treatment in T47D cells and MDA-MB-231 derived ABC28 cells, suggesting that TRIM22 was involved in the progesterone pathway.

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1.8.4 Functions of TRIM22

TRIM22 as an E3 ubiquitin ligase

It has been found that many proteins containing the RING finger domain have the ability to bind to E2 ubiquitin conjugase and the substrate, acting as E3 ubiquitin ligase. *In vitro* ubiquitination assay using TRIM22 recombinant protein, ubiquitin and reticulocyte lysate (as a source of E1 and E2 enzymes) showed that TRIM22 was capable of self ubiquitination. Further screening using different recombinant E2 ubiquitin conjugase (UbcH2, UbcH5B, UbcH7, UbcH10, UbcH10, and hCDC34) showed that TRIM22 is an E3 ubiquitin ligase that works specifically with UbcH5B. Deletion of RING domain or point mutation of the conserved cysteine residue (C15A) in the RING domain abolished the E3 ligase activity of TRIM22, demonstrating that RING finger domain is crucial for this function (Duan et al., 2008).

Antiviral property of TRIM22

The antiviral property of TRIM22 has been extensively investigated and many of these studies have focused on TRIM22's action against HIV infection. TRIM22 inhibits the transcriptional activity directed by HIV long terminal repeats promoter by 60-90% in reporter gene assay (Tissot and Mechti, 1995). Its expression is induced in HIV-1 infected monocyte-derived macrophages (MAC). Overexpression of TRIM22 inhibited the HIV-1 infection by 50-90% in 293T CD4/CCR5 cells as well as in MAC (Bouazzaoui et al., 2006). Further studies demonstrated that TRIM22 is mainly involved in the anti-HIV response induced by type I IFN. It disrupts the trafficking of HIV Gag protein to the plasma membrane and blocks the budding of HIV virions. TRIM22 knock down allows

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the production of HIV particles in the presence of IFN- β . The E3 ubiquitin ligase activity of TRIM22 is needed for this antiviral activity (Barr et al., 2008).

Studies using cell lines expressing Latency-associated nuclear antigen (LANA) or replication and transcription activator (RTA) of Kaposi's sarcoma-associated Herpesvirus showed that TRIM22 expression level increases both during latency and during switching from latency to lytic cycle (Renne et al., 2001; Zhang et al., 2005). In addition, TRIM22 expression can also be stimulated in Epstein-Barr virus (EBV), Human Papillomavirus 16 (HPV16) and Hepatitis B virus (HBV) infected cells (Javanbakht et al., 2006; Pett et al., 2006; Wieland et al., 2004; Zhang et al., 2004). Transient and stable expression of Flag-TRIM22 confers protection against encephalomyocarditis virus (EMCV) in HeLa cells by promoting the ubiquitination and degradation of viral 3C protease (Eldin et al., 2009).

In addition to their association with IFN pathway, TRIM22 also acts as an activator for NF- κ B. It activates NF- κ B transcriptional activity in reporter gene assay and its overexpression stimulates the secretion of pro-inflammatory cytokines and participates in innate immune response. RING domain and SPRY domain are important for this activator activity (Yu et al., 2011).

TRIM22 in tumorigenesis

TRIM22 may play a role in cancer development with and without viral infection. Increase in TRIM22 expression is found to associate with latent infection and cellular transformation. EBV infection leads to various types of cancers such as lymphoma. Induction of TRIM22 expression by the Latent membrane protein 1 (LMP-I) of EBV is

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associated with the establishment of EBV latency and transformation of primary B cells *in vitro* (Zhang et al., 2004). TRIM22 level is higher in EBV-infected Akata Burkitt B lymphoblasts than in uninfected Akata cells (Javanbakht et al., 2006). Kaposi's sarcoma (KS) is commonly associated with latency infection by human herpesvirus 8 and LANA is one of the few viral genes expressed during latency and detected in KS patients. It is shown be important in the maintenance of episomal viral DNA in the cells during division and it can regulate transcription of cellular genes (Ballestas et al., 1999; Cotter and Robertson, 1999). An *In vitro* study shows that TRIM22 expression is upregulated when cells overexpress LANA. Further testing using Kaposi's sarcoma patient tissue showed that TRIM22 mRNA is indeed higher in the patient tissues (Renne et al., 2001). In HPV16 infection, integration of viral genome and disruption of viral transcriptional regulator E2 expression are necessary for the establishment of cervical carcinoma (Daniel et al., 1997; Romanczuk and Howley, 1992). When culturing cervical keratinocyte cell line W12 that was infected by HPV16 for long term and observing the changes during cervical carcinogenesis, Pett *et al* demonstrated that TRIM22 level increased as there was an increase in integration of the viral genome and a decrease in E2-expressing episomes in the cells. TRIM22 levels dropped back to the basal level or lower when E2-expressing episomes were completely lost. This suggests that expression of TRIM22 is important in the selection of cells containing only integrated HPV16 (Pett et al., 2006).

TRIM22 expression level is found to be associated with certain cancer. Wilms tumor is the most common malignant renal tumor in children. Microarray experiments comparing the gene expression in 63 primary Wilms tumors showed that TRIM22 expression is

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higher in relapse-free Wilms tumor than in relapsed Wilms tumor (Zirn et al., 2006). A later study by the same group using a larger sample size further validated that TRIM22 expression is lower in the relapse tumor. Downregulation of TRIM22 expression level correlates with Wilms tumor with fatal outcome (Wittmann et al., 2008). Furthermore, TRIM22 level is lower in neuroblasts than in neuroblastoma cells (De Preter et al., 2006).

TRIM22 in cell proliferation and differentiation

As a downstream target of p53, TRIM22 exerts an anti-proliferative effect on cells. Overexpression of TRIM22 in U-937 cells resulted in reduced clonogenic growth of the cells on soft agar (Obad et al., 2004). TRIM22 expression may correlate inversely to differentiation in other cases. It is highly expressed in CD34⁺ human bone marrow progenitor cells but decreases in mature populations (Obad et al., 2007).

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1.9 Scope of study

Recent progress on the studies of protein methylation has shown that it is an important PTM in regulating the activity of proteins such as p53 and FOXO (Kurash et al., 2008; Shi et al., 2007; Yamagata et al., 2008). Many have reported on the effect of methylation of SHR signaling by methylating coregulators. For instance, methylation of SRC-3 by CARM1 in response to E2 treatment not only leads to the dissociation of SRC-3 from the coactivator complex, it also increases the rate of SRC-3 degradation. Mutating the arginine residues and blocking the methylation by CARM1 result in enhanced coactivator activity for SRC-3 (Feng et al., 2006; Nacem et al., 2007b). Methylation of CBP at R742 by CARM1 is needed for its coactivating activity in steroid hormone induced gene expression (Chevallard-Briet et al., 2002). However, knowledge on how protein methylation may directly affect SHR activity is still very limited. Only 1 lysine methylation site and 1 arginine methylation site have been reported for ER, whereas 2 lysine methylation sites are reported for AR. All the lysine sites identified are methylated by SET7/9 and the arginine methylation is carried out by PRMT1 (Gaughan et al., 2010; Ko et al., 2011; Le Romancer et al., 2008; Subramanian et al., 2008). We have conducted a comprehensive mapping of ER PTM by mass spectrometry and identified several putative arginine methylation sites that have not been reported previously and do not share the Arg-Gly-Gly-rich (RGG) motif. We hypothesized that PRMTs other than PRMT1 are involved in ER methylation to regulate ER activity.

The objectives of this study are to:

1. Screen all 8 PRMTs with known methyltransferase activity to identify PRMT(s) that can methylate ER.

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2. Locate the arginine sites that are targeted for methylation.
3. Elucidate the mechanism of regulating ER activity through arginine methylation.

TRIM22 is a downstream target of p53 and it has been found to associate with virus induced tumorigenesis (Obad et al., 2004; Renne et al., 2001). While its anti-retroviral activity has been studied intensively, not much is known about its role in tumor development (Gao et al., 2009; Kajaste-Rudnitski et al., 2011; Singh et al., 2011). As TRIM22 is one of the most highly upregulated genes in our microarray analysis using RNA collected from progesterone treated breast cancer cells and it has been reported to inhibit the growth of leukemic cells, it may be involved in promoting the growth inhibitory effect of progesterone in breast cancer (Obad et al., 2004). In part two of my study, I seek to characterize the TRIM22 induction by different pathways, the regulation of TRIM22 expression in breast cancer tissues and its prognostic value.

The objectives of this study are to:

1. Examine the expression pattern of TRIM22 in normal mammary tissue and breast cancer tissue.
2. Study how TRIM22 is regulated by IFN and p53 pathways.
3. Investigate the possible involvement of TRIM22 in progesterone receptor signaling.

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CHAPTER 2

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2.1 Chemicals

5-aza-2'-deoxycytidine (5-aza-dC), β -mercapthoethanol, bovine serum albumin (BSA), curcumin, cycloheximide, dextran, human insulin, hydrocortisone, hydroxyurea, cholera toxin, mitomycin C, propidium iodide, RNase A, pepstatin A, leupeptin, aprotinin, Tris base, sodium vanadate, phenylmethylsulphonylfluoride (PMSF), ethidium bromide, formaldehyde, diethylpyrocarbonate (DEPC), sodium fluoride (NaF), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 17β -estradiol, progesterone, dexamethasone, dideoxytestosterone were obtained from Sigma Chemical Co (St. Louis, MO, USA). Triton-X-100, ethanol, isopropanol and methanol were from Merck KGaA (Darmstadt, Germany). Acrylamide, Tetramethylethylenediamine (TEMED), ammonium persulfate (APS), gel-drying solution were purchased from bio-rad. EGF, IFN γ and IFN β was purchased from ProSpec-Tany TechnoGene Ltd (Rehovot, Israel). S-adenosyl-L-[methyl- 3 H] methionine (SAM) (5-15 Ci/ml), H 3 -methionine, progestin (R5020) and ENLIGHTENING solution were purchased from Perkin Elmer. Taxol, camptothecin, nocodazole were gifts from Dr Joe Yoon (Nanyang Technological University, Singapore). VP16 was a gift from Dr Li Hoi Yeung (Nanyang Technological University, Singapore).

2.2 Cell culture

2.2.1 Cell lines

MCF7 and MDA-MB-231 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) in 1995 at passage 147 and 28 respectively. T47D, MDA-MB-435 and CRL-1500 cells were obtained from Dr. Suet Feung Chin from the University of Cambridge (UK). ABC28 cells were generated from MDA-MB-231 cells by

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overexpression human PR-A and PR-B (Lin et al., 1999). COS7 cells and HeLa cells were obtained from Dr. Koh Cheng Gee and Dr. Li Hoi Yeung from Nanyang Technological University (Singapore).

All these cells were routinely maintained in phenol red-containing Dulbecco's modified Eagle's medium (DMEM) (PAA laboratories) supplemented with 7.5% fetal bovine serum (FBS) (Sigma), 2 mM L-glutamine, and 40 mg/liter gentamicin (PAA laboratories). CRL-1500 cells were routinely maintained in phenol red-containing RPMI 1650 medium supplemented with 7.5% FBS, 2 mM L-glutamine, and 40 mg/liter gentamicin.

MCF10A cells were obtained from Dr. Ann Lee from the National Cancer Centre (NCC; Singapore). MCF-12A cells were obtained from Dr. Bay Boon Huat from the National University of Singapore. These cells were routinely maintained in DMEM:F12 (50:50) medium supplemented with 5% FBS, 2 mM L-glutamine, and 40 mg/liter gentamicin, 2 µg/ml human insulin and 0.5 µg/ml hydrocortisone, 0.1 µg/ml cholera toxin and 0.01 µg/ml EGF.

Table 2.1 List of mammary epithelial cell lines used

Cell Line	Malignancy	ER status	PR status
H16N2	N	-	-
MCF10A	N	-	-
MCF-12A	N	-	-
Cama-1	Y	+	+
CRL-1500	Y	+	+
MCF7/MCF7-Ras	Y	+	+

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MDA-MB-231 /ABC28	Y	-	-
MDA-MB-361	Y	+	+
MDA-MB-435	Y	-	-
MDA-MB-436	Y	-	-
SK-BR-3	Y	-	-
T47D	Y	+	+

2.2.2 Hormone treatment

All hormone treatment experiments with cells were conducted using phenol red-free DMEM supplemented with 2 mM L-glutamine, 40 µg/ml gentamycin and 5% dextran-coated charcoal-treated FBS (DCC-FBS). FBS was treated with dextran-coated charcoal overnight to remove the steroid hormones present in serum. For all hormone treatment experiments, cells were grown in this medium for 48 hrs before treatment to remove the residual effect of hormone from serum.

2.2.3 Transfection and luciferase assay

Transfection of siRNA for gene silencing was carried out using Lipofectamine 2000 (Invitrogen) according to manufacturer's instruction. siRNAs used for knock down in this study were purchased from Ambion. Negative control siRNA was Ambion silencer select negative control siRNA 1 (4390844, sequence not revealed). TRIM22 siRNA sequence is 5'GCACCUGCACAUUUAAGAAtt (sense) and 5'UUCUAAAUGUGCAGGUGCgt (antisense). PRMT6 siRNA 1 sequence is 5'GAGGCAAGACGGUACUGGAtt (sense) and 5'UCCAGUACCGUCUUGCCUCgc (antisense). PRMT6 siRNA 2 sequence is

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5'GAAAUAGUAUGGAUUUUUAtt (sense) and 5'UAAAAAUCCAUAUAUUUCtt (antisense). Two reagents were used for the transfection of DNA vectors. Polyethylenimine (PEI) (Polysciences, Inc) dissolved in Milli-Q water at 2 mg/ml and pH adjusted to 7.0 was used for the transfection into HeLa and COS7 cells at a ratio of PEI (1.5 μ l): plasmid (1 μ g) according to the cellIntec advanced cell systems transfection protocol for PEI. Lipofectamine 2000 was used for the transfection of other cell lines at a ratio of Lipofectamine (4 μ l): plasmid (1 μ g) according to manufacturer's instruction.

For luciferase reporter assay, HeLa cells in 60mm dishes were transfected with 1.5 μ g of PRE-luciferase or ERE-luciferase, 5 ng of SHRs, with various amounts of TRIM22 or PRMT6 plasmids, depending on the experiment design, empty vector controls (mock) were used to balance the amount of plasmid used in all experiments. All plasmid amounts were scale to 1/3 for transfection using 6-well plate. Cells were treated with 10 nM hormone for 24 hrs after transfected for 24 hrs. Lysate was collected using Promega Dual Luciferase assay kit and firefly luciferase signal was read using Tecan Safire 2 microplate reader.

PRMT1 to PRMT 8 were subcloned from the various vectors into pcDNA3.1+/hygro plasmid using primers containing Flag tag in the forward sequence. PRMT2, 4, 5, 6 and 8 in GFP vector were gifts from Dr. Mark Bedford (The University of Texas M.D. Anderson Cancer Center), PRMT1, 3 and 7 MGC clones were given by Dr. Tobias Cornvik (Nanyang Technological University, Singapore). Flag-GR was generated from pSG5-GR plasmid given by Dr. Ravi Kambadur (Nanyang Technological University,

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Singapore). PRB (indicated as PR in the text as this study only focus on PRB) and ER α (indicated as ER as this study only focus on ER α but not ER β) truncations constructs were generated by PCR using specific primers targeting different PR and ER domains and ligate into pcDNA3.1+/hygro vector. Gene inserts were amplified using Taq DNA polymerase (Fermentas), restriction digested with NEB restriction enzymes and ligated into cut vector backbone with T4 DNA ligase (Fermentas).

Table 2.2 List of primers for cloning

Gene	Primer sequences
PRMT1-F	5'CCGGGATCCACCACCATGGACTACAAGGACGACGATGATAAGGAGGTGCTCTGTGGCCAGG
PRMT1-R	5'ATTGCGGCCGCTCAGCGCATCCGGTAGTCGG
PRMT2-F	5'CCGGGATCCGCCACCATGGACTACAAGGACGACGATGATAAGGCAACATCAGGTGACTGTCCC
PRMT2-R	5'ATTGCGGCCGCTCATCTCCAGATGGGGAAGAC
PRMT3-F	5'CCGGGATCCGCCACCATGGACTACAAGGACGACGATGATAAGTGCTCGTTAGCGTCAGGCGC
PRMT3-R	5'ATTGCGGCCGCTCAC'GGAGACCATAAGTTTGAG
PRMT4-F	5'CCGAAGCTTGCCACCATGGACTACAAGGACGACGATGATAAGGCAGCGGGCGGACGACG
PRMT4-R	5'ATTGCGGCCGCTAACTCCCATAGTGCATGGTG
PRMT5-F	5'CCGGGATCCGCCACCATGGACTACAAGGACGACGATGATAAGGCGGCGATGGCGGTCGGG
PRMT5-R	5'ATTGCGGCCGCTAGAGGCCAATGGTATATGAG
PRMT6-F	5'CCGGGATCCGCCACCATGGACTACAAGGACGACGATGATAAGTCGAGCCCAAGAAAAGAAAGC
PRMT6-R	5'ATTGCGGCCGCTCAGTCCTCCATGGCAAAGTCT
PRMT7-F	5'CCGGGTACCGCCACCATGGACTACAAGGACGACGATGATAAGAAGATCTTCTGCAGTCGGGCC
PRMT7-R	5'ATTGCGGCCGCTCAGTCTGGGGTATCTGCATG
PRMT8-F	5'CCGGGTACCGCCACCATGGACTACAAGGACGACGATGATAAGGGCATGAAACACTCCTCCCG
PRMT8-R	5'ATTGCGGCCGCTAACGCATTTTGTAGTCATTAGA
ER-F	5'CCGGCTAGCCACCATGGACTACAAGGACGACGATGATAAGACCATGACCCTCCACACCAAA
ER(VR)F	5'CCGGGTACCGCCACCATGGCAGTGTGCAATGACTATGCTT

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ER(LBD)-F	5'GCTAGCCACCATGGACTACAAGGACGACGATGATAAGCACATGATCAACTGGGCGAAG
ER-R	5'ATTGGATCCTCAGACCGTGGCAGGGAAAC
ER(VR)-R	5'ATTGGTACCTCAACAGTAGCGAGTCTCCTTGGC
ER(141)-R	5'ATTGGTACCTCACACCGTGTAGCCGCTGGGC
ER(161)-R	5'ATTGGTACCTCAGCCACCCTGGCGTCGATTAT
ER(182)-R	5'ATTGGTACCTCAAGTCTCCTTGGCAGATTCCATA
PR-F	5'CCGGCTAGCGCCACCATGGACTACAAGGACGACGATGATAAGACTGAGCTGAAGGCAAA
PR(VR)-F	5'CTAGCTAGC-CACCATGTACAGCTTCGAGTCATTACCT
PR(BUS)-no stop-R	5'GGGGTACCGAGCGGGGACAAACCCCG
PR(Hinge)-F	5'CCGGGTACCGGTCGAAAATTTAAAAAGTTCAATAAA
PR(Hinge)-R	5'CCGTCTAGATCATGGTGAATCAACTGTATGTCTT
PR(LBD)-F	5'CCGGGTACCCTGATCAACCTGTTAATGAGCAT
PR(NLS)-no stop-R	5'GCGGTACCGACTTTTATTGAAC'TTTTAAATTTTC
PR(NLS)-R	5'GCTCTAGATCAGACTTTTATTGAAC'TTTTAAATTTTC
PR-R	5'GCTCTAGATCACTTTTATGAAAGAGAAGGG
GR-F	5'CCGGGTACCGCCACCATGGACTACAAGGACGACGATGATAAGGACTCCAAAGAATCATT
GR-R	5'GCTCTAGATCACTTTTATGAAAGAGAAGGG

Table 2.3 List of plasmids used in transfection

gene insert	plasmid backbone	Cloned by/Given by
Flag-PRMT1	pcDNA3.1+/Hygro	Sun Yang
Flag-PRMT2	pcDNA3.1+/Hygro	Sun Yang
Flag-PRMT3	pcDNA3.1+/Hygro	Sun Yang
Flag-PRMT4	pcDNA3.1+/Hygro	Sun Yang
Flag-PRMT5	pcDNA3.1+/Hygro	Sun Yang
Flag-PRMT6	pcDNA3.1+/Hygro	Sun Yang
Flag-PRMT7	pcDNA3.1+/Hygro	Sun Yang

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Flag-PRMT8	pcDNA3.1+/Hygro	Sun Yang
Flag-GR	pcDNA3.1+/Hygro	Sun Yang
flag-AR	pCMV5	Gift from Dr. Edwin Cheung
Flag-ER	pCMV5	Gift from Dr. Edwin Cheung
Flag-PRB	pcDNA3.1+/Hygro	Raj Mohan
TRIM22	pEF1-Myc/HisA	Sun Yang
Flag-TRIM22	pXJ-Flag	Gayathri Sivaramakrishnan
Flag-TRIM22(C15/18A)	pXJ-Flag	Gayathri Sivaramakrishnan
GFP-TRIM22	pEGFP-N1	Gayathri Sivaramakrishnan
Flag-TRIM22(Δ RING)	pXJ-Flag	Gayathri Sivaramakrishnan
PRB	pcDNA3.1+/Hygro	Zheng Zeyi
PRA	pcDNA3.1+/Hygro	Zheng Zeyi
PR(BUS+NLS)	pcDNA3.1+/Hygro	Zheng Zeyi
PR(BUS-Hinge)	pcDNA3.1+/Hygro	Zheng Zeyi
PR (BUS-DBD+NLS)	pcDNA3.1+/Hygro	Zheng Zeyi
PR(VR+DBD+NLS)	pcDNA3.1+/Hygro	Zheng Zeyi
PR(VR+NLS)	pcDNA3.1+/Hygro	Sun Yang
PR(BUS+DBD-LBD)	pcDNA3.1+/Hygro	Sun Yang
PR(BUS+NLS+LBD)	pcDNA3.1+/Hygro	Sun Yang
Flag-ER(Δ VR)	pcDNA3.1+/Hygro	Sun Yang
ER(Δ VR)	pcDNA3.1+/Hygro	Sun Yang
Flag-ER(LBD)	pcDNA3.1+/Hygro	Sun Yang
Flag-ER(VR)	pcDNA3.1+/Hygro	Sun Yang
Flag-ER(Δ LBD)	pcDNA3.1+/Hygro	Sun Yang
Flag-ER(1-141)	pcDNA3.1+/Hygro	Sun Yang
Flag-ER(1-161)	pcDNA3.1+/Hygro	Sun Yang
Flag-ER(1-182)	pcDNA3.1+/Hygro	Sun Yang

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ERE-Luciferase	pGL4	Gift from Dr. Edwin Cheung
PRE-Luciferase	pGL4	Gift from Dr. Ming-Jer Tsai
p53 wt	pCMV-Neo-Bam	Bert Vogelstein. Addgene plasmid 16434

2.3 Cell lysate isolation, cell fractionation and Western blotting

Cells plated in 6-well plate were lysed with cold lysis buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 5 µg/ml pepstatin A, 5 µg/ml leupeptin, 2 µg/ml aprotinin, 1 mM PMSF, 100 mM sodium fluoride, and 1 mM sodium vanadate (pH 7.5)) and incubated on ice for 5 mins. Cells were further broken down using plastic scraper and transferred into 1.5 ml eppendorf tube, homogenized by passing through 29 gauge syringes for 5 times. Protein supernatants were then collected by centrifugation at 13,800 rpm for 12 mins at 4 °C. 63 breast tumors and 63 matching control breast tissue samples were obtained from the Tissue Repository at the Singapore National Cancer Centre (CIRB number 2010/361/B). Mashed tissues were homogenized in the presence of 500 µl of cold lysis buffer and spun down at 13,800 rpm for 12 mins at 4 °C. Protein concentration was analyzed using BCATM Protein Assay Kit (PIERCE). Twenty micrograms of the protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes using a wet-transfer system (Biorad-Laboratories, Inc, Hercules, CA, USA). Membranes were incubated with specific primary antibodies in 5% skimmed milk (listed in table 2.4). Anti-mouse and anti-rabbit HRP conjugated secondary antibodies purchased from GE healthcare were used in 1:1000 and 1:2000 dilutions respectively. Signal detection was carried out by using either enhanced chemiluminescence (ECL) or ECL plus system (GE healthcare, UK) or Imbillion (Millipore) followed by exposing to X-ray films (Eastman Kodak Co., New Haven, CT).

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For cellular fractionation, cells were trypsinized and spun down at 800 rpm at room temperature. Cell pellet was re-suspended in buffer S1 (10 mM Hepes pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5% NP-40, 10 mM DTT, 5 mg/ml pepstatin A, 5 mg/ml leupeptin, 2 mg/ml aprotinin), incubated on ice for 10 mins and passed through a 21G syringe 10 times. Cytoplasmic fraction was obtained by centrifugation at 1000 rpm for 10 mins. Nuclear pellet was washed once with buffer S1 and re-suspended in buffer S2 (10 mM Hepes pH7.9, 1.5 mM MgCl₂, 0.4M KCl, 0.5% NP-40, 10 mM DTT, 5 mg/ml pepstatin A, 5 mg/ml leupeptin, 2 mg/ml aprotinin), passed through a 29G syringe 10 times and nuclear fraction was obtained by centrifugation at 13,800 rpm for 15 min. Protein was quantitated and used for Western blotting analysis.

Table 2.4 List of antibodies

Antibody	Catalog number	Company
PR	MS-298 (ab8)	Lab vision
PR	Sc-7208 (H190)	Santa cruz
p294PR	PPS018	R&D systems
p400PR	Gift from Dr. Carol Lange. University of Minnesota Cancer Center	
p53	OP43	Calbiochem
ER (rabbit)	Sc-543	Santa cruz
ER (mouse)	Sc-002	Santa cruz
p167ER	2514	Cell signaling
		Santa cruz (gift from Dr. Ravi kambadur.
GR	Sc-8992	Nanyang Technological University)
Hsp70	Sc-24	Santa cruz

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Hsp90	Sc-13119	Santa cruz
PRMT6	Sc-271744	Santa cruz
Flag	F1804	Sigma
β -actin	Sc-47778	Santa cruz
GAPDH	AM4300	Ambion

2.4 Co-immunoprecipitation (Co-IP)

500 μ g of total protein lysate collected using cold lysis buffer were incubated with 1 μ g of antibody overnight at 4 °C with rotation. 30 μ l of protein A/G agarose beads suspension (Santa Cruz) was then added and rotate for additional 2-3 hrs. Beads were spun down at 1000 rpm for 2 mins at 4 °C. Beads was washed three times with lysis buffer before adding in 15 μ l of 2X sample buffer and heated at 100 °C for 8 mins for elution. For Co-IP using Flag ab affinity gel agarose beads (Flag beads) (Sigma), 12 μ l of Flag beads were added to the protein lysate for overnight incubation. Beads were then spun down at 5000 rpm for 30 sec and washed three times with lysis buffer before adding in 10 μ l of 2X sample buffer and heated at 100 °C for 8 mins for elution. Both beads and supernatant were loaded for Western blotting analysis as described in 2.3.

2.5 RNA extraction, cDNA synthesis and real-time PCR

Total RNA was extracted using TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD, USA), chloroform:isoamyl-ethanol (24:1) and phenol:chloroform:isoamyl-ethanol (50:24:1), precipitated using isopropanol and washed with 75% ethanol in DEPC-treated water before re-suspending in 0.2% DEPC-treated water. RNA was quantitated using NanoDrop and purity determined by Abs260nm/Abs280nm and Abs260nm/Abs230nm.

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RNA integrity was determined by running 0.5 µg of total RNA on a 1% ethidium bromide-stained agarose gel. The band intensity of 28S should be about double of 18S.

cDNA was synthesized from 1-5 µg of total RNA using random primer (Promega) and SuperScript II TM reverse transcriptase according to manufacturer's instructions (Life Technologies, Inc., Gaithersburg, MD, USA). Briefly, 1-5 µg total RNA was incubated with 0.25 µg random primer in DEPC-treated sterile double-distilled water at 70 °C for 10 mins. After the reaction was cooled on ice for 3 mins, it was incubated with a mixture containing 5X first strand buffer, 0.01 mM dithiothreitol, and 0.4 mM dNTPs at 25 °C for 10 mins, then 42 °C for 2 mins before incubating with reverse transcriptase for additional 50 mins. The reverse transcriptase was inactivated by heating at 72 °C for 10 mins.

Real-time PCR was performed using KAPA SYBR Green PCR reagents on an ABI Prism 7000 and 7500 Sequence Detection System (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's protocol. PCR for each gene was performed in triplicates. The relative amount of PCR products generated from each primer set was determined on the basis of the threshold cycle (Ct) number. Housekeeping gene 36B4 was used as control to normalize the amount of cDNA used. Relative expression = $2^{[Ct(\text{control})\text{gene X} - Ct(\text{treatment})\text{gene X}] - [Ct(\text{control})36B4 - Ct(\text{treatment})36B4]}$. List of primers used is shown in table 2.5. All the primers have annealing temperature of 60 °C.

Table 2.5 List of primers for real-time PCR

Gene	Primer sequences
TRIM22	Forward primer: GGATGCCAGCACGCTCATCTCAG Reverse primer: TTCAGCATCACGTCCACCCAGTAG

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PR	Forward primer: CGCGCTCTACCTGCACTC Reverse primer: TGAATCCGGCCTCAGGTAGTT
PRMT1	Forward primer: ATCGTCAAAGCCAACAAGTTAGA Reverse primer: TGGACTCGTAGAAGAGGCAGTAG
PRMT2	Forward primer: AAGAGTACTTCGGCAGCTATGG Reverse primer: CCGTCAGGGATTCTTTATTCTG
PRMT3	Forward primer: CAATTTCCTCAGGATTTTGTA Reverse primer: GCCCGTATGAGCTGAAATAAAC
PRMT4	Forward primer: TGTTCCTTCTACAACATCCTGAAA Reverse primer: GACAGGTAGCCATAAACTGGAAG
PRMT5	Forward primer: TCTCCCCACTAGCATTTTCCT Reverse primer: ATGATGAACTGCACCTCCAAC
PRMT6	Forward primer: CTCCCCCTTTTCCCTCATAG Reverse primer: TAAGCACGCTGTATCCGTIG
PRMT7	Forward primer: CACGATGACTACTGCGTATGGTA Reverse primer: GTTCTGTCCTGGTCATTGATCTC
PRMT8	Forward primer: GAGGAAATCTACGGGACCATATC Reverse primer: AGCTGTCCCTTAAAATCCAAGTC
36B4	Forward primer: GATTGGCTACCCAAGTGTGCA Reverse primer: CAGGGGCAGCAGCCACAAAGGC

2.6 Site-directed mutagenesis

ER mutants were generated using Stratagene site-directed mutagenesis XL II kit (Stratagene, La Jolla, CA, USA). 100 ng of wild type ER vector was used as a template in a PCR with PAGE-purified sense and anti-sense primers, fresh dNTPs, reaction buffer and Pfu *Turbo* polymerase using the following cycling conditions: Initial denaturation at 95 °C for 2 mins, 18 cycles of 95 °C for 50 sec, 75 °C for 50 sec, 68 °C for 18 min followed final cycle of 68 °C for 7 mins to amplify the plasmid incorporating the desired mutation. Methylated parental template DNA was digested for 1 hr at 37 °C by DpnI (10 units/μl) and 10 μl of resultant product transformed into 45 μl of XL Gold competent cell

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Transformed bacteria were spread on LB-amp plates and colonies sequenced to identify clones carrying the desire mutations.

Table 2.6 List of primers for site directed mutagenesis

Gene	Primer sequences
PRMT6	sense primer: : ACTGCGAGGCAAGACGAAGCTGGCAGTGGGCGCGGGCACCG
(V86K/D88A)	antisense primer: CGGTGCCCCGCGCCCACTGCCAGCTTCGTCTTGCCTCGCAGT
ER(R233K)	sense primer: : GTGCACCATTGATAAAAACAAGAGGAAGAGCTGCCA
	antisense primer: TGGCAGCTCTTCCTCTTGTTTTATCAATGGTGCAC
ER(R234K)	sense primer: CAGTGCACCATTGATAAAAACAAGAAGAAGAGCTGCCAGGCC
	antisense primer: GGCCTGGCAGCTCTTCTTCTTGTTTTATCAATGGTGCAGT
ER(R233/234K)	sense primer: : ACCATTGATAAAAACAAGAAGAAGAGCTGCCAGGCC
	antisense primer: GGCCTGGCAGCTCTTCTTCTTGTTTTATCAATGGT
ER(R234F)	sense primer: : AGTGCACCAATTGATAAAAACAGGTTCAAGAGCTGCCAGGCCTGC
	antisense primer: GCAGGCCTGGCAGCTCTTGAACCTGTTTTATCAATGGTGCAGT
ER(R260K)	sense primer: : GTGGGATACGAAAAGACCGAAAGGGAGGGAGAATGTTGAAA
	antisense primer: TTTCAACATTCTCCCTCCCTTTCGGTCTTTTCGTATCCCAC

2.7 Mass spectrometry sample preparation

Endogenous TRIM22 in MCF10A was pulled down using TRIM22 conditioned medium containing TRIM22 monoclonal antibody, Flag tagged ER was overexpressed in COS7 cells and pulled down using Flag beads. Protein bands were separated by SDS-PAGE and stained by coomassie blue. Specific ER and PR gel strips were cut out, reduced by dithiothreitol (10 mM) for 1 hr at 60 °C , alkylated by adding iodoacetamide (55 mM) for 1 hr in the dark at room temperature, in-gel digested with trypsin at 1:100 (trypsin:protein) mass ratio overnight at 37 °C. Peptides were then extracted by sonication and sent for liquid chromatography-mass spectrometry (LC-MS) analysis. LC-MS analysis and

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Mascot search were carried out by Dr. Newman Sze. Possible peptide hits were further validated by in vitro methyltransferase assay.

2.8 Immunostaining and proximity ligation assay (PLA)

Cells on cover slip were fixed with 3.7% formaldehyde for 10 mins and permeabilized with 0.2% triton-X 100 for 10 mins. After washing with PBS, cells were blocked with 2% FBS/PBS at room temperature for 1 hr, and incubated with primary antibodies in 2% FCS/PBS for 2 hrs at 37 °C (antibody dilution 1:200 to 1:1000). Cells were subsequently incubated with secondary antibodies and 4',6-diamidino-2-phenylindole (DAPI) (0.1 µg/ml) (Invitrogen, Inc.) in 2% FBS/PBS for 2 hrs at 37 °C before mounting on glass slides using anti-fade fluorescence mounting media (Invitrogen, Inc.). The secondary antibodies used for immunostaining were: Dylight 594 Goat anti-mouse IgG (H+L) (115-515-166): from Jackson ImmunoResearch and Alexa Fluor 488 nm anti-rabbit IgG (H+L) (A11034): from Invitrogen Molecular Probes.

For PLA, cells were plated on nunc latek chamber slide, fixed and permeabilized. After overnight primary antibody incubation at 4 °C, samples were processed using Duolink II PLA kit (Olink biosciences) according to the kit manual. Briefly, samples were incubated with anti-mouse and anti-rabbit PLA probes for 1 hr at 37 °C, add in ligation reaction mix and incubate for additional 30 mins at 37 °C. Signal was amplified using amplification-polymerase for 100 mins at 37 °C. The slide was then washed and mounted for viewing. Detection reagent orange (ex/em 554/579) was used, it appeared red under the microscope. Red dots represented positive interactions. Images for immunostaining were acquired with an Olympus Manual Reflected Fluorescence System (Olympus Singapore Pte Ltd,

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Singapore). Images for PLA were acquired using DeltaVision microscope with softWorx software for image acquisition and processing with help from Dr. Rachel Kraut's lab (Applied Precision).

2.9 *In vitro* methyltransferase assay

In vitro methyltransferase assay for PRMT6 was performed in methylation buffer containing 100 mM of Tris pH 8.0, 25 mM MgCl₂ and 0.004 M DTT. 0.55 µCi of H³-SAM was used for each sample. Flag tagged SHRs and PRMT6 were transfected into COS7 cells and pulled down with Flag beads. Beads were incubated in the methylation buffer for 1 hr at 37 °C with mixing every 15 mins. For assay using peptides, 1 µg of peptides were incubated with Flag-PRMT6 beads or 200 ng of recombinant PRMT6 (51049, BPSbioscience). Reaction was quenched using protein loading dye and boiled at 100 °C for 8 mins. Samples were separated by SDS-PAGE and signal was enhance using ELIGHTENING solution (Perkin Elmer). Gels were dried in GelAir dryer (bio-rad) and dried gels were kept with hyperfilm MP (GE healthcare) for 1-3 weeks at -80 °C to detect the radioactivity.

2.10 *In vivo* methyltransferase assay

In vivo methyltransferase assay was carried out by overexpressing Flag-ER in the presence and absence of Flag-PRMT6. 24 hrs after transfection, cells were treated with 20 µM Adenosine, periodate oxidised (AdOx, sigma) for 24 hrs to inhibit protein methylation. Cells were then pretreated with 100 µg/ml of cycloheximide and 40 µg/ml of chloramphenicol for 30 mins to inhibit protein synthesis followed by labeling with H³-

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methionine for additional 3 hrs. Lysates were collected for immunoprecipitation with Flag beads and samples were loaded for SDS-PAGE, processed as mentioned in session 2.9.

2.11 Enzyme-Linked Immunosorbent Assay (ELISA)

All the incubation was done in a humid box to avoid drying out of plate during the procedure. Antigen protein was prepared in PBS (containing 0.05% w/v NaN₃) at 4 µg/ml. The day before the assay, 50 µl antigen solutions were added into each well of the 96-well plate and incubated overnight at 4 °C. On the day of assay, the coating antigen solution was decanted, followed by blocking the plate with 200 µl/well blocking buffer (1% w/v BSA, 0.3% v/v Tween 20 and 0.05% w/v NaN₃ in PBS) at 37 °C for 2 hrs. During blocking, antisera from mice were diluted in blocking buffer. After blocking, 100 µl diluted primary antibody was added into each well and incubated for 1 hr at room temperature. As a negative control, pre-immune serum was included at 1:50 dilution. The plate was then washed once in ELISA washing buffer and another time in ddH₂O. 100 µl of Anti-mouse-IgG-HRP (GE Healthcare) was added into each well at 1:5000 dilutions in PBS. After 1 hr incubation at room temperature, the plate was washed in ELISA washing buffer once and another time with ddH₂O. 100 µl of substrate (Sigma) was added to each well. The plate was allowed to develop for 5 mins and reaction was inactivated by 0.9 M HCl, OD read at 450 nm in the dark.

2.12 Bisulfite Sequencing

Genomic DNA were extracted from cell cultures using protein kinase digestion buffer containing proteinase K (Fermentas Life Sciences, USA) with 2 hrs incubation at 55 °C. The genomic DNA were then twice extracted with phenol:chloroform:isoamyl alcohol

Materials and methods

(25:24:1), and precipitated with 100% ethanol. Precipitated genomic DNA was washed with 70% ethanol, and dissolved in sterile water at 4 °C overnight. Bisulfite treatment of genomic DNA was done with CpGenome™ Fast DNA modification kit (Chemicon International, USA), and sodium hydroxide (Merck, Germany). PCR of bisulfite-treated DNA were carried out with primers (designed using MethPrimer (Li and Dahiya, 2002)) specific for the 3 CpG islands as listed in Table 2.7. The PCR products were then cloned into a pCR®2.1-TOPO® vector from TOPO TA Cloning® Kit (Invitrogen, USA) and sequenced. Analysis of bisulfite sequencing results was done using the online software Quantification tool for Methylation Analysis (QUMA).

Table 2.7 List of Primers for bisulfite sequencing

CpG-1 Forward primer	5' TTTAGTTTGTAGGGAGGTGTGGAGG 3'
CpG-1 Reverse primer	5' TTAACACAACAACCTACAACCCAAATACT 3'
CpG-2 Forward primer	5' ATAGGATTTATGAATAGAAAAGGAGTTGTA 3'
CpG-2 Reverse primer	5' AAATAACTAAAACCAACAAACACCC 3'
CpG-3 Forward primer	5' GGTGGATTATGAGGTTAGGAGATTGAGATT 3'
CpG-3 Reverse primer	5' CACCACACACAATTTATCACCAAAATAAAA 3'

2.13 Statistical analysis

Charts were plotted using mean value with standard error mean (SEM) indicated as error bars. Significance was calculated by unpaired Student's *t*-test (two-tailed, equal variance) and *p* values less than 0.05 was considered significant. Pearson correlation was employed to examine the relationship between p53 and TRIM22 expression.

PRMT6 and SHRs

CHAPTER 3

METHYLATION OF STEROID HORMONE RECEPTORS BY PRMT6

3.1 Detection of methylated arginines in ER

To date, arginine 260 is the only arginine residue shown to be methylated in ER (Le Romancer et al., 2008). To identify additional arginine sites methylated in ER (referring to ER α only and it is the focus of this study), LC-MS technique was adopted to detect modifications in ER. As post-translational modifications are usually only detectable in LC-MS when there is sufficient amount of protein sample used, instead of pulling down endogenous ER from mammary epithelial cell lines or overexpress ER in mammary epithelial cell lines, COS7 cells which can be transfected at high efficiency was chosen to express high level of ER protein for MS analysis. In addition, study of progesterone receptor in our lab has revealed that almost identical post-translational modifications on progesterone receptor are detected in COS7 and breast cancer cells. Full length Flag tagged ER expressed in COS7 cells was immunoprecipitated and processed for MS analysis to search for methylation sites. ER band intensity for the coomassie stained gel is shown in Figure 3.1A. Two independent sets of ER samples were sent for analysis and the modifications detected were highly consistent. The sequence coverage was 58-60% and the same regions were detected from LC-MS for both sets of data (Figure 3.1B). This was most probably due to the limitation that trypsin only cuts after arginine and lysine residues; some of the regions without these two residues would not be digested and regions rich in these two residues would be cut into fragments too small to be captured well in the LC-MS analysis.

Since exogenously expressed ER protein was used in LC-MS analysis and it may not have been modified the same way as endogenous ER, the PTMs detected here were compared

PRMT6 and SHRs

against PTMs published in previous studies. Many published ER PTM sites in breast cancer cells such as serine 154 phosphorylation, serine 294 phosphorylation and lysine 299 acetylation were also present in our MS data, suggesting that ER expressed in COS7 were post-translationally modified at same residues as in breast cancer cells and the MS detection was accurate and reliable (Atsriku et al., 2009; Britton et al., 2008; Wang et al., 2001a).

Multiple arginine sites were identified to be methylated and highlighted in yellow in Figure 3.1B. MS spectra for R233 and R234 are shown in Figure 3.1C & D. Comparison of the spectra for unmethylated R233 peptide and methylated R233 peptides showed a mass shift of 14 Da which was caused by addition of one methyl group (Figure 3.1C). Spectra for methylated R234 peptide was also included showing the mass shift of 14 Da as compared to theoretical mass for the fragment (Figure 3.1D). Knowing that PRMT1 can regulate ER activity by methylating its arginine residue 260, these methylated arginines detected in the LC-MS may also contribute to the regulation of ER activity (Le Romancer et al., 2008).

PRMT6 and SHRs

PRMT6 and SHRs

R233

#	b	b ⁺⁺	b ⁺	b ⁺⁺⁺	b ⁰	b ⁰⁺⁺	Seq.	y	y ⁺⁺	y ⁺	y ⁺⁺⁺	y ⁰	y ⁰⁺⁺	#
1	88.0393	44.5233			70.0287	35.5180	S							22
2	201.1234	101.0653			183.1128	92.0600	I	2552.1075	1276.5574	2535.0810	1268.0441	2534.0969	1267.5521	21
3	329.1819	165.0946	312.1554	156.5813	311.1714	156.0893	Q	2439.0234	1220.0154	2421.9969	1211.5021	2421.0129	1211.0101	20
4	386.2034	193.6053	369.1769	185.0921	368.1928	184.6001	G	2310.9649	1155.9861	2293.9383	1147.4728	2292.9543	1146.9808	19
5	523.2623	262.1348	506.2358	253.6215	505.2518	253.1295	H	2253.9434	1127.4753	2236.9169	1118.9621	2235.9328	1118.4701	18
6	637.3052	319.1563	620.2787	310.6430	619.2947	310.1510	N	2116.8845	1058.9459	2099.8579	1050.4326	2098.8739	1049.9406	17
7	752.3322	376.6697	735.3056	368.1565	734.3216	367.6645	D	2002.8416	1001.9144	1985.8150	993.4111	1984.8310	992.9191	16
8	915.3955	458.2014	898.3690	449.6881	897.3850	449.1961	Y	1887.8146	944.4109	1870.7881	935.8977	1869.8041	935.4057	15
9	1062.4309	531.7191	1045.4044	523.2058	1044.4204	522.7138	M	1724.7513	862.8793	1707.7247	854.3660	1706.7407	853.8740	14
10	1222.4616	611.7344	1205.4350	603.2212	1204.4510	602.7291	C	1577.7159	789.3616	1560.6893	780.8483	1559.7053	780.3563	13
11	1319.5143	660.2608	1302.4878	651.7475	1301.5038	651.2555	P	1417.6852	709.3463	1400.6587	700.8330	1399.6747	700.3410	12
12	1390.5515	695.7794	1373.5249	687.2661	1372.5409	686.7741	A	1320.6325	660.8199	1303.6059	652.3066	1302.6219	651.8146	11
13	1491.5991	746.3032	1474.5726	737.7899	1473.5886	737.2979	T	1249.5954	625.3013	1232.5688	616.7880	1231.5548	616.2960	10
14	1605.6421	803.3247	1588.6155	794.8114	1587.6315	794.3194	N	1148.5477	574.7775	1131.5211	566.2642	1130.5371	565.7722	9
15	1733.7006	867.3540	1716.6741	858.8407	1715.6901	858.3487	Q	1034.5048	517.7560	1017.4782	509.2427	1016.4942	508.7507	8
16	1893.7313	947.3693	1876.7047	938.8560	1875.7207	938.3640	C	906.4462	453.7267	889.4196	445.2135	888.4356	444.7214	7
17	1994.7790	997.8931	1977.7524	989.3798	1976.7684	988.8878	T	746.4155	373.7114	729.3890	365.1981	728.4050	364.7061	6
18	2107.8630	1054.4352	2090.8365	1045.9219	2089.8525	1045.4299	I	645.3678	323.1876	628.3413	314.6743	627.3573	314.1823	5
19	2222.8900	1111.9486	2205.8634	1103.4353	2204.8794	1102.9433	D	532.2838	266.6455	515.2572	258.1323	514.2732	257.6402	4
20	2350.9849	1175.9961	2333.9584	1167.4828	2332.9744	1166.9908	K	417.2568	209.1321	400.2303	200.6188			3
21	2465.0279	1233.0176	2448.0013	1224.5043	2447.0173	1224.0123	N	289.1619	145.0846	272.1353	136.5713			2
22							R	175.1190	88.0631	158.0924	79.5498			1

R233me

#	b	b ⁺⁺	b ⁺	b ⁺⁺⁺	b ⁰	b ⁰⁺⁺	Seq.	y	y ⁺⁺	y ⁺	y ⁺⁺⁺	y ⁰	y ⁰⁺⁺	#
1	88.0393	44.5233			70.0287	35.5180	S							22
2	201.1234	101.0653			183.1128	92.0600	I	2566.1232	1283.5652	2549.0966	1275.0519	2548.1126	1274.5599	21
3	329.1819	165.0946	312.1554	156.5813	311.1714	156.0893	Q	2453.0391	1227.0232	2436.0126	1218.5099	2435.0285	1218.0179	20
4	386.2034	193.6053	369.1769	185.0921	368.1928	184.6001	G	2324.9805	1162.9939	2307.9540	1154.4806	2306.9700	1153.9886	19
5	523.2623	262.1348	506.2358	253.6215	505.2518	253.1295	H	2267.9591	1134.4832	2250.9325	1125.9699	2249.9485	1125.4779	18
6	637.3052	319.1563	620.2787	310.6430	619.2947	310.1510	N	2130.9001	1065.9537	2113.8736	1057.4404	2112.8896	1056.9484	17
7	752.3322	376.6697	735.3056	368.1565	734.3216	367.6645	D	2016.8572	1008.9322	1999.8307	1000.4190	1998.8467	999.9270	16
8	915.3955	458.2014	898.3690	449.6881	897.3850	449.1961	Y	1901.8303	951.4188	1884.8037	942.9055	1883.8197	942.4135	15
9	1062.4309	531.7191	1045.4044	523.2058	1044.4204	522.7138	M	1738.7669	869.8871	1721.7404	861.3738	1720.7564	860.8818	14
10	1222.4616	611.7344	1205.4350	603.2212	1204.4510	602.7291	C	1591.7315	796.3694	1574.7050	787.8561	1573.7210	787.3641	13
11	1319.5143	660.2608	1302.4878	651.7475	1301.5038	651.2555	P	1431.7009	716.3541	1414.6743	707.8408	1413.6903	707.3488	12
12	1390.5515	695.7794	1373.5249	687.2661	1372.5409	686.7741	A	1334.6481	667.8277	1317.6216	659.3144	1316.6376	658.8224	11
13	1491.5991	746.3032	1474.5726	737.7899	1473.5886	737.2979	T	1263.6110	632.3091	1246.5845	623.7959	1245.6005	623.3039	10
14	1605.6421	803.3247	1588.6155	794.8114	1587.6315	794.3194	N	1162.5633	581.7853	1145.5368	573.2720	1144.5528	572.7800	9
15	1733.7006	867.3540	1716.6741	858.8407	1715.6901	858.3487	Q	1048.5204	524.7638	1031.4939	516.2506	1030.5098	515.7586	8
16	1893.7313	947.3693	1876.7047	938.8560	1875.7207	938.3640	C	920.4618	460.7346	903.4353	452.2213	902.4513	451.7293	7
17	1994.7790	997.8931	1977.7524	989.3798	1976.7684	988.8878	T	760.4312	380.7192	743.4046	372.2060	742.4206	371.7139	6
18	2107.8630	1054.4352	2090.8365	1045.9219	2089.8525	1045.4299	I	659.3835	330.1954	642.3570	321.6821	641.3729	321.1901	5
19	2222.8900	1111.9486	2205.8634	1103.4353	2204.8794	1102.9433	D	546.2994	273.6534	529.2729	265.1401	528.2689	264.6481	4
20	2350.9849	1175.9961	2333.9584	1167.4828	2332.9744	1166.9908	K	431.2725	216.1399	414.2459	207.6266			3
21	2465.0279	1233.0176	2448.0013	1224.5043	2447.0173	1224.0123	N	303.1775	152.0924	286.1510	143.5791			2
22							R	189.1346	95.0709	172.1081	86.5577			1

PRMT6 and SHRs

D

#	b	b ⁺⁺	b ⁺	b ⁰	b ⁰ ++	Seq	y	y ⁺⁺	y ⁺	y ⁰ ++	y ⁰	y ⁰ ++	#
1	88.0393	44.5235		70.0287	35.5180	S							23
2	201.1234	101.0653		183.1128	92.0600	I	2706.2294	1353.6183	2689.2028	1345.1050	2688.2188	1344.6130	22
3	329.1819	165.0946	312.1554	156.5813	311.1714	Q	2593.1453	1297.0763	2576.1187	1288.5630	2575.1347	1288.0710	21
4	386.2034	193.6053	369.1769	185.0921	368.1928	G	2465.0867	1233.0470	2448.0602	1224.5337	2447.0761	1224.0417	20
5	523.2623	262.1348	506.2358	253.6215	505.2518	H	2408.0653	1204.5363	2391.0387	1196.0230	2390.0547	1195.5310	19
6	637.3052	319.1563	620.2787	310.6430	619.2947	N	2271.0063	1136.0065	2253.9798	1127.4935	2252.9958	1127.0015	18
7	752.3322	376.6697	735.3056	368.1565	734.3216	D	2156.9634	1078.9853	2139.9369	1070.4721	2138.9528	1069.9801	17
8	915.3955	458.2014	898.3690	449.6881	897.3850	Y	2041.9365	1021.4719	2024.9099	1012.9586	2023.9259	1012.4666	16
9	1046.4360	523.7216	1029.4095	515.2084	1028.4254	NI	1878.8731	939.9402	1861.8466	931.4269	1860.8626	930.9349	15
10	1206.4667	603.7370	1189.4401	595.2237	1188.4561	C	1747.8327	874.4200	1730.8061	865.9067	1729.8221	865.4147	14
11	1303.5194	652.2633	1286.4929	643.7501	1285.5089	P	1587.8020	794.4046	1570.7755	785.8914	1569.7914	785.3994	13
12	1374.5565	687.7819	1357.5300	679.2686	1356.5460	A	1490.7492	745.8783	1473.7227	737.3650	1472.7387	736.8730	12
13	1475.6042	738.3057	1458.5777	729.7925	1457.5936	T	1419.7121	710.3597	1402.6856	701.8464	1401.7016	701.3544	11
14	1589.6471	795.3272	1572.6206	786.8139	1571.6366	N	1318.6644	659.8359	1301.6379	651.3226	1300.6539	650.8306	10
15	1717.7057	859.3465	1700.6792	850.8432	1699.6952	Q	1204.6215	602.8144	1187.5950	594.3011	1186.6110	593.8091	9
16	1877.7364	939.3718	1860.7098	930.8585	1859.7258	C	1076.5629	538.7851	1059.5364	530.2710	1058.5524	529.7798	8
17	1978.7840	989.8957	1961.7575	981.3824	1960.7735	T	916.5323	458.7698	899.5057	450.2565	898.5217	449.7645	7
18	2091.8681	1046.4377	2074.8416	1037.9244	2073.8575	I	815.4846	408.2459	798.4581	399.7327	797.4741	399.2407	6
19	2206.8951	1103.9512	2189.8685	1095.4379	2188.8845	D	702.4006	351.7039	685.3740	343.1906	684.3900	342.6956	5
20	2334.9900	1167.9986	2317.9635	1159.4854	2316.9794	K	587.3736	294.1904	570.3471	285.6772			4
21	2449.0329	1225.0201	2432.0064	1216.5068	2431.0224	N	459.2786	230.1430	442.2521	221.6297			3
22	2605.1341	1303.0707	2588.1075	1294.5574	2587.1235	R	345.2357	173.1215	328.2092	164.6082			2
23						R	189.1346	95.0709	172.1051	86.5577			1

PRMT6 and SHRs

Figure 3.1 Detection of methylated arginine in ER by LC-MS.

(A) Flag-ER were expressed in COS7 and pulled down by Flag beads. Distinct ER band can be seen on the coomassie blue stained gel. This band was cut out for in gel digest and LC-MS analysis. (B) Full ER protein sequence shown with the regions detected in LC-MS marked as red and methylated arginine highlighted in yellow. The sequence coverage for this detection was 60%. (C) Sequence and MS spectra for unmethylated and methylated R233 peptides. Mass/charge ration (m/z) for y3 (KNR) fragment showed that addition of one methyl group to R233 lead to mass shift of 14 Da. Tables showing the lists of peptide fragments corresponding to the peaks detected are also included; molecular weights of the detected fragments are marked in red. (D) Sequence and MS spectra of methylated R234 peptide. m/z for y3 (NRR) fragment showed that addition of one methyl group to R234 lead to mass shift of 14 Da as compared to theoretical mass. A table showing the list of peptide fragments corresponding to the peaks detected is also included; molecular weights of the detected fragments are marked in red.

PRMT6 and SHRs

3.2 PRMT6 interacts with steroid hormone receptors ER, PR and AR

3.2.1 Expression level of PRMT1 to PRMT8 in mammary epithelial cell lines

To gain insight on the functional significance of PRMTs in breast cancer cell biology, the relative expression level of various PRMTs were evaluated by real-time PCR in three commonly used breast cancer cell lines (MCF7, MDA-MB-231 and T47D) and in a non-malignant human mammary epithelial cell line MCF10A. All the PRMTs tested were expressed at detectable levels except PRMT8, which has been found to express specifically in the brain (Lee et al., 2005a). Expression level of PRMT1 and PRMT5 were higher than the other PRMTs in general. PRMT2 level was higher in MCF10A but lower in the breast cancer cells. PRMT 3, 4 and 7 expressions were similar in the four cell lines tested, whereas PRMT6 expression was higher in MCF7 but much lower level in the other three cell lines (Figure 3.2).

Figure 3.2 PRMT expressions in mammary epithelial cell lines.

Total RNA from MCF7, MDA-MB-231, T47D and MCF10A were collected and reverse transcribed into cDNA. mRNA expressions of individual PRMTs were analyzed by real-time PCR using specific primers in triplicates. 36B4 was used as the internal control. Relative mRNA expression presented here was obtained by normalization with 36B4.

*PRMT6 and SHRs***3.2.2 Screening the interaction between ER/PR and 8 PRMTs**

Since direct protein-protein interaction is important for PRMTs to methylate their target proteins, I set out to screen the physical interaction between ER/PR and eight PRMTs to identify the PRMT that can bind and hence may be able to methylate the receptors (Chan et al., 2009). Full length PRMT1 to PRMT8 coding sequences were inserted into pcDNA3.1+/hygro vector with Flag tag at the N terminus which enables detection with Flag antibody (Figure 3.3A). Individual PRMT was co-expressed with ER or PR in COS7 cells for Co-IP. As shown in Figure 3.3B, all eight PRMT proteins could be pulled down by Flag antibody specifically, but ER was only pulled down by PRMT2 and PRMT6. Similarly for PR, only PRMT2 and 6 could interact and Co-IP PR well (Figure 3.3C). I chose to focus on PRMT6 as the enzymatic activity of PRMT2 was yet to be demonstrated. Further validation for the interaction with PRMT6 was carried out with reverse IP using ER and PR antibody. As shown in Figure 3.4, PRMT6 was pulled down by either ER or PR antibody specifically.

A

Figure 3.3 PRMT2 and PRMT6 interact with ER and PR.

(A) Full length PRMT1 to PRMT8 were cloned into pcDNA3.1+/hygro vector with Flag tag at the N terminus. Number of amino acids for each PRMT is indicated on the right. (B&C) ER or PR was co-transfected with different PRMTs in COS7 cells for 48 hrs. Lysates were collected for co-IP with Flag antibody conjugated beads by overnight rotation. Western blotting analysis showed that PRMT1 to PRMT8 were detected well with Flag antibody and 2% input control showed that ER and PR were expressed in all samples, but ER and PR IP bands were only seen in the presence of PRMT2 and PRMT6.

PRMT6 and SHRs

Figure 3.4 Reverse IP for PRMT6 using ER or PR antibody.

(A) Pull down of PRMT6 by ER. PRMT6 was co-transfected with vector control or ER into COS7 cells for 48 hrs and lysate was collected for co-IP using ER antibody. (B) Pull down of PRMT6 by PR. PRMT6 was co-transfected with vector control or PR into COS7 cells for 48 hrs and lysate was collected for co-IP using PR antibody. 2% input showed that all proteins were expressed well; ER and PR antibody can pull down the ER/PR proteins efficiently.

*PRMT6 and SHRs***3.2.3 PRMT6 interacts with non-chaperone bound ER, PR and AR but not GR both in the presence and absence of hormone treatment**

Having shown that PRMT6 can bind to both ER and PR, I investigated if PRMT6 can interact with all SHRs in general and if this interaction was regulated by hormone treatment. Co-IP was performed using COS7 cells overexpressing ER, PR, AR or GR with pcDNA3.1 vector control or with PRMT6. As shown in Figure 3.5, PRMT6 antibody pulled down PRMT6 protein well and it also co-immunoprecipitated ER, PR and AR. However, no GR protein could be pulled down by PRMT6. As demonstrated by the densitometry analysis of protein band intensity, the amount of ER/PR/AR pulled down by PRMT6 was higher without hormone treatment compared with hormone treatment, although there was no significant decrease in the input protein level after 3 hrs of hormone treatment. These interactions were repeated in three independent experiments.

It is well known that SHRs complex with hsp70 and hsp90 and several other proteins in the absence of ligand binding and this complex formation is critical for receptor folding and maturation (Kimmins and MacRae, 2000; Pratt and Toft, 1997b, 2003b; Smith and Toft, 1993). Since PRMT6 binds to the unliganded receptors better, I checked if PRMT6 was also part of this pre-activation chaperone complex. Probing the IP blot with specific antibodies against hsp70 and hsp90 proteins showed that endogenous hsp70/90 were expressed at high levels in the input control, but no pull down by PRMT6 was seen despite the presence of ER, PR and AR proteins co-immunoprecipitated (Figure 3.5A-C). PRMT6 interacts specifically to the non-chaperone bound receptors both in the presence and absence of hormone treatment.

PRMT6 and SHRs

The downregulation in interaction by hormone was further demonstrated by reverse IP with PR antibody. Although similar amount of PR was directly pulled down by PR antibody with 6 hrs of 0.1% ethanol or 10nM progesterin R5020 treatment, the level of PRMT6 pulled down with PR was reduced by more than half in the presence of R5020 (Figure 3.6, last two lanes). Phosphorylations of PR serine 294 and 400 have been found to be upregulated during PR activation by hormone (Daniel et al., 2005; Pierson-Mullany and Lange, 2004b). Interaction of PRMT6 with these phospho-PR proteins was examined to evaluate if PRMT6 binds to ligand activated PR. Detection using antibody against PR phosphorylated at serine 294 and 400 showed that PRMT6 could interact with phospho-PR proteins, suggesting that its interaction with liganded receptor was weakened but not abolished (Figure 3.6).

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Figure 3.5 PRMT6 interacts directly with ER, PR and AR but not GR.

COS7 cells were transfected with (A) ER, (B) PR, (C) AR or (D) GR, in combination with pcDNA3.1 vector control or Flag-PRMT6 plasmid for 24 hrs. The cells were treated with 0.1% ethanol or 10 nM steroid hormones (17 β -estradiol (E2) for ER, progesterin R5020 for PR, Dihydroxytestosterone (DHT) for AR and dexamethasone (Dex) for GR) for 3 hrs before total cell lysate were collected. Samples were incubated with 1 μ g of PRMT6 antibody overnight with rotation, followed by conjugation with protein A/G agarose beads. 2% of the total lysate was used as input control to show that the amount of proteins used in the IP was similar between all the samples for the same experiment. Western blotting analysis showed that ER, PR and AR were pulled down only in the presence of PRMT6, but not GR. No endogenous hsp70 and 90 proteins were pulled down by PRMT6 with ER/PR/AR. Protein band intensities were analyzed by Densitometer and the relative protein intensity for ER, PR and AR were indicated at the bottom of the blots. Band intensities for ER/PR/AR input controls with ethanol treatment were taken as 1 in the normalization. Consistent results were obtained in three independent experiments.

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Figure 3.6 PRMT6 interacts with phosphorylated PR.

Lysate for COS7 cells overexpressing PR with or without PRMT6 were split equally for co-IP using PRMT6 antibody or PR antibody. Samples were analyzed using Western blotting with antibodies targeting phospho294PR, phospho400PR, total PR and PRMT6. PRMT6 can pull down both forms of phosphorylated PR. The amount of PR pulled down by PRMT6 decreased with R5020 treatment (6 hrs). Similarly, PRMT6 pulled down by PR also reduced with R5020 treatment.

3.2.4 PRMT6 binds to ER and PR at different domains

To locate the site of interaction between PRMT6 and ER/PR, truncation constructs containing different ER/PR domain structures were generated using specific primers. First, the variable region of ER (amino acid 1-185) was removed and tested if there were any reduction in the interaction. As shown in Figure 3.7A, PRMT6 bind to ER (Δ VR) at a high level as compared to full length ER, suggesting that VR might not be involved in the interaction. Vectors containing VR only (amino acid 1-185), VR to hinge region (amino acid 1-355) and LBD to region F (amino acid 356-595) were then constructed for further testing. As expected, VR alone could not interact with PRMT6 and no pull down was seen in the Co-IP. Extending VR to hinge region did not help in the interaction with PRMT6, it still did not bind to PRMT6. However, LBD to region F alone was sufficient to interact with PRMT6. Although the protein expression level for LBD to region F was lower than

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the other domains, it could be pulled down by PRMT6 very well, at a level that was higher than the input control (Figure 3.7B).

To identify the site of interaction for PR, similar truncations were generated for PR. Unfortunately, C-terminal domains of PR (from DBD onwards) were not stable and the protein could not express well. Even though the mRNA transcript expressions could be detected in real-time PCR for all PR truncation constructs, no protein or an extremely low protein level was expressed for all of them (Figure 3.7E). In order to stabilize these domains, BUS region was added at their N-terminus. This enables the expression of DBD and low expression for DBD-LBD and LBD region. Removal of BUS from PRB (became equivalent of PRA) did not have a significant effect on the amount of protein pulled down by PRMT6. Further truncation of Hinge-LBD region (left with VR+DBD) also did not abolish the interaction with PRMT6, but the level pulled down was reduced, suggesting that Hinge-LBD region might help in the protein folding to facilitate binding to PRMT6 (Figure 3.7C). Bands for BUS alone and BUS with LBD overlapped with the light and heavy IgG bands and could not be seen clearly if they could be pulled down. But since BUS with DBD-LBD and BUS with DBD did not interact with PRMT6, I deduced that BUS or LBD most probably did not interact with PRMT6 as well (Figure 3.7D). Multiple bands were present for BUS because this region is heavily phosphorylated (Hagan et al., 2011a). This identification of interaction sites provides us a clue of where PRMT6 may acts on ER and PR to methylate them. The differences in the sites of interaction also suggest that PRMT6 may play different role in the regulations of SHR activities, since it binds to C-terminal AF-2 region for ER but interacts with N-terminal AF-1 region for PR.

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Figure 3.7 PRMT6 binds to ER (LBD-region F) and PR (VR-DBD).

Full length ER/PR or their truncation constructs were co-transfected with PRMT6 vector into COS7 cells for 24 hrs. Lysates were collected for co-IP using PRMT6 antibody. (A) Removal of variable region (VR) did not affect interaction with PRMT6. Both ER full length and ER (Δ VR) could be pulled down by PRMT6. (B) PRMT6 interacts with ER at C-terminus LBD-region F. PRMT6 did not pull down N-terminus VR or VR-Hinge (1-355) domains. It only pulled down LBD-region F. Bands were numbered and labeled at the side for easier identification. (C) PRMT6 interacts with PR at VR-DBD region. PRB, PRA and VR-DBD could all be pulled down by PRMT6. (D) No interaction was detected between PRMT6 and C-terminal domains of PR. PRMT6 did not pull down DBD-LBD, DBD or LBD with BUS tagged at the N-terminus. Bands were numbered and labeled at the side for easier identification. All co-IP experiments were repeated three times for confirm the results. (E) Poor protein expression but normal mRNA expression of C-terminal domains of PR. Full length PR and various PR truncations constructs were transfected into COS7 cells for 24 hrs, total lysates were collected for Western blotting analysis and total RNA was collected for reverse transcription and real-time PCR analysis. Protein expressions were detected for full length PR, BUS+NLS+LBD and BUS+DBD-LBD but not the rest. mRNA level of those PR truncation constructs which did not have protein expression or low protein expression showed high mRNA level in real-time PCR with full length PR as the control. Real-time PCR was carried out in triplicates. Relative mRNA expression was obtained by normalizing with 36B4 internal control and the error bars represent standard deviation for the fold changes.

3.2.5 PRMT6 interacts with ER in the nucleus

To further validate the interaction between PRMT6 and ER in breast cancer cells, MCF7 stable cell lines transfected with pcDNA3.1 vector control or Flag-PRMT6 vector were established. Both immunostaining and the proximity ligation assay (PLA) were carried out using these stable cell lines as the endogenous level of PRMT6 was not high enough for the immunostaining and the commercial PRMT6 antibody available was not good enough for immunostaining. Immunostaining showed that without E2 treatment, PRMT6 protein mainly localized in the nucleus, while ER had both nuclear and cytoplasmic staining, with the majority in the nucleus. Image overlay demonstrated high level of colocalization for PRMT6 and ER (Figure 3.8A, top). After E2 treatment, PRMT6 remained in the nucleus, whereas ER protein showed decreased cytoplasmic staining. These two proteins still colocalized in the nucleus to give the yellow staining for the overlaid image (Figure 3.8A, middle). For MCF7 transfected with pcDNA3.1 control vector, only ER staining was detected, showing that the antibodies used in the immunostaining were highly specific (Figure 3.8A, bottom). Close proximity of PRMT6 and ER was further demonstrated by PLA, red dots represent positive signal amplification which could only happen if the two proteins were very close (<40 nm). In the negative control with pcDNA3.1 transfected, none or very few red dots were seen after PLA staining, showing that the assay is highly specific. In the presence of overexpressed PRMT6, high number of red dots in the nucleus was seen for both ethanol and E2 treated samples, indicating that PRMT6 interacted with endogenous ER specifically in the nucleus, similar to what was observed in the immunostaining. There was also overall decrease in PLA signal intensity after E2 treatment (Figure 3.8B). When the number of

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red dots were counted for 30 cells in 6 different fields taken for both ethanol and E2 treated cells, the number of red dots is significantly higher in ethanol treated cells than in E2 treated cells with a p value less than 0.0001 (Figure 3.8C). Protein expression of ER and PRMT6 for the stable cell lines were shown in Figure 3.8D. Exogenous Flag-PRMT6 was expressed at high level as compared to the pcDNA3.1 control but endogenous ER level in the two stable cell lines were similar, the ER protein level decreased by about half after treated with 10 nM E2 for 1hr, which might be responsible for the observed decrease in PLA signal after E2 treatment.

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Figure 3.8 PRMT6 interacts with ER exclusively in the nucleus.

(A) PRMT6 and ER colocalized in the nucleus. MCF7 cells overexpressing Flag-PRMT6 were treated with 0.1% ethanol (EtOH) or 10 nM E2 for 1 hr and fixed for immunostaining with Flag and ER antibodies. MCF7 cells transfected with pcDNA3.1 control was used as control. No Flag-PRMT6 staining was seen in this control but endogenous ER was detected normally. Images were taken at 60X objective. (B) PRMT6 and ER interacted in the nucleus. MCF7-pcDNA3.1 or MCF7-PRMT6 cells were fixed and incubated with Flag and ER primary antibody, followed by incubation with PLA probes, ligation and signal amplification. Amplified signal appeared as red dots under the microscope. Minimal numbers of red dots in MCF7-pcDNA3.1 cells showed that the assay was specific. Red dots in the MCF7-PRMT6 cells appear exclusively in the nucleus demonstrated presence of interaction between PRMT6 and ER in the nucleus. Images were taken at 40X objective. (C) Number of red dots in each cell was counted for 30 cells for both ethanol treated control cells and E2 treated cells in six different fields taken. Average numbers of red dots were plotted with standard deviation shown as error bar. The number of red dots is significantly higher in Ethanol control treated cells than in E2 treated cells with p value less than 0.0001 by unpaired Student's t test. (D) ER and PRMT6 protein levels in the stable cell lines. Cell lysates were collected concurrently with immunostaining and PLA and expression level of ER and Flag-PRMT6 were analyzed using Western blotting with GAPDH used as loading control.

3.3 Differential regulations of SHR ligand-dependent and ligand-independent activities by PRMT6

After characterizing the interaction between PRMT6 and SHRs, the effect of PRMT6 on SHR activity was examined using luciferase reporter assay in HeLa cells, as it is a human cervical cancer cell line and hence a better model to study cellular activity of steroid hormone receptors. It has been reported that PRMT6 increases the hormone induced transcriptional activity of ER, PR and GR (Harrison et al., 2010). I tested if the same observation could be made in our hands and if it worked similarly for AR. It turned out that PRMT6 upregulated hormone induced transcriptional activities of PR, AR and GR, similar to what has been reported (Figure 3.9 A-C). For PR, AR and GR which has very low level of constitutive ligand-independent activity, 60-100% increase in ligand induced activities were observed with 0-50 ng of PRMT6 plasmids used in the transfection, without significant effect on their protein expression (Figure 3.9 A-C, E). In addition, when PRMT6 was co-transfected with PR (Δ LBD) which is constitutively active, PRMT6 inhibits PR (Δ LBD) activity in a dose-dependent manner (Figure 3.9D).

For ER which has much higher ligand-independent activity than the other receptors, in addition to the enhancement of ligand-induced activity on ER by PRMT6 reported, I observed that ligand-independent ER activity was also upregulated in the presence of PRMT6 (Harrison et al., 2010). When ERE-luciferase activities were tested with either wild type PRMT6 or inactive PRMT6 mutant overexpressed, increase in the ER ligand-independent activity was seen with both wild type and mutant PRMT6, despite the absence of methyltransferase activity for the mutant. In contrast, the ligand-induced ER

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activity was only upregulated by wild type PRMT6 but not the inactive mutant, demonstrating that methyltransferase activity of PRMT6 was needed for its coactivation of ligand-induced activity (Figure 3.10 A). Knock down of endogenous PRMT6 in MCF7 cells showed downregulation for both ligand-independent and ligand-dependent ER activities without significant effect on the ER protein level; further demonstrate that PRMT6 was important for ER activation (Figure 3.10B). In addition, the level of serine 167 phosphorylation, which is a marker for ligand-independent ER activation, was also decreased by PRMT6 knock down (Figure 3.10B).

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Figure 3.9 Coactivation of PR, AR and GR transcriptional activities by PRMT6.

Transcriptional activities of SHRs in the presence of PRMT6 were examined using luciferase reporter assay. (A-C) HeLa cells were co-transfected with PRE-luciferase (for PR, AR and GR) and 5 ng of expression vectors for PR and AR (no GR transfected as it is expressed endogenously in HeLa) and different amount of PRMT6 (0, 25, 50 ng) for 24 hrs, treated with 10 nM of their respective hormone for additional 24 hrs before collection. (D) HeLa cells were co-transfected with PRE-luciferase and 5 ng of PR (Δ LBD) with 0, 10 and 20 ng of PRMT6 for 48 hrs without treatment. Lysates were collected for analysis with illuminometer. Fold induction in luciferase activity was calculated using luciferase signal with hormone treatment over EtOH control after normalizing with empty vector control. Relative luciferase activity was calculated using receptor luciferase signal over empty vector control signal. Results shown for panel A to E are from a single experiment using triplicates, which is representative of three independent experiments, error bar represents standard error of mean and the p values were obtained by unpaired student's t test. * $p < 0.05$; ** $p < 0.01$. (E) Protein expression of SHRs in the presence of PRMT6. Total protein lysates were collected concurrently with the luciferase assays following the same condition for Western blotting analysis. PRMT6 overexpression did not affect the SHR expressions significantly. GAPDH was used as the loading control.

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Figure 3.10 PRMT6 enhances both ligand-independent and ligand-dependent ER activities and PRMT6 knock down downregulates both activities.

(A) Transcriptional activities of ER in the presence of PRMT6 were examined using ERE-luciferase assay in HeLa cells. 50 ng of pcDNA3.1 vector control or wild type PRMT6 or PRMT6 (V86K/D88A) mutant were transfected with 5 ng of ER plasmid or pCMV control for 24 hrs followed by 24 hrs treated with E2 (10 nM). Relative luciferase activity was calculated using receptor luciferase signal over empty vector control signal after normalizing with protein concentration for each sample. (B) PRMT6 specific siRNA was transfected into MCF7 cells to knock down PRMT6 expression. ERE-luciferase plasmid was transfected 24 hrs following the knock down. 48 hrs after knock down cells were treated for additional 24 hrs before collected for illuminometer reading. Relative ERE-luciferase activity is the firefly luciferase signal normalized with protein concentration for each sample. Results shown for both panel A and B are from a single

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experiment using triplicates, which is representative of three independent experiments, error bar represents standard error of mean and the p values were obtained by unpaired student's t test. *p<0.05; ** p<0.01. Protein lysate was collected concurrently for Western blotting analysis for both experiments and GAPDH was used as the loading control.

3.4 PRMT6 methylates steroid hormone receptors

Since PRMT6 interacts with the SHRs and regulates their transcriptional activities, I examined whether SHRs are direct targets for PRMT6 methylation.

3.4.1 PRMT6 methylates full length ER, PR, AR but not GR

In vitro methyltransferase assay was carried out using exogenous Flag tagged PRMT6 and Flag tagged SHRs co-expressed in COS7 and pulled down by Flag conjugated beads. Proteins attached to the Flag beads were at close proximity which facilitated the methylation reaction to happen. Flag-PRMT6, Flag-ER and Flag-AR were pulled down well, less Flag-PR was pulled down and the band for Flag-GR was not distinguishable as the size of Flag-GR overlapped with one non-specific band between 75 and 100 kDa. Beads were incubated with methylation buffer and H³-SAM for labeling at 37 °C for 1hr. Proteins were separated by SDS-PAGE and stained with G250 coomassie blue. Dried gel was kept for developing at -80 °C for 1 week. Autoradiogram showed that PRMT6 was capable of self-methylation. No radioactivity was detected in the absence of PRMT6. Bands corresponding to the size of AR, PR and ER were seen but no specific band for GR was detected. ER methylation band was the strongest and PR methylation band was much weaker and appeared just below one background band (Figure 3.11A).

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To further demonstrate that the labeling was indeed caused by the methyltransferase activity of PRMT6, ER methylation in the presence of inactive PRMT6 mutant (V86K/D88A) was also tested. As shown in Figure 3.11B, although both ER and PRMT6 mutant protein levels were high in the coomassie blue staining, no ER methylation band could be seen in the autoradiogram, confirming that presence of active PRMT6 was needed for the labeling to occur.

In vitro methylation labels target proteins after they are released into reaction buffer by cell lysis. These proteins were brought close together by immunoprecipitation and the reaction condition used might not resemble cellular condition well. However, *in vivo* methyltransferase assay allows the examination of methylation carried out in live cells. After 24 hrs of treatment with methylation inhibitor adenosine dialdehyde (AdOx) to enrich unmethylated proteins, *In vivo* labeling with H³-methionine in the presence of protein synthesis inhibitor was then carried out for ER with and without PRMT6 overexpression. Cell lysates were collected after 3 hrs of labeling and proteins were pulled down using Flag beads and loaded for SDS-PAGE. Amount of ER and PRMT6 proteins detected was lower due to the treatments done. After 2 weeks developing at -80 °C, no bands appeared in the absence of PRMT6. ER bands could be seen only in the presence of PRMT6 and PRMT6 still specifically methylated itself. There was no significant change in ER methylation band intensity in the 3 hrs of E2 treatment during labeling (Figure 3.11C).

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Figure 3.11 PRMT6 methylates full length AR, PR and ER.

(A) *In vitro* methyltransferase assay was carried out by pulling down full length Flag tagged PRMT6 alone or together with Flag tagged AR/GR/PR/ER overexpressed in COS7 cells. Flag beads were incubated with PRMT6 methylation buffer with H³-SAM for 1 hr at 37 °C for methylation to occur. In addition to the self-methylation bands for PRMT6, specific bands corresponding to the size of AR, PR and ER were detected for both coomassie blue stained gel and autoradiogram. GR protein band overlap with one non-specific background band and its methylation could not be determined. Similar results were obtained in at least two independent experiments. * Marks the position for the receptors, in this case, they are AR, GR, PR and ER. (B) Specificity of ER methylation by PRMT6 was further demonstrated with enzymatically inactive PRMT6 mutant (V86K/D88A). *In vitro* methyltransferase assay were carried out by pulling down full length Flag tagged wild type PRMT6 or PRMT mutant with Flag tagged ER overexpressed in COS7. ER methylation band on autoradiogram could only be seen in the presence of wild type PRMT6 but not inactive PRMT6 mutant. (C) *In vivo* methyltransferase assay was carried out in COS7 cells overexpressing PRMT6 alone, ER alone or PRMT6 with ER. Cells were treated with AdOx to inhibit protein methylation for 24 hrs, followed by labeling with H³-methionine in the presence of protein synthesis inhibitors for 3 hrs, with EtOH or E2. Proteins pulled down using Flag beads were separated by SDS-PAGE and dried for developing for 2 weeks. Autoradiogram showed that radioactivity was only detectable in the presence of PRMT6. ER was methylated specifically with PRMT6 coexpressed.

3.4.2 PRMT6 methylates multiple sites in ER

As the methylation signal for ER was much stronger than the other receptors, I suspected that ER had multiple sites methylated by PRMT6. *In vitro* methyltransferase assay was carried out using Flag tagged ER truncation constructs containing VR only, LBD-region F (356-595) or with VR cut out (Δ VR). As shown in Figure 3.12, strong methylation band was detected for VR but absent for LBD-region F. Since the expression level of LBD-region F was low and much less protein was pulled down, further optimization to improve the amount of protein pulled down would be necessary to confirm the absence of methylation in this region. Δ VR could also be methylated by PRMT6 specifically, suggesting that DBD or Hinge might be methylated by PRMT6. As both VR and Δ VR

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were well methylated by PRMT6, it proved that there were more than one arginine residue methylation by PRMT6 in ER, at least one in VR and one in DBD to region F.

Figure 3.12 PRMT6 methylates multiple domains in ER.

In vitro methyltransferase assay was carried out with Flag-PRMT6 or Flag-ER (truncations) overexpressed in COS7 cells. Proteins pulled down by Flag beads were incubated in methylation buffer at 37 °C for 1 hr with H³-SAM. Labeled samples were separated using SDS-PAGE and dried gels were kept in -80 °C freezer for developing for 1 week. Radioactivity was only detectable in the presence of PRMT6. Specific

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methylation band for both Flag-ER (VR) and Flag-ER (Δ VR) could be seen but not for Flag-ER (356-595). * Marks the position for the bands of interest, in this case, they are Flag-PRMT6, Flag-ER (356-595), Flag-ER (VR) and Flag-ER (Δ VR). Experiments have been repeated to confirm the results.

3.4.3 Screening of PRMT6 methylation targets from residues identified by MS analysis

As shown in Figure 3.1, multiple arginine residues outside VR were identified to be methylated in ER. To test which of these arginine residues were methylated by PRMT6; short peptides were synthesized containing those sites for *in vitro* methyltransferase assay with recombinant PRMT6 protein, with H3R2 used as the positive control. Besides the positive control, R233 and R234 came out as two of the strongest methylation signals on the autoradiogram, removal of either arginine did not abolish the signal, suggesting both residues were methylated (Figure 3.13A). Further validation was carried out using ER R233K and R234K mutants generated by site directed mutagenesis with the arginine residue removed. *In vitro* methyltransferase assay for full length wild type ER and mutant ER showed that there was no obvious reduction in the methylation signal when R233 and R234 were mutated, indicating that R233 and R234 may not be the major PRMT6 methylation sites for ER in the absence of treatments (Figure 3.13B). Luciferase reporter gene assay was also used to elucidate the possible function of R233 and R234 methylation. No significant change was observed for R233K mutation, whereas R234K caused drastic increase in the ligand-independent activity of ER, with proportional increase in ligand-independent activity. Similar effect was seen for R233K/R234K double mutant (Figure 3.13C). When R234F mutant was used to mimic the methylated state of R234, ligand-independent activity of ER was abolished completely, while the ligand induced activity

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remained higher than the wild type ER (Figure 3.13D). Protein expressions for all the mutants used were at similar level as ER wild type (Figure 3.13E).

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Figure 3.13 R234 mutation enhances both ligand-dependent and independent activity of ER.

(A) *In vitro* methyltransferase assay was performed using short peptides (12mer) containing these arginine residues identified to be methylated in MS analysis and incubated with recombinant PRMT6 protein. H3R2/17 was used as positive control and negative control was a non-arginine containing peptide. Dried gel was exposed for 1 week at -80 °C. Radioactivity detected for positive control, R233, R234 and R412. (B) *In vitro* methyltransferase assay was performed using ER R233 and R234 mutants. PRMT6 and ERwt or mutants were coexpressed in COS7 cells and pulled down using Flag beads. Samples were separated using SDS-PAGE after methylation reaction and dried gel was developed for 1 week at -80 °C. Radioactivity is detected for both ERwt and mutants. (C) R234K mutation enhances ER ligand-independent activity. HeLa cells were co-transfected with ERE-luciferase and 5 ng of expression vectors for control, ERwt or ER mutants for 24 hrs, treated with 10 nM E2 for additional 24 hrs before collection. Lysates were collected for analysis with illuminometer. Drastic increase in luciferase activity was obtained in the presence of R234K mutation. (D) R234K and R234F showed opposite change in ER activity. HeLa cells were co-transfected with ERE-luciferase and 5 ng of expression vectors for control, ERwt, ER (234K) or ER (R234F) for 24 hrs, treated with 10 nM E2 for additional 24 hrs before collection. Lysates were collected for analysis with illuminometer. R234K increased luciferase signal whereas R234F decreased ligand-independent activity. Relative luciferase activity was obtained by normalizing with empty vector control (pCMV EtOH). Results shown are from a single experiment in triplicates, which is representative of at least two independent experiments. Fold change induced by hormone treatment is indicated on top of two bars. ** $p < 0.01$; NS: non-significant. (E) ER mutants expressed at similar protein level as wild type ER. ER wild type and mutant constructs were transfected into HeLa cells using the same condition as luciferase assay. Protein expressions of ER mutants used in the luciferase assay were analyzed by Western blotting using GAPDH as loading control.

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3.4.4 PRMT6 methylates R260 with conserved PRMT recognition motif

As the MS analysis could not cover the whole ER sequences, we looked at the ER amino acid sequences in detail and identified arginine 260 in the RGG PRMT recognition motif as a possible target of PRMT6. ER (R260K) mutant was generated for *in vitro* methylation and the signal was compared with wild type ER. As shown in Figure 3.14, although PRMT6 could still methylate ER (R260K), the methylation signal was weakened by 28% after normalization with protein level, suggesting that ER is methylated by PRMT6 at multiple sites and R260 is one of the targets for PRMT6.

Figure 3.14 Downregulation of ER methylation signal with R260K mutation.

R260 is present in the RGG conserved PRMT recognition motif. *In vitro* methyltransferase assay was carried out by pulling down exogenous PRMT6 with wild type ER or ER (R260K) mutant in COS7 and incubated them in methylation buffer. Relative protein levels were shown by coomassie blue staining and autoradiogram showed methylation labeling on ER. Relative methylation signals for ER and R260K mutant analysed by densitometer are also indicated.

3.5 Discussion

The PTMs on protein enable the rapid changes in protein activity without the need for new protein synthesis. Multiple PTMs allows fine-tuning of various aspects of protein activity in response to the changing cellular environment. This study demonstrated that ER, PR and AR bind to PRMT6 and are direct targets for PRMT6 methylation. The interaction and methylation are hormone-independent; suggesting PRMT6 is involved in the ligand-independent activity of SHRs. PRMT6 regulates ER ligand-independent and ligand-induced activities via distinct mechanisms. Methyltransferase activity is not required for enhancement of ligand-independent activity but is essential for it to upregulate ligand-induced activity. This study also identified R234 in the DBD and R260 in the Hinge of ER as possible targets of PRMT6 methylation.

Although PRMT1, PRMT2, PRMT4 and PRMT6 have been identified as coactivators of SHR signaling (Chen et al., 2000b; Frietze et al., 2008; Harrison et al., 2010; Majumder et al., 2006; Meyer et al., 2007; Qi et al., 2002; Wang et al., 2001b), only PRMT1 has been reported to methylate ER directly. This study highlights a broader importance of PRMT6 as a PRMT in regulating both the ligand-dependent and ligand-independent activities of SHRs.

3.5.1 PRMT6 as a novel regulator of ER ligand-independent activity

Similar to the study by Harrison *et al* which showed that PRMT6 upregulates ligand-dependent activity of ER, PR and GR, I observed the similar enhancement effect for ER, PR, GR and AR (Harrison et al., 2010). In addition, ligand-independent activity of ER was highly upregulated by PRMT6 and ligand-independent activity of PRALBD was

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downregulated by PRMT6. In the study performed by Harrison *et al*, the ligand independent activity of ER α was very low as compared to what have been reported in many studies (Tremblay *et al.*, 1998; Webb *et al.*, 1998). This absence of ligand-independent activity in their assay, possibly due to the medium condition and serum concentration that they used, may be the reason they were not able to detect the effect of PRMT6 on ER ligand-independent activity. It has been proposed by their study that PRMT6 acts as a coactivator for the hormone induced transcriptional activity of SHRs. Since there was no direct interaction between PRMT6 and GR and no hormone induction was required for PRMT6 to bind to ER, PR and AR, most probably this coactivation of ligand-induced activity involved methylation of coregulators by PRMT6. PRMT6 have been found to interact with coactivator SRC-1, hence it may enhance SHR activity through methylation of coregulatory proteins such as SRC-1 (Harrison *et al.*, 2010).

Activation of ER in the absence of hormone, possibly by growth stimulating factors, mainly involves the action on AF1. AF1 located in the N-terminal VR domain is an important regulator of both ligand-independent and ligand-dependent SHR activity. Unlike AF2 whose activation relies on ligand binding, AF1 is constitutively active and it can be activated further by peptide growth factors and other cell signaling molecules such as cAMP (Bunone *et al.*, 1996; Kumar and Litwack, 2009; McEwan, 2004; Takimoto *et al.*, 2003). Full ligand induced activity of SHR also requires the synergy between AF1 and AF2 (Benecke *et al.*, 2000; Ikonen *et al.*, 1997; Kraus *et al.*, 1995; Mostaqul Huq *et al.*, 2006; Tetel *et al.*, 1999). AF1 exerts its function by recruiting coregulators just like AF2, but they often recognize different domains on the coregulators (Bevan *et al.*, 1999;

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Christiaens et al., 2002; Khan et al., 2011; Lavery and McEwan, 2005; Onate et al., 1998; Webb et al., 1998). AF1 activity is also closely regulated by PTMs. There are six known phosphorylation sites found on ER AF1 (S102, S104, S106, S118, S154 and S167), they enable the selective activation of ER target genes in a promoter cell context specific manner and they also regulate ER stability (Arnold et al., 1994; Atsriku et al., 2009; Britton et al., 2008; Campbell et al., 2001; Castano et al., 1997; Chen et al., 2000c; Duplessis et al., 2011; Joel et al., 1998; Medunjanin et al., 2005; Park et al., 2011; Rogatsky et al., 1999; Sheeler et al., 2003; Thomas et al., 2008).

As PRMT6 can bind to ER without hormone stimulation and its methyltransferase activity is not needed to enhance ER ligand-independent activity, it probably regulates the ligand-independent AF1 activity of ER via physical interacting with ER and modulating the ER binding to coregulators. The weakening of interaction between ER and PRMT6 suggests that either the binding is only important for the regulation of ligand-independent ER activity or the interaction is transient with ligand-bound ER. Additional proofs were provided by the observation that PRMT6 binds to non-hsp70/hsp90 bound ER, which are well-known components of chaperone complex associated with inactive SHRs (Pratt, 1998; Pratt and Toft, 1997a, 2003a). It is very likely that PRMT6 caused the dissociation of chaperones from ER and facilitated coactivator binding. This is further supported by the finding that PRMT6 binds to C-terminus LBD-F domains of ER, the same region responsible for hsp90 and ER interaction (Aumais et al., 1997; Chambraud et al., 1990). Possible competitive binding at ER LBD results in the release of ER from chaperones and allows it to be activated in the absence of hormone.

3.5.2 Cross-talk between methylation by PRMT6 and ER phosphorylation

This study provides evidence that PRMT6 also plays an important role in ligand-independent ER signaling via cross-talk between arginine methylation and serine phosphorylation. R234 is identified as a possible target for PRMT6 methylation. R233 and R234 are next to the S236 phosphorylation site and form part of the PKA recognition motif R₂₃₃R₂₃₄KS₂₃₆ (R/KRXS/T). Phosphorylation of S236 by PKA activates ER activity in ligand-independent signaling (Chen et al., 1999b). When R233 was mutated for lysine and can no longer be methylated, PKA recognition was not affected and there was no significant change in ER transcriptional activity. This suggests that R233 may not be methylated or the methylation at this site was not critical for recognition. However, when R234 was mutated to lysine, ligand-independent activity of R234K mutant increased to six times of the ER wild type. In contrast, when R234 was changed to phenylalanine which is bulkier than arginine and mimics the methylated state of arginine, the ligand-independent activity was completely abolished. This suggests that the bulkiness at the R234 site may be unfavorable for PKA binding. Increase in bulkiness due to PRMT6 methylation inhibits PKA binding and S236 phosphorylation, hence blocking ER binding to DNA and transcriptional activation of target genes. As there was no obvious reduction in ER methylation level after R234 mutation, further experiment is needed to confirm if R234 is indeed methylation by PRMT6 and if it only occurs only under specific stimuli. Since PRMT6 showed overall enhancement on ligand-independent activity of ER, R234 methylation may not be the predominant mechanism for the regulation of ER activity by PRMT6. It is possible that it helps to fine-tune ER activity under specific stimuli or cellular conditions.

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3.5.3 Possible overlapping function between PRMT1 and PRMT6

R260 is the only arginine residue on ER that is reported to be methylated so far. PRMT1 methylation on ER Arg 260 activates ER cytoplasmic signaling by promoting ER binding to PI3K signaling molecules and activates Akt. It is a rapid and transient process. Upon E2 treatment, the level of ER methylated at Arg 260 increases within 5 mins after E2 but decreases by 15 mins and disappears after 24hrs (Le Romancer et al., 2008). In this study, we showed that R260 mutation to lysine reduced ER methylation signal by PRMT6, showing that R260 is also a target of PRMT6. PRMT6 may be involved in the regulation of ER cytoplasmic signaling. Since PRMT1 expression level is higher than PRMT6 in normal tissues, maybe it is the main regulator of this ER non-genomic signaling pathway. When PRMT6 level increases, such as in the case of breast cancer, it may contribute to the cytoplasmic activation of ER as well (Phalke et al., 2012).

Although PRMT6 mainly localizes in the nucleus, its involvement in the regulation of ER cytoplasmic signaling can be made possible by the nuclear export of R260 methylated ER. Arginine methylation regulated nuclear export has been reported for proteins such as receptor interacting protein 140 (RIP140) (Mostaqul Huq et al., 2006). RIP140 methylation by PRMT1 enhances its interaction with exportin 1 and hence exports RIP140 into the cytoplasm, suppresses the corepressor effect of RIP140 on nuclear receptors. After ER is methylated at R260 by PRMT6 in the nucleus, methylated ER may be exported in a similar way that allows it to interact with cytoplasmic factors.

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3.5.4 Ability of PRMT6 to methylate arginine residues outside the binding site

It is important to note that while PRMT6 binds to ER at its C-terminus, it has the ability to methylate N-terminus VR domain when they are put together, demonstrating that close proximity but not direct binding on the methylation site is needed for the transfer of methyl group to occur. Although PRMT6 could not bind to ER (VR), by tagging both with Flag sequence and pulled them down at the same time using Flag antibody conjugated beads, Flag-PRMT6 was positioned next to the Flag-ER (VR) and that made methylation of ER (VR) possible. In the cellular system, interaction between N- and C-terminal of receptors has long been proposed to be important for the synergistic effect of AF1 and AF2 for full activation of receptors (Benecke et al., 2000). The ligand induced interaction between the N- and C-terminal domains of PR has been demonstrated both *in vitro* and *in vivo* (Tetel et al., 1999). Coactivators such as SRC-1 can bind to both AF1 and AF2 and helps to enhance their interaction (Bevan et al., 1999; Onate et al., 1998). TIF2 can also interact with the AF1 and AF2 of ER or AR simultaneously using different sites to synergize their transcriptional activity (Benecke et al., 2000; Phalke et al., 2012). Hence, it is tempting to speculate that ER can be folded in a conformation that enables interaction of its N- and C-terminus, allows the PRMT6 sitting at the C-terminal domain to reach arginine residues at the VR and methylate it. This observation of PRMT's ability to methylate arginine without direct binding at that site has also been made for CARM1. CARM1 physically interacts with the p160 family of coactivators at their activation domain 2 (AD2), which is from amino acid 1097 to 1304 for the case of SRC-3 (Chen et al., 1999a). However, it has been found that CARM1 methylates SRC-3 at both arginine 839 and 1171, with R839 out of the AD2 (Feng et al., 2006; Naeem et al., 2007a). This

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further supports the presence of a mechanism for PRMT to interact with target protein at one site and methylates arginine at nearby position.

In addition, preliminary testing showed that PRMT6 did not methylate LBD-region F of ER. This region has been identified to be the site of interaction between PRMT6 and ER, absence of methylation at this region further supports that PRMT6 does not necessary methylate within its binding site, it has the ability to methylate arginine closed to it in conformation. It would also be interesting to look if PRMT6 methylates C-terminal residues in the presence of hormone. It may be possible that even though PRMT6 binds to LBD-region F in the absence of hormone, hormone induced conformational change for AF2 is needed to enable the methylation to occur.

3.5.5 Clinical relevance of ER methylation by PRMT6 in breast cancer

About 70% of breast cancers are ER positive and dependent on estrogen for their growth (Ali and Coombes, 2002). Treatment of breast cancer involves either direct inhibition of ER using antagonists such as tamoxifen or inhibition of estrogen production by targeting aromatase (Johnston and Dowsett, 2003; MacGregor and Jordan, 1998). However, efficacy of endocrine treatment is often limited by intrinsic or acquired therapeutic resistance (Musgrove and Sutherland, 2009). Correlation between ER modifications with prediction of treatment outcomes have been demonstrated by several studies. High phosphorylation of ER S167 in breast tumors may be indicative of functional ER and it associates with better outcome with endocrine treatments and longer survival in these studies (Generali et al., 2009; Jiang et al., 2007; Murphy et al., 2004; Skliris et al., 2010; Yamashita et al., 2005; Yamashita et al., 2008). But functional studies for ER

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phosphorylation carried out under *in vitro* conditions observed that S167 phosphorylation in ER promotes breast cancer cell proliferation and may associate with increased tamoxifen resistance (Guo et al., 2009; Maruani et al., 2012; Sun et al., 2001; Yamnik et al., 2009; Yamnik and Holz, 2010). In addition, detection of S305 phosphorylated ER is also predictive of tamoxifen resistance in breast cancer (Holm et al., 2009; Michalides et al., 2004). ER with A908G point mutation which results in K303R amino acid mutation and inhibition of acetylation is detected in 5.7% of invasive breast tumor in the Carolina breast cancer study (Conway et al., 2005). Studies using antibody targeting ER methylated at R260 showed that about 55% of the ER-positive breast tumors express this form of methylated ER, suggesting the possibility that ER is hypermethylated in breast cancer (Le Romancer et al., 2008).

Knowing that PTMs present on ER can affect ER activity greatly, detection of new modifications and a better understanding of their functions will improve the prognosis and development of new treatments for ER-positive breast cancers. It has been reported that PRMT6 expression is generally low in normal tissue but overexpressed in a significant number of cancer tissues including breast tumors. Serum level of asymmetrical demethylarginine is also elevated in breast cancer patients, which may be partly contributed by higher PRMT6 activity in cancer (Yoshimatsu et al., 2010). Ligand-independent activation of ER is generally caused by cross-talk with growth factor signaling and it cannot be inhibited by tamoxifen treatment which targeted E2 induced ER activation specifically (Arpino et al., 2008; Fagan and Yee, 2008; Gaben et al., 2012). It has been reported that PRMT6 knock down is associated with gene expression signature

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for better overall relapse-free and distant metastasis-free survival in the ER+ breast cancer subgroup (Dowhan et al., 2012). Meaning that overexpression of PRMT6 is associated with worse prognosis specifically for ER+ breast cancers. This study identified PRMT6 as a new enhancer of ER ligand-independent activity. We suspect that the increase aggressiveness of breast tumor with PRMT6 overexpression is caused by the enhancement of ligand-independent ER activation by PRMT6, which promotes hormone-independent tumor growth and this pathway is resistant to tamoxifen treatment. The study provides new insight into the regulation of ER activity by PRMT and demonstrates that PRMT6 is an important factor to consider in the choice of treatments and the prediction of treatment outcomes. As PRMT6 expression is low in all normal tissues except testis, the higher level of PRMT6 in breast tumors may be a good target for molecular therapy and specific inhibitor for PRMT6 may be effective in breast cancer treatment (Yoshimatsu et al., 2010).

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CHAPTER 4

REGULATION OF TRIM22 EXPRESSION IN BREAST CANCER

4.1 Generation of TRIM22 monoclonal antibody

4.1.1 Immunization of Balb/c mice and hybridoma clone screening

To detect the endogenous TRIM22 protein, we need to generate our own TRIM22 antibody as there was no good quality commercial TRIM22 antibody available. His-tagged full length TRIM22 protein (purified by Gayathri Sivaramakrishnan) was used to immunize four male *Balb/c* mice, mice tail blood (100 μ l) was collected after each booster to test the level and specificity of the response. Three out of four mice responded after the third booster and mouse 3 was identified to produce antibody specific to TRIM22 with the least background. It was chosen to proceed with monoclonal antibody production (Figure 4.1A). The mouse 3 was dissected in the tissue culture hood and the splenocytes isolated were used for fusion with myeloma cells to form antibody-secreting hybridoma cells (with the help of Dr. Klaus Kajanlinen). These hybridoma cells were then plated into 96-well plates for culturing and screening.

Initial screening was carried out using ELISA with either TRIM22-(His)₆ purified protein or TRIM22 unique peptide (KISSLNKRKSSGFAFDPSVNYSKV). Conditioned media which gave positive result in this assay were further verified by Western blot analysis using MCF10A cell lysate collected with and without TRIM22 knock down. As each well contains multiple hybridoma clones in the first plating, serial dilution was carried out for the cells from the well we picked and plated into 96-well again to achieve one cell per well. These clones were further screened using Western blot analysis. Hybridoma clone 8 that recognize TRIM22 well were identified. Western blotting result for the different

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dilutions of conditioned medium from clone 8 is shown in Figure 4.1B. They all gave a specific TRIM22 band near 58 kDa.

Figure 4.1 Generation of TRIM22 monoclonal antibody.

(A) Bleed testing after third booster. Tail blood was collected from four mice after the third booster and spun down at 1,500 rpm to obtain the antiserum. Specificity of the antibody was tested in Western blotting using lysates from (1) MCF10A cells transfected with control siRNA or (2) TRIM22 siRNA to knock down TRIM22 expression, (3) MCF7 treated with IFN- β which stimulate TRIM22 expression. High band intensity in 1 & 3, low band intensity in 2 indicates specific recognition of TRIM22. (B) Conditioned medium from antibody-producing hybridoma clone 8 was tested in Western blotting using different dilutions and compared against the result obtained by probing with antiserum control (mouse 3). Lysates from MCF10A cells transfected with control siRNA and TRIM22 siRNA were used. 1:30 dilution of the conditioned medium produced the similar result as the antiserum control.

4.1.2 Validate specificity of TRIM22 antibody by immunoprecipitation and mass spectrometry

The binding affinity of Clone 8 antibody was further tested by immunoprecipitation of TRIM22 protein from control siRNA or TRIM22 siRNA transfected MCF10A lysates under non-denaturing condition. Without knock down, a significant amount of TRIM22 protein was pulled down using Clone 8 conditioned medium (Mab8) which is 6.6 times of the 10% input control. There was less pulled down with knock down, which was 8 times of the 10% input control, indicating that this antibody was capable of concentrating TRIM22 protein from the lysate. Clone 57 which did not detect TRIM22 in Western blot was used as a negative control. As expected, Clone 57 conditioned medium did not contain antibodies and therefore failed to pull down any proteins (Figure 4.2A).

The ID of the protein pulled down by monoclonal antibody Mab8 was also investigated by LC-MS. Immunoprecipitation product was separated using SDS-PAGE and the band at the predicted molecular weight of TRIM22 was cut out and trypsin in-gel digestion of protein was carried out. The digested peptides were eluted out and sent for analysis (by Dr. Newman Sze). As shown in figure 4.2B, TRIM22 band appeared just above 50kDa ladder mark after coomassie blue staining, a non-specific protein pulled down by the beads also showed up near 75 kDa.

The identity of TRIM22 immunoprecipitated by mAb-C8 was verified with LC-MS, with a high log (e) score of -133.2 (Table 4.1). It ranked third in the list of proteins identified, just after bovine albumin and keratin which are very common contaminants during sample

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processing. The sequence coverage was calculated to be 25.7% (Figure 4.1C). In addition, no post-translational modifications such as phosphorylation or methylation were found in the sequence detected, possibly due to the low abundance of the protein, and the amount pulled down was not sufficient for detection of modifications.

Log (e)	Accession No.	Protein Name	Protein MW
-322.7	sp ALBU_BOVIN	Bovine albumin	69.2
-136.3	ENSP00000252244	Keratin 1	66
-133.2	ENSP00000369299	TRIM22	56.2

Table 4.1 List of proteins detected by LC-MS analysis

Top three ranking proteins identified by LC-MS are listed in the table. Highly negative log(e) value for TRIM22 reflects high confidence for the detection in MS.

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4.2 Expression pattern of TRIM22 in cell lines and breast cancer tissue and its association with the development of breast cancer

To elucidate the possible association of TRIM22 with breast cancer development, I examined the TRIM22 protein expression level in both breast cancer cell lines and patients' breast cancer tissue samples using the TRIM22 antibody I generated.

4.2.1 TRIM22 expression is suppressed in breast cancer cell compared to non-malignant mammary epithelial cells

Western blotting analysis performed on a panel of mammary epithelial cell lines (3 non-malignant mammary epithelial cell lines and 11 breast cancer cell lines) revealed that TRIM22 expression is high in non-malignant mammary epithelial cells but hugely downregulated in malignant breast cancer cell lines (Figure 4.3A). Its protein level was hardly detectable in breast cancer cells. Comparison of the TRIM22 transcript levels in non-malignant mammary epithelial cell lines MCF10A and MCF-12A with breast cancer cell line MCF7 also revealed that TRIM22 mRNA level is 240 and 677 times higher in MCF10A and MCF-12A respectively than that in MCF7 cells (Figure 4.3B).

4.2.2 Higher TRIM22 protein level detected in adjacent normal breast tissue as compared to breast cancer tissue

Since TRIM22 level was lower in breast cancer cell lines, we seek to examine if TRIM22 was similarly downregulated in the breast cancer tissues. Breast tumor biopsies were obtained from National Cancer Center (Singapore). Whole protein lysates were isolated from 63 pairs of breast tumors and the adjacent normal breast tissues and the protein expressions were analyzed by Western blotting. All SDS-PAGE gels were run under the same condition and the membranes were arranged together for antibody incubation and film exposure at the same time in order to ensure consistency in handling. 20 μ g of total cell lysate from TRIM22 transfected MCF7 cells was used as the positive control and β -actin which expressed at normal level in epithelial cells but low in adipose tissue was probed as the loading control. Result for 7 pairs of the tissues is shown in Figure 4.4A. Although 20 μ g of total tissue lysates were used for both normal and tumor tissue, the band intensities of β -actin were consistently lower in the normal adjacent tissue than in the tumor tissue. Using the MCF7-TRIM22 β -actin level as standard for comparison, β -actin for the tumor tissue was similar to or slightly lower than the β -actin band for MCF7-TRIM22 but the β -actin for the normal tissue was much lower than the MCF7-TRIM22 band. This was likely caused by the presence of less epithelial cells and more fat cells in the normal adjacent tissue, proteins from the adipose tissue also contributed to the protein quantitation. The adjacent control breast tissues contain much higher amount of adipose tissues than the breast tumor biopsy. TRIM22 protein expression was not detectable in these adipose tissues but their protein contents contribute to total protein concentration during quantitation. The β -actin expression which is very low in adipose tissue but much

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more consistent in other cell types allows us compare the TRIM22 level expressed in non-adipose cells. Hence, even though TRIM22 band intensity appeared lower for the normal tissue, it was caused by the lower amount of epithelial cells in the normal tissue used and it was reflected well by the β -actin level. In the analysis, band intensities for the immunoblots were quantified by densitometry and relative TRIM22 protein expression in epithelial cells was obtained by normalization with the level of β -actin followed by normalization with MCF7-TRIM22 β -actin level to eliminate variations between different immunoblots (Figure 4.4B). Comparison of the relative TRIM22 level showed that for these 63 pairs of tissue, 57 of them have higher TRIM22 protein level in the adjacent normal tissue ($R > 1$, $R = N/T$, Figure 4.4C), 46 of them have at least double in protein level for normal tissue than in tumor tissue ($R > 2$, Figure 4.4C). Paired t-test statistical analysis shows that this difference in TRIM22 level is statistically significant ($p < 0.00001$), TRIM22 protein level was consistently downregulated in the breast cancer tissues tested.

4.2.3 TRIM22 protein level correlates positively with p53 protein level in adjacent normal tissue

As TRIM22 is a downstream target of p53 (Obad et al., 2004), the correlation between TRIM22 protein expression and p53 protein expression in the patient tissues were investigated. Immunoblot for p53 level in 7 pairs of the tissues is shown in Figure 4.4A. Relative p53 protein level after normalization with β -actin is shown in Figure 4.5A. The analysis showed that out of 63 pairs of tissue, 51 of them have higher p53 protein levels in tumor tissue than adjacent normal (Figure 4.5A) and the difference in p53 protein level between breast tumor tissue and the adjacent normal tissues was statistically significant ($p < 0.00001$).

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After Log base 10 transformation of the relative expression of TRIM22 and p53, when $\text{Lg}(\text{TRIM22 expression})$ was plotted against $\text{Lg}(\text{p53 expression})$, we found that TRIM22 expression is positively correlated (Pearson's correlation $R=0.64$, $p<0.001$) with the p53 protein expression in normal tissue (Figure 4.5B). However, this correlation coefficient dropped to 0.42 ($p<0.001$) in breast tumor tissues (Figure 4.5C).

Figure 4.4 TRIM22 protein level in 63 pairs of breast cancer patient tissues.

(A) Western blotting analysis of protein lysates collected from breast tumor tissue and adjacent normal tissue. Blots for patient 1 to 7 are shown here. Samples were analyzed using antibody specific to TRIM22 and p53. MCF7 cell was transfected with TRIM22 and 20 μg of the total lysate was loaded as a positive control. β -actin was used as the loading control. (B) TRIM22 band intensity was quantified and normalized with β -actin. In total,

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63 patient tissue pairs are presented. The higher TRIM22 protein expression observed in the adjacent normal breast tissue than in the tumor tissue is significant ($P < 0.00001$) via the paired t-test. (C) Relative ratio of TRIM22 protein level in adjacent normal tissue (N) vs. tumor tissue (T) shows that out of 63 patients, 57 of them have higher TRIM22 expression in normal tissue than in tumor tissue.

Figure 4.5 Correlation between TRIM22 and p53 protein level in patient tissues.

(A) p53 band intensity from Western blotting was quantified and normalized with β -actin. In total, 63 patient tissue pairs are presented. (B) The Log base 10 of the relative expression of TRIM22 is positively correlated with the Log base 10 of the relative expression of p53 is significant with correlation coefficient (R) of 0.64 with $p < 0.001$. (C) Weak correlation was found between the Log base 10 relative expression of TRIM22 and p53 in the tumor tissues ($R=0.42$) with $p < 0.001$.

*TRIM22 and breast cancer***4.3 Mechanisms regulating TRIM22 expression in breast cancer cells**

Having shown that TRIM22 expression was consistently lower in breast cancer tissues, possible mechanisms responsible for downregulating TRIM22 expression in breast cancer were examined.

4.3.1 TRIM22 level in MCF7 can be upregulated by DMNT inhibitor but not HDAC inhibitors

Both DNA hypermethylation and histone deacetylation are common causes of gene expression downregulation during tumor development. When MCF7 cells were treated with HDAC inhibitor trichostatin A (TSA), no change in TRIM22 protein level was observed (Figure 4.6A). However, when cells were treated with DNA methyltransferase (DMNT) inhibitor 5-aza-2'-deoxycytidine (5-aza-dC), there was drastic increase in both TRIM22 protein and mRNA level. Increase in TRIM22 protein level was detectable at 48 hrs post-treatment, with further increase at 72 hrs (Figure 4.6B). TRIM22 mRNA level also responded in a time-dependent manner, reached about 300 folds by 120 hrs (Figure 4.6C). This increase in TRIM22 mRNA level was not inhibited in the presence of CHX, demonstrated that *de novo* protein synthesis was not required for the induction by 5-aza-dC (Figure 4.6D). A similar effect was observed for T47D and HeLa cell. After treating with 5-aza-dC for 120 hrs, TRIM22 mRNA level in T47D and HeLa cells increased by 11 and 4 fold respectively. However, no increase in TRIM22 level could be detected in MDA-MB-231 cells after treatment. To our surprise, MCF10A and MCF-12A cells which already express high level of TRIM22 also responded to the treatment. TRIM22 mRNA level in MCF10A and MCF-12A increased by 6 and 12 fold after 5-aza-dC treatment (Figure 4.6E).

4.3.2 Bisulfite sequencing for putative CpG islands in TRIM22 promoter region

To study the methylation pattern in TRIM22 promoter, bisulfite genomic sequencing was used to elucidate the methylation status of individual cytosine in putative CpG dinucleotides in 10 kb genomic sequence upstream of TRIM22 transcription start site, which is likely to contain the TRIM22 promoter region. EMBOSS CpGPlot software was used to predict the CpG islands in this 10 kb region. 3 CpG islands were identified and named as such: CpG-1 (the region located furthest from the transcription start site); CpG-2 (the region located second furthest from the transcription start site); and CpG-3 (the region located nearest to the transcription start site) (Figure 4.7A). MCF7 and MCF10A cells which express different levels of TRIM22 were used in bisulfite sequencing to analyze if there is any difference in their methylation status corresponding to the different expression level.

Genomic DNA from MCF7 and MCF10A cells were treated with sodium bisulfite for 14 hrs. The region containing putative CpG islands were then amplified using primers specific for bisulfite converted genomic DNA. PCR products were cloned into TOPO vector for sequencing. At least 3 clones were sequenced for each CpG island. The result for sequences which could align with genomic sequence is shown in Figure 4.7B. After the analysis of the three CpG islands, CpG island two is most heavily methylated. All twelve cytosine residues were methylated. CpG island one had about 50% cytosine methylated whereas CpG island three only had two cytosines that were consistently methylated. No obvious difference in the extent of DNA methylation was detected in this region between MCF7 and MCF10A cells.

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Figure 4.6 Upregulation of TRIM22 expression in breast cancer cells by DNA methyltransferase inhibitor 5-aza-dC.

(A) No upregulation in TRIM22 level was observed after TSA treatment. MCF7 cells were treated with two different doses of TSA for 12 and 24 hrs, cell lysate were collected for Western Blotting analysis. (B,C) TRIM22 protein and transcript level were up-regulated by 5-aza-dC in a time-dependent manner in MCF7 cells. MCF7 cells were treated with 1 μ M 5-aza-dC and collected at 48 to 120 hrs. Increase in TRIM22 level after treatment was shown by Western blotting and real-time PCR. Real-time PCR analysis was carried out using triplicates. Error bar represents standard error of mean. (D) TRIM22 was induced by 5-aza-dC without de novo protein synthesis. MCF7 cells were treated with 5-aza-dC for 120 hrs, protein synthesis inhibitor CHX was added for the last 72 hrs. Presence of CHX was unable to block the increase in TRIM22 mRNA level. Real-time PCR analysis was carried out using triplicates. Error bar represents standard error of mean.

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(E) MDA-MB-231, T47D, MCF10A, MCF-12A and HeLa cells were all treated with 5-aza-dC for 120 hrs and TRIM22 level were analyzed by real-time PCR. Increase in TRIM22 transcript level was observed for all cell lines except MDA-MB-231. Real-time PCR analysis was carried out using triplicates. Error bar represents standard error of mean.

Figure 4.7 Methylation status of TRIM22 gene promoter in MCF7 and MCF10A cells.

(A) Predicted location of 3 CpG islands in the 10 kb genomic region upstream of TRIM22's transcription start site. The 3 CpG islands were predicted using the online software EMBOS CpGPlot. Nucleotide position of each predicted CpG islands relative to TRIM22's transcription start site is also indicated. (B) Methylation status of the three CpG islands in MCF7 and MCF10A. Bisulfite sequencing of each putative CpG island was done to determine the methylation status of each island in MCF7 and MCF10A cells. Each horizontal string represent methylation pattern of cytosine sequenced from one clone. Analysis of methylation status from sequencing results was performed using Quantification tool for Methylation Analysis (QUMA) (Kumaki et al., 2008).

*TRIM22 and breast cancer***4.4 Mechanism of TRIM22 regulation by interferon and p53**

Both IFN and p53 have been implicated to be important in cancer development and treatment. IFN can promote host immune response against tumor, exerts anti-proliferative action on tumors and have been widely used in cancer treatments (Belardelli et al., 2002; Bromberg et al., 1996; Dighe et al., 1994; Dunn et al., 2006; Gao et al., 2010b; Gresser, 2007; Gutterman, 1994; Kaplan et al., 1998; Park et al., 2006; Street et al., 2001). On the other hand, p53 is widely known as the guardian of the genome and controls cell fate in response to stress stimuli (Benchimol, 2001; Giono and Manfredi, 2006; Linke et al., 1996; Pellegata et al., 1996; Shen and White, 2001; Toillon et al., 2002; Zhang et al., 2010b). In addition, IFN has also been shown to boost p53 response in cancer cell and enhance tumor suppression (Cho and Pyo, 2010; Kim et al., 2009; Pestka, 2003; Takaoka et al., 2003; Yuan et al., 2007; Zhang and Sriram, 2009). Since TRIM22 level is downregulated in breast cancer and its expression can be induced by both IFN and p53 activation (Obad et al., 2004; Tissot and Mechti, 1995), I investigated the mechanism of TRIM22 induction by IFN and p53, and if TRIM22 contributes to their tumor suppressing function.

4.4.1 IFN induction of TRIM22 expression independent of protein synthesis and p53 status

To examine if TRIM22 is a direct target of IFN in breast cancer cells, T47D cells were treated with 500 international units (IU) of IFN- β or IFN- γ in the presence or absence of protein synthesis inhibitor CHX (10 μ g/ml). Real-time PCR analysis of TRIM22 transcript level showed that TRIM22 expression increased by 45 fold and 29 fold with IFN- β and IFN- γ treatments respectively. This increase was not inhibited by CHX

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treatment, suggesting *de novo* protein synthesis was not needed for the induction of TRIM22 expression by either IFN- β or IFN- γ in T47D cells (Figure 4.8A). TRIM22 protein level was not increased by IFN in the presence of CHX, showing that CHX was effective in inhibiting protein synthesis (Figure 4.8B).

Next, being a common protein regulated by both IFN and p53, I looked to see if there was any correlation between IFN induction of TRIM22 and p53 status in breast cancer cells and normal mammary epithelial cell line MCF-10A. MCF10A and MCF7 express wild type p53, whereas MDA-MB-231 and T47D express mutated p53. As shown in Figure 4.8C, IFN- β was able to upregulate TRIM22 protein level in all four cell lines, despite the absence of functional p53 in T47D and MDA-MB-231. More interestingly, TRIM22 was also induced by IFN in p53 null cell line H1299. Transfection of p53 into H1299 did not have a significant effect on the level of TRIM22 induced (Figure 4.8D). This implies that, although TRIM22 levels positively correlate with p53 expression normally as is shown in Figure 4.5B, TRIM22 responds to IFN independent of p53 status in the cells.

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Figure 4.8 IFNs upregulate TRIM22 expression in breast cancer cells independent of protein synthesis and p53 status.

(A) TRIM22 mRNA level can be upregulated by IFN in the presence of CHX. T47D cells were pre-treated with 10 μ g/ml of CHX for 1hr followed by IFN- β or IFN- γ treatment (500IU) with or without CHX for additional 16 hrs. RNA was collected and reverse transcribed. TRIM22 transcript level was analyzed by real-time PCR using specific primer. 36B4 was used as internal control. Real-time PCR analysis was carried out using triplicates. Error bar represents standard error of mean. 30-45 fold increase in TRIM22 mRNA level by IFN- β and IFN- γ can be detected both in the presence and in the absence of CHX. (B) Protein lysate was collected concurrently to show the inhibitory effect of CHX on the induction of TRIM22 protein level. (C) IFN- β upregulated TRIM22 protein level in all the mammary epithelial cell lines tested. MCF10A, MDA-MB-231, MCF7 and T47D cells were treated with IFN- β for 24 hrs and cell lysate was collected for Western blot analysis. GAPDH was used as loading control. (D) TRIM22 protein level was upregulated both the presence and absence of p53 by IFN- β . p53-null H1299 cells were transfected with empty vector control or p53 vector for 24hrs followed by IFN- β treatment for additional 24 hrs. Lysate was collected for Western blotting analysis.

4.4.2 Induction of TRIM22 by genotoxic drugs is cellular context-dependent

The p53 gene is often activated in response to stress, mediating cell cycle arrest or apoptosis of the cells. It achieves this by activating transcription of a specific set of downstream genes depending on the nature of the stimuli and the tissue/cell type. Many factors have been suggested to be involved in the control of target gene selection, including post-translational modifications of p53 and actions of cofactors. Phosphorylation of Serine 46 of p53 was associated with activation of p53-regulated Apoptosis-Inducing Protein 1 (p53AIP1) and acetylation of lysine 120 which recruits p53 to pro-apoptotic genes such as Puma and bax, thus promoting apoptosis (Oda et al., 2000; Sykes et al., 2006). On the other hand, cofactor hematopoietic zinc finger (Hzf) binds to the DNA binding domain of p53 and facilitates activation of pro-arrest genes, whereas expression of POU domain transcription factor brn-3b is associated with bax expression and apoptosis induction (Budhram-Mahadeo et al., 2006; Das et al., 2007).

It has been found that TRIM22 is a downstream target of p53 and it contains the p53 response element in the first intron (Obad et al., 2004). I was interested to see if TRIM22 was a direct target gene of p53 during DNA damage and if it act to promote cell cycle arrest or apoptosis. MCF10A and MDA-MB-231 cells were treated with various genotoxic drugs and TRIM22 protein level was analyzed using Western blotting. In MCF10A cells which encode wild type p53, DNA crosslinker mitomycin C (MC), topoisomerase inhibitors camptothecin (CPT) and etoposide (VP16) all caused DNA damage and stimulated both p53 and TRIM22 protein expression. Taxol which has been reported to cause both mitotic and G1 arrest also induced a minor increase in p53 and

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TRIM22. Nocodazole, which interferes with polymerization of microtubules and leads to apoptosis, did not upregulate p53 and TRIM22 protein level (Figure 4.9A). In MDA-MB-231, which carries a p53 point mutation, no significant change in TRIM22 level was detected in all treatments (Figure 4.9B).

At this point, TRIM22 induction appeared to be p53-dependent in the cell. However, additional testing with MCF7 cells showed that there were factors other than p53 needed for TRIM22 induction. Although MCF7 encodes wild type p53 and its p53 was responsive to the genotoxic drug treatments, no TRIM22 upregulation was detectable despite the obvious increase in p53 level with all the treatments (Figure 4.9C). p53-null cell line H1299 was used to check if re-expression of p53 alone was sufficient to induce TRIM22 during DNA damage. No increase in TRIM22 level was detected in H1299 after treating with mitomycin C and camptothecin (Figure 4.9D). Expression of exogenous p53 into H1299 cells was not able to upregulate TRIM22 expression in response to DNA damaging drugs (Figure 4.9E). p53 transfected into H1299 could be stabilized in response to CPT treatment as shown by increased p53 protein, but TRIM22 induction was not detected. Furthermore, no increase in TRIM22 mRNA level was detected in H1299 cells transfected with p53 and treated with CPT (Figure 4.9F). Therefore, it appeared that activation of p53 was not sufficient to induce TRIM22 during stress response. Instead, the TRIM22 antibody detected an induced protein band that appeared smaller than the TRIM22, Judging by the TRIM22 protein band induced with IFN treatment (Figure 4.9E). Intriguingly, this smaller protein was increased with p53 overexpression in H1299 and

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further enhanced with CPT treatment. The identity of the p53-induced protein is not known but it is likely a TRIM family protein whose level is not affected by IFN treatment.

As the activation of p53 can result in either cell cycle arrest or apoptosis, we explored if TRIM22 is involved more in cell cycle arrest or apoptosis. Hydroxyurea (HU) is an inhibitor of ribonucleotide reductase. It induces cell cycle arrest at S phase by inhibiting DNA synthesis (Koc et al., 2004; Marusyk et al., 2007). Reactivation of p53 and induction of tumor cell apoptosis (RITA) is a small molecule that has been shown to selectively induce apoptosis in various cell lines in a p53 dependent manner. It blocks the binding of p53 to human double minute -2 (HDM-2) prevents the degradation of p53 and induces massive apoptosis without significant increase in cell cycle arrest (Issaeva et al., 2004). HU and RITA were used to selectively induce cell cycle arrest and apoptosis in MCF10A (Figure 4.10A). MCF10A cells were treated with various dosages of HU and RITA and the induction of TRIM22 was analyzed. A range of HU and RITA drug dosage was chosen based on publications and was tested to see the effect on TRIM22 induction. HU at 15 mM and RITA at 10 μ M were the highest concentrations to use that can induce TRIM22 expression well and yet were not too toxic to the cells which made 48 hrs of treatment possible. Western blotting analysis and mRNA analysis of treated samples showed that earlier and higher induction of TRIM22 was observed with HU treatment as compared to RITA, but the difference was marginal and not sufficient to conclude if TRIM22 induction occurs predominantly during cell cycle arrest (Figure 4.10B,C).

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Figure 4.9 TRIM22 expression upregulated by genotoxic drugs in MCF10A but not MDA-MB-231, MCF7 and H1299 cells.

(A) MCF10A cells and (B) MDA-MB-231 cells were treated with different dosages of taxol, mitomycin C (MC), nocodazole, camptothecin (CPT) and VP16 for 24 hrs. TRIM22 and p53 protein level was analyzed using Western Blotting. MCF10A expressing wild type p53 enabled the upregulation of TRIM22 by DNA damaging drugs mitomycin C, camptothecin and VP16. No obvious change in TRIM22 level was observed with taxol

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and nocodazole treatments which targets microtubules. No increase in TRIM22 was detected in all treatments in MDA-MB-231 which express mutant p53. (C) No TRIM22 induction by DNA damaging signal in MCF7. MCF7 cell were exposed to UV to short duration and lysates were collected 8 hrs later. Treatments with CPT and MC were carried out for 16 hrs and lysates were collected for Western blotting analysis. (D) p53 null cells H1299 were treated with MC and CPT for 24 hrs. No induction of TRIM22 was detected. (E) Exogenous p53 expression in H1299 upregulated the expression of a different form of TRIM22 and the expression level was further enhanced with CPT treatment. H1299 cells were transfected with pCMV vector control (mock) or p53 vector for 24 hrs, cell were treated with 0.1% DMSO or 1 μ g/ml CPT for additional 16 hrs before lysate collection. IFN treatment was performed to show the normal TRIM22 size. GAPDH was used as the loading control. (F) No TRIM22 mRNA increase observed in H1299 transfected with p53. H1299 cells were transfected with pCMV control or p53 vector for 24 hrs followed by 16 hrs CPT (1 μ g/ml) treatment. RNAs were collected for reverse transcription and real-time analysis with TRIM22 primer. 36B4 was used as the internal control.

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Figure 4.10 Higher TRIM22 induction in response to cell cycle arrest.

(A) HU induced growth arrest and RITA induced apoptosis in MCF10A. Live cell images (at 10X objective) were taken after treating with 5 mM HU or 1 μM RITA for 16 hrs. (B) Higher increase in TRIM22 protein level was detected with HU treatment than with RITA treatment. MCF10A cells were treated different dosage of HU and RITA for 24 and 48 hrs. TRIM22 and p53 protein level was analyzed by Western blotting. β-actin was used as loading control. Band intensities for TRIM22 had been quantified by densitometer and normalized with beta-actin band intensities and indicated below the Western blots. Relative TRIM22 expression was obtained by comparing with control treated sample in each case. Control treated sample was taken as 1 in each case. (C) Higher induction in TRIM22 mRNA expression with HU treatment than with RITA treatment. MCF10A cells were treated 15 mM HU or 10 μM RITA for 16 hrs. RNA was collected and reversed transcribed for real-time PCR analysis. TRIM22 mRNA fold induction was calculated using TRIM22 level for drug treated sample over TRIM22 level for control vehicle treated sample. 36B4 was used as internal control. Real-time PCR analysis was carried out using triplicates. Error bar represents standard error of mean.

4.5 Enhancement of PR signaling by TRIM22

SHR signaling has been found to play a major role in breast cancer development and treatment. TRIM22 was one of the most highly upregulated genes induced by progesterone treatment in our microarray analysis using T47D breast cancer cells or PR transfected MDA-MB-231 cells. The gene was also specifically down regulated in breast tumors and I was interested to investigate how TRIM22 may affect breast cancer progression by regulating PR signaling.

4.5.1 TRIM22 overexpression increased PR expression and PRE transcriptional activity in a dose-dependent manner

When PR was co-transfected with TRIM22, there was an enhancement of PR expression was detected with TRIM22 overexpression. For this study equal amount of PR expression vector was co-transfected with 10, 50 and 100 ng of pEF1-TRIM22 expression vector (amount balanced by empty vector control to keep the total amount of DNA used in the transfection equal). Western blotting analysis showed that protein level of PR increased with increasing amount of TRIM22 plasmid used (Figure 4.11A). Highest PR level was seen with 50 ng of TRIM22 vector transfected and no further increase was seen for the 100 ng of TRIM22 plasmid used; most probably due to saturation effect at 50 ng dose. This upregulation in PR expression was independent of R5020 treatment. Instead, PR level was slightly lower after treatment with TRIM22 coexpression, which might suggest faster turnover rate for PR in the presence of TRIM22. A similar result was obtained with Flag tagged TRIM22 expression vector, except the increase in PR protein level is higher with 10 ng of Flag-TRIM22 vector but decreased with 50ng of Flag-TRIM22 (Figure 4.11B). This was most probably because the protein expression of Flag-TRIM22 was

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higher under the control of human cytomegalovirus (CMV) promoter which is a stronger promoter than the elongation factor 1 (EF1) promoter present in the pEF1-TRIM22 vector.

The increase in PR expression level by TRIM22 lead to a corresponding dose dependent increase in progestin R5020 induced PRE transcriptional activity. The increase in PRE transcriptional activity by 2-5 ng of pEF1-TRIM22 was small but statistically significant (18-35%, $p < 0.01$) (Figure 4.11C). 50 ng of pEF1-TRIM22 expression vector, which had maximum effect on PR protein expression, increased the PRE transcriptional activity by 50%. Slight decrease in the fold induction was obtained with 100 ng of TRIM22 vector as compared to 50 ng, similar to what we observed for the PR protein level (Figure 4.11D). TRIM22 upregulates the expression and transcription activity of PR in a dose-dependent manner and it reached a plateau with higher amount of TRIM22 transfected.

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Figure 4.11 TRIM22 enhances PR expression and transcriptional activity in a dose dependent manner.

(A) TRIM22 overexpression increased PR protein level in HeLa cells. 100 ng of pEF1/myc-hisA empty vector control or 10, 50 and 100 ng of pEF1-TRIM22 plasmid were transfected into HeLa cells with or without PRB plasmid. Cells were treated with 10 nM of R5020 after 24 hrs transfection. Lysate were collected at 48 hrs post-transfection (with 24 hrs treatment) for Western blotting analysis. GAPDH was used as loading control. (B) Flag tagged TRIM22 caused similar increases in PR protein level. 50 ng of pxj-Flag empty vector control or 10, 50 ng of Flag-TRIM22 plasmid were transfected into HeLa with or without PRB plasmid. Cells were treated with 10 nM of R5020 after 24 hrs of transfection. Lysates were collected 48 hrs post-transfection (24 hrs treatment) for Western blotting analysis. GAPDH was used as loading control. (C,D) Dose-dependent increases in PR transcriptional activity by TRIM22. HeLa cells were co-transfected with PRE-luciferase and vectors for PR alone, or with 2 ng, 5 ng, 50 ng or 100 ng of pEF1-TRIM22. Cells were treated with 10 nM of R5020 for additional 24 hrs after transfected for 24 hrs. Lysates were collected for analysis with illuminometer. Fold induction by R5020 treatment were calculated by luciferase signal with R5020 treatment over EtOH control after normalizing with luciferase signal of empty vector control. For panel C and D, triplicates were used for all luciferase reporter assays and the error bar represents standard error of mean, the statistical test used for the calculation of p value was unpaired

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student's t test. Results shown are from a single experiment, which is representative of three independent experiments. ** $p < 0.01$.

4.5.2 TRIM22 enhances the transcriptional activity of PR independent of E3 ubiquitin ligase activity but intact RING domain is needed for this function

The function of TRIM22 protein has been frequently associated with its RING finger domain and its E3 ubiquitin ligase activity (Duan et al., 2008; Eldin et al., 2009; Gao et al., 2009; Kajaste-Rudnitski et al., 2011). Here the involvement of TRIM22 E3 ubiquitin ligase activity and RING domain in the regulation of PR expression level and activity was examined. Flag-TRIM22 (C15A/C18A) mutant which loses its E3 ubiquitin ligase activity and Flag-TRIM22 (Δ RING) truncation construct which has its RING domain removed were generated by Gayathri Sivaramakrishnan (Figure 4.12A). PR protein level and transcriptional activity were analyzed in the absence of Flag-TRIM22 plasmid, in the presence of Flag-TRIM22 wild type (wt) or mutant plasmid. Wild type TRIM22 doubled the PR transcriptional activity after R5020 treatment. Higher increase of PR activity was seen when E3 ubiquitin ligase deficient mutant TRIM22 C15A/C18A were tested, even though the protein expression of C15A/C18A mutant was much lower than the TRIM22 wild type. No significant change in PR activity was observed in the presence of TRIM22 Δ RING (Figure 4.12B). Corresponding increase in PR protein level was observed when co-transfected with Flag-TRIM22 wt and C15A/C18A mutant. No increase in PR level was observed when RING domain was absent (Figure 4.12C).

4.5.3 TRIM22 upregulates PR mRNA expression

To investigate if the increase in PR protein level in the presence of TRIM22 was a result of increase protein stability, time point treatment with protein synthesis inhibitor CHX was carried out. Although PR protein level was higher in the presence of TRIM22 at 0hr, the speed of PR degradation was higher with TRIM22 overexpression. A similar PR level was detected at 12hrs time point (Figure 4.13A). In addition, no direct interaction between PR and TRIM22 was detected using Co-IP, suggesting that the upregulation in PR protein level by TRIM22 was not through direct binding of TRIM22 to PR protein (Figure 4.13B). RNA was collected concurrently at a 0 hr time point to study the PR mRNA level. Real-time PCR analysis with PR primer showed that on average, mRNA level of PR was 1.43 folds in the presence of TRIM22 as compared to the mock transfected samples. The average increase in PR mRNA level was less than 1.5 folds but it was consistent in three independent experiments (Figure 4.13C). This increase in PR mRNA is similar in magnitude to the increase of PR protein levels so it is likely responsible for the higher levels of PR protein expression in response to TRIM22 co-transfection.

4.5.4 TRIM22 downregulates PR ligand-independent transcriptional activity

In addition to its effect on hormone induced transcriptional activity, TRIM22 also play a role in regulating the ligand-independent activity of PR. PR Δ LBD truncation construct can activate PRE constitutively. Presence of TRIM22 decreased the constitutive PR Δ LBD transcriptional activity by more than 80% without affecting the protein level of PR Δ LBD (Figure 4.14).

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Figure 4.12 E3 ubiquitin ligase activity is dispensable for TRIM22's effect on PR expression level and transcriptional activity.

(A) TRIM22 wt, C15A/C18A double mutant or RING truncation were cloned using pxj-Flag vector to generate Flag tagged TRIM22 expression vectors. (B) TRIM22 RING domain but not E3 ubiquitin ligase activity was needed for enhancement of PR transcriptional activity. HeLa cells were co-transfected with PRE-luciferase plasmid and 10ng expression vectors for PR with 10 ng of control vector or TRIM22 wt, C15A/C18A mutant or Δ RING truncation constructs. Cells were treated with 10 nM of R5020 for additional 24 hrs after transfected for 24 hrs. Fold induction by R5020 treatment were calculated by luciferase signal with R5020 treatment over EtOH control after normalizing with luciferase signal of empty vector control. Triplicates were used for all luciferase reporter assays and the error bar represents standard error of mean, the statistical test used for the calculation of p value was unpaired student's t test. Results shown are from a single experiment, which is representative of two independent experiments. * $p < 0.05$; **

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$p < 0.01$, NS: non-significant. (C) TRIM22 wt and C15A/C18A mutant upregulated PR expression level but not Δ RING truncation construct. Total lysates were collected concurrently with the luciferase assay for Western blotting analysis. GAPDH was used as loading control.

Figure 4.13 TRIM22 does not increase PR protein stability but increases PR mRNA expression.

(A) Faster PR degradation observed in the presence of TRIM22. HeLa cells were transfected with 50 ng of pEF1/myc-hisA empty vector (mock) or pEF1-TRIM22 plasmid and 5 ng of PR expression vectors for 24 hrs in a master dish and split into 35 mm dishes for protein and RNA collection. This ensured equal transfection efficiency in all the samples for better comparison. Cells were treated with 20 μ g/ml of CHX after overnight attachment and PR expression level were analyzed by Western blotting. Band intensity for PR protein was measured by densitometry and normalized with GAPDH band intensity. Line plot for the PR normalized band intensity showed that PR protein degraded faster with TRIM22 overexpressed. (B) pcDNA3.1 or PR vector were co-transfected with TRIM22 vector into COS7 cells for 24 hrs, followed by 6 hrs of treatment with EtOH

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(0.1%) or R5020 (10 nM) before lysate collection. Lysates were incubated overnight with 1 μ g PR antibody and conjugated with protein A/G agarose beads for another 3 hrs. Samples were analyzed by Western blotting. As shown in the input control (Lane 1 to 4), TRIM22 and PR were expressed well according to transfection. However, PR antibody only pulled down PR protein well (Lane 7 & 8), but no specific co-IP of TRIM22 protein could be seen. (C) TRIM22 overexpression upregulated PR mRNA level. Total RNA for untreated samples was collected concurrently with the CHX experiment to study the PR transcript level. 1.37 to 1.55 folds increase in PR mRNA level was detected in three independent experiments. Real-time PCR analysis was carried out using triplicates. Error bar represents standard error of mean.

Figure 4.14 TRIM22 inhibits PR ligand-independent transcriptional activity.

(A) TRIM22 downregulated constitutive transcriptional activity by PRΔLBD. 10 ng of flag-TRIM22 plasmid or vector control were co-transfected with equal amount of PRΔLBD plasmid and PRE-luciferase for 48 hrs without hormone treatment. Lysates were collected for analysis with illuminometer. Relative luciferase activity was calculated by PRΔLBD luciferase signal over pcDNA3.1 vector control luciferase signal. Triplicates were used for the luciferase reporter assays and the error bar represents standard error of mean, the statistical test used for the calculation of p value was unpaired student's t test. Results shown are from a single experiment, which is representative of two independent experiments. ** $p < 0.01$. (B) Total lysates were collected concurrently with the luciferase assay for Western blotting analysis. No significant change in PRΔLBD protein level was seen with and without TRIM22 overexpression. GAPDH was used as loading control.

4.6 Nuclear localization of TRIM22 protein

After characterizing the regulation of TRIM22 in breast cancer, the subcellular localization of TRIM22 protein was investigated by both fluorescence microscopy and cellular fractionation to identify its site of action. Transfection of TRIM22 with GFP tag into MCF7 cells allowed direct viewing of TRIM22 localization. As shown in Figure 4.15A, GFP-TRIM22 localized mainly in the nucleus as distinct nuclear bodies. Cell fractionation for IFN- β and mitomycin C treated MCF10A cell lysate showed that in the control, TRIM22 protein localized mainly in the nucleus, only very low level was detected in the cytoplasm. Upon treatment, there was a minute increase in the cytoplasmic fraction but massive increase in TRIM22 protein level in the nuclear fraction (Figure 4.15B, C).

Figure 4.15 TRIM22 localizes predominantly in the nucleus.

(A) GFP-TRIM22 fusion protein localized in the nucleus. MCF7 cells transfected with GFP-TRIM22 construct were fixed and stained with DAPI to mark the position of nucleus. GFP-TRIM22 proteins were seen as nuclear bodies under microscope (under 20X objective). Cytoplasmic and nuclear lysate were isolated from IFN- β (B) and Mitomycin C (C) treated MCF10A cells, changes in endogenous TRIM22 protein level in each fraction were analyzed using western blotting. GAPDH was used as cytoplasmic marker. (C: cytoplasmic fraction; N: nuclear fraction)

4.7 Discussion

TRIM22 is known for its antiviral activity and its association with virus-induced tumorigenesis, but not much is known about its role in breast cancer (Barr et al., 2008; Bouazzaoui et al., 2006; Singh et al., 2011; Wittmann et al., 2008; Zhang et al., 2004). In this study, I analyzed the change in expression pattern of TRIM22 during breast cancer development by comparing its level in breast tumor tissue and adjacent normal breast tissue. I also characterized the induction of TRIM22 by IFN and p53, presenting evidence suggesting TRIM22's regulatory role in PR signaling. Abnormality in expression of many TRIM family proteins has been found to associate with various types of cancers, but none has yet been reported for TRIM22 (Hatakeyama, 2011). With the TRIM22 antibody generated, I showed that the endogenous TRIM22 protein expression was consistently downregulated in both breast cancer cell lines and tumor tissues, making it a possible candidate as a prognostic marker in breast cancer. Loss of heterozygosity (LOH) and hypermethylation are common causes for gene inactivation in cancer and may also be responsible for this decrease in TRIM22 expression.

4.7.1 LOH and hypermethylation may be involved in TRIM22 silencing

LOH is associated with many types of cancers such as breast cancer and gliomas (Boulay et al., 2009; Karnik et al., 1998; Lam et al., 2002; Schiebe et al., 2001; Zikan et al., 2011). It is a somatic event where the loss of one allele happens after the other allele of that gene has already been inactivated in the germline and it happens at a higher frequency to imprinted genes (Anderson et al., 1999; Sarquis et al., 2006). It is often observed in tumor suppressor gene loci, inactivating genes such as cancer type I susceptibility protein

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(BRCA1) and retinoblastoma (Rb) and leads to tumor progression (Gras et al., 2001; Okada et al., 2012).

TRIM22 is located in chromosome 11p15, a region which has been extensively studied for its high incidence of LOH and its association with cancer development. Analyses of gastric carcinoma, lung cancer and esophageal squamous cell carcinomas showed that 40-62% of them have LOH at 11p15 (Baffa et al., 1996; Lam et al., 2002; O'Briant and Bepler, 1997). In the case of breast cancer, 43 cases of 11p15 LOH were detected when 115 ductal carcinomas of the breast were analyzed in one study (Lichy et al., 1998). Comparison of 94 matched normal and breast tumor samples in another study showed that 11p15 LOH was found in 35-45% of them using 17 marker and LOH in 11p15.5-p15.4 was associated with metastatic breast cancer (Karnik et al., 1998). TRIM3 is one example of genes that resides in 11p15 and gets deleted during tumor progression. TRIM3 locus at 11p15.5 is deleted in 24% of 70 malignant gliomas analyzed due to LOH (Boulay et al., 2009). The possible connection between lower TRIM22 expression and LOH at 11p15 can be made based on studies done on Wilms tumor. 11p15 LOH is the most genetic abnormality found in Wilms tumors and it associates with relapse in very low-risk Wilms tumors (VLRWT) (Coppes et al., 1992; Perlman et al., 2011; Yuan et al., 2005). Consistent with this, it is also reported that TRIM22 expression is higher in relapse-free Wilms tumor than in relapsed Wilms tumor and lower TRIM22 expression correlates with Wilms tumor with fatal outcome (Wittmann et al., 2008; Zirn et al., 2006). This suggests that TRIM22 expression may be lost in some cases due to 11p15 LOH and give rise to the similar correlation with tumor condition.

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Besides LOH, promoter hypermethylation is also a possible factor contributing to downregulation of TRIM22 expression in breast cancer. Chromosome 11p15 contains a cluster of imprinted genes including paternally expressed genes (IGF-II and KCNQ1 opposite strand/antisense transcript 1 (KCNQ1OT1)) and maternally expressed genes (cyclin-dependent kinase inhibitor 1C (CDKN1C) and H19, a non-coding RNA) (Reik and Walter, 2001). Expression of those genes are under the control of imprinting centers, which are cis-acting sequences carrying parent-specific epigenetic marks (Soejima and Wagstaff, 2005). DNA methylation or H3K9 dimethylation for histone bound to the imprinting center can result in the suppression of gene expression and it has been found to associate with pathological conditions such as Beckwith–Wiedemann syndrome (Chung et al., 1996; Gaston et al., 2001; Schwienbacher et al., 2000). Loss of imprinting (LOI) of IGF-II (23.5%) and H19 (6.7%) genes was also detected in ovarian cancer (Chen et al., 2000a).

Although TRIM22 is not imprinted, it is located in this cluster of imprinted genes in 11p15 and allelic variation in its gene expression has also been demonstrated. It has been found single-nucleotide polymorphism (SNP) can directly affect DNA methylation as part of CpG dinucleotide or indirectly affect local methylation pattern and contribute to monoallelic expression (Hellman and Chess, 2010). In a human transcribed SNPs array using kidney and liver cDNA collected from seven individuals, TRIM22 was found to be heterozygous for 3 individuals in kidney and 4 in liver. Monoallelic expression of TRIM22 for the heterozygous individual was observed, with only one allele preferentially expressed, more than four times the frequency of the other allele (Lo et al., 2003). In this

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study, DNMT inhibitor 5-aza-dC was able to increase TRIM22 expression in MCF10A, MCF-12A, MCF7 and T47D but not MDA-MB-231 cells, suggesting that DNA methylation in TRIM22 promoter is one of the mechanisms regulating TRIM22 expression in both non-malignant and malignant mammary epithelial cells. This is further supported by the detection of high level of methylated cytosines in two putative CpG islands located in the 10kb region upstream of TRIM22 transcriptional start site for both MCF10A and MCF7 cell lines, demonstrating that TRIM22 expression is tightly controlled by DNA methylation. Global increase in CpG methylation in promoters of cancer cells are observed in many neoplasias, indicating that DNA methylation is a common mechanism downregulating gene expression in cancer (Ruike et al., 2010; Smiraglia et al., 2001; Szyf, 2012). Mutation in the TRIM22 regulatory sequence may contribute to change in epigenetic marks and differential expression of TRIM22 in cancer. In addition, the level of TRIM22 expression upregulated by 5-aza-dC treatment was different for each cell line, it would be interesting to look at the allelic variations in these cell lines and how it may affect TRIM22 expression differently. Further study on the methylation pattern including more distance nucleotide sequence would be necessary to elucidate the link between DNA methylation and TRIM22 expression in cancer, as it has been reported that imprinting center can regulate expression of gene that is located up to 1Mb away (Soejima and Wagstaff, 2005). Also we would also consider other factors such as histone modifications. 5-aza-dC is not only an inhibitor of DNA methyltransferase, it can also inhibit histone methylation which lead to gene activation or inactivation depending on the specific histone sites (Wozniak et al., 2007).

4.7.2 Dysregulation of p53 and its association with TRIM22 expression in breast cancer

P53 is a well-known cell cycle check point coordinating cellular response to stress and it is often inactivated during the development of cancer (Lane, 1992). In normal cells, p53 level is tightly controlled and maintained at a low level by E3 ubiquitin ligase such as Mdm2 which targets it for degradation in the absence of external stimuli (Piette et al., 1997). P53 function is deregulated in more than half of human cancers, either by direct p53 gene mutation or disruption of the mechanisms regulating its expression and stability (Dai and Gu, 2010; Gasco et al., 2002; Giaccia and Kastan, 1998; Kaeser et al., 2004; Soussi and Lozano, 2005; Xu-Monette et al., 2012; Yang et al., 2004). Gain of function mutations can even turn it into an oncogene and contributes to cancer progression (Acin et al., 2011; Bossi et al., 2006; Liu et al., 2010; Oren and Rotter, 2010; Solomon et al., 2011; Vikhanskaya et al., 2007). Elevated p53 protein expression is observed in many neoplasias such as gastric carcinoma, squamous cell carcinomas, leukemia and hepatocellular carcinoma (Burns et al., 1993; Koeffler et al., 1986; Martin et al., 1992; Zhang et al., 2009). The increase in p53 protein expression is contributed by many factors, including p53 point mutations that stabilize the protein or disruption of the p53 regulatory pathway. 70-80% of p53 point mutations in its core DNA-binding domain result in increased p53 stability. P53 overexpression has been reported in different cancer tissues including breast cancer (Cripps et al., 1994; Dai and Gu, 2010; Soong et al., 1996). In my study, p53 protein was also higher in the tumor tissues as compared to the adjacent normal tissues, consistent with the published results and confirming the validity of the sample processing and analysis (Singh et al., 1993; Varley et al., 1991).

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Positive correlation between TRIM22 and p53 protein level with correlation coefficient of 0.79 ($p < 0.001$) was found in the normal mammary tissue I examined, meaning when TRIM22 expression is higher in the tissue with higher p53 level. In non-malignant epithelial cells, p53 is generally functional and is responsive to stimuli such the DNA damaging signals. Being a p53 downstream gene, it is expected that TRIM22 will be upregulated when p53 is activated and this explains the positive correlation in their protein level. The correlation is weaker in tumor tissues; this may be partly caused by the high mutation rate of p53 in cancer which caused it to lose its function and altered its expression (Sorlie et al., 2001). In this case, high p53 expression may be caused by stabilization of protein due to mutation. It no longer reflects activation by external stimuli and there will not be induction of TRIM22 expression. It has been reported that downregulation of p53 target gene p21^{WAF/Cip1} expression associates with more aggressive tumors and poor survival in multiple types of cancers including colorectal cancer, gastric cancer and breast cancer (Caffo et al., 1996; Ogawa et al., 2001; Polyak et al., 1996). Higher expression of p21^{WAF/Cip1} in lung cancer and pancreatic adenocarcinoma associates with better prognosis and longer survival (Dergham et al., 1997; Komiya et al., 1997). TRIM22 being downstream of p53 may play a similar role as p21. It would be interesting to see if p53-high/TRIM22-high represents tumors with functional p53 and if it associates with better prognosis, or p53-high/TRIM22-low may associate with poor prognosis.

4.7.3 TRIM22 regulation by wild type p53 disrupted in cancer cells

TRIM22 expression can be regulated by eISRE next to the transcription start site and p53 response element in intron 1, which enables its induction by IFN and p53 activation (Gao et al., 2010a; Obad et al., 2004). While I showed that IFN can induce TRIM22 expression in non-malignant and malignant mammary epithelial cells tested regardless of their p53 status and without the need for *de novo* protein synthesis, regulation of TRIM22 by p53 seemed to be only normal in the non-malignant MCF10A cells but disrupted in all the breast cancer cells. Even expression of exogenous p53 into H1299 cells was not able to rescue the induction of TRIM22 by p53 during DNA damage. This suggests that IFN and p53 may induce TRIM22 through independent mechanisms and IFN pathway remained intact whereas p53 induction of TRIM22 is disrupted in tumorigenesis. One of the possible causes for the loss of p53 regulation may be a specific defect in the recruitment of p53 or binding of p53 to the TRIM22 promoter site. Analysis has shown that SNPs in the p53 response element can alter p53 binding during DNA damage in doxorubicin treated lymphoblastoid and U2OS osteosarcoma cells (Bandelet et al., 2011). Allele specific induction of TRIM22 expression by p53 is observed in the study. P53 does not bind well to the weak TRIM22 allele; result in reduction in TRIM22 gene expression. Similar SNPs may be present in the p53 response element for TRIM22 in breast cancer cell lines that reduce the p53 binding. In addition, abnormality for the proteins involved in the p53 signaling pathways in cancer cells can also contribute to the absence of TRIM22 induction. For instance, MCF7 expresses wild type p53 but does not have caspase 3; cell death caused by drug treatment occurs without DNA fragmentation (Kagawa et al., 2001).

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Our finding of the absence of TRIM22 induction by DNA damage stimuli in cancer cells contradicts the previous published result that UV can induce TRIM22 expression in MCF7 cells (Obad et al., 2004). This may be caused by the different forms of TRIM22 we are working with. In that study, only RNA level was studied and the TRIM22 mRNA they studied was only 2.8kb which encodes for 442 amino acids (GI:15208661) (Tissot and Mechti, 1995). Later, this transcript was replaced by a longer 3.1kb TRIM22 sequence that encodes for 498 amino acids, which is what I used for antibody generation (GI:313760627). The alignment of these two forms are shown in Figure 4.15, the main difference is the extension of C-terminal sequence for the longer form. I detected a smaller TRIM22-like protein induced by CPT treatment in H1299 cells expressing exogenous p53. This TRIM22-like protein has a size of 50kDa, similar to the predicted molecular weight of the 442 amino acid TRIM22 identified earlier, suggesting that the presence of a splice variant of TRIM22 in the cancer cells that is responsive to p53, but this needs further validation. This smaller TRIM22-like protein can also be one of TRIM22 paralogs. On chromosome 11p15, besides TRIM22, there are six other TRIM proteins identified, namely, TRIM3, 5 α , 6, 21, 34 and 68 (Billaut-Mulot et al., 2001; El-Husseini et al., 2001; Frank et al., 1993; Sawyer et al., 2007). TRIM21 (475 amino acids), TRIM34 (488 amino acids) and TRIM68 (485 amino acids) which exhibit sequence homology with TRIM22 but smaller than TRIM22 are possible hits for this unknown protein. This disruption of TRIM22 induction by p53 pathway in cancer and the consistent downregulation of TRIM22 expression in breast cancer tissues suggest that TRIM22 expression is specifically suppressed during breast carcinogenesis and it may be a possible tumor suppressor in breast cancer.

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TRIM-498      MDFSVKVDIEKEVTCPICLELLTEPLSLDCGHSFCQACITAKIKESVIISRGESSCPVCO 6
TRIM-422      MDFSVKVDIEKEVTCPICLELLTEPLSLDCGHSFCQACITAKIKESVIISRGESSCPVCO 6
*****

TRIM-498      TRFQPGNLRPNRHLANIVERVKEVKMSPQEGQKRDVCEHHGKKLQIFCKEDGKVICWVCE 1
TRIM-422      TRFQPGNLRPNRHLANIVERVKEVKMSPQEGQKRDVCEHHGKKLQIFCKEDGKVICWVCE 1
*****

TRIM-498      LSQEHQGHQTFRINEVVKECQEKIQVALQRLIKEDQEAEKLEDDIRQERTAWKNYIQIER 1
TRIM-422      LSQEHQGHQTFRINEVVKECQEKIQVALQRLIKEDQEAEKLEDDIRQERTAWK----IER 1
*****

TRIM-498      QKILKGFNEMRVILDNEEQRELQKLEEGEVNVLNLAATDQLVQQRQDASTLISDLQRR 2
TRIM-422      QKILKGFNEMRVILDNEEQRELQKLEEGEVNVLNLAATDQLVQQRQDASTLISDLQRR 2
*****

TRIM-498      LRGSSEMLQDVVIDVMKRSESWTLKKPKSVSKKLKSVFRVPDLSGMLQVLKELTDVQYYW 3
TRIM-422      LTGSSEMLQDVVIDVMKRSESWTLKKPKSVSKKLKSVFRVPDLSGMLQVLKELTDVQYYW 2
* *****

TRIM-498      VDVMLNPGSATSINVAISVDQRQVKTVRTCTFKNSNPCDFSAFGVFGCQYFSSGKYYWEVD 3
TRIM-422      VDVMLNPGSATSINVAISVDQRQVKTVRTCTFKNSNPCDFSAFGVFGCQYFSSGKYYWEVD 3
*****

TRIM-498      VSGKIAWILGVHSHKISSLNKRKSSGFAPDPSVNYSKVYSRYRPQYGYWVIGLQNTCEYNA 4
TRIM-422      VSGKIAWILGVHSHKISSLNKRKSSGFAPDPSVNYSKVYSRYRPQYGYWVIGLQNTCEYNA 4
*****

TRIM-498      FEDSSSSDPKVLTLFMAVPPCRITGVFLDYFAGIVSFFNVTNHGALIYKFSGCRFSRPAYP 4
TRIM-422      FEDSSSSDPKVLTLFMAVLPVVLGFS----- 4
*****

TRIM-498      YFNPWNCLVPMTVCPPSS 498
TRIM-422      -----

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Figure 4.16 Alignment of TRIM22 proteins

Alignment of TRIM22 protein identified earlier (442 amino acids, GI:117938315) and full length TRIM22 protein sequence identified later (498 amino acids, GI:313760627). The main difference is the last 52 amino acids in the C-terminus.

4.7.4 TRIM22 regulates PR signaling

While the role of TRIM22 in cancer has been much associated with viral infection, IFN and p53 pathways, I identified the TRIM22 as a novel regulator of PR signaling. Co-transfection of TRIM22 increases the expression level of full length PR and its hormone induced transcriptional activity. TRIM22 also downregulated PR Δ LBD's ligand-

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independent activity without affecting its protein level. For TRIM22's effect on full length PR, there was no increase in PR protein stability when it was tested. This narrows down the mechanism of regulation to either increase of translation, increase of transcription or increase of mRNA stability. Increase of translation or transcription is unlikely because exogenous vector was used in this study; PR translation and transcription were regulated by the vector backbone. It is more likely that increase in mRNA stability is responsible for the observed increase in expression. In addition, increase PR mRNA level was detected in the presence of TRIM22. TRIM22 contains multiple zinc finger binding motifs which have the ability to interact with protein, RNA and DNA (Brown, 2005; Hall, 2005; Klug, 1999; Matthews and Sunde, 2002). Radioactive-RNA end labeling of IP from mouse embryonic lysate using TRIM32 antibody shows that TRIM32 is present in a complex with 34 microRNAs of about 21 nucleotides in length and RNase Argonaute 1 (Schwamborn et al., 2009). It enhances the activities of these interacting microRNAs including let-7 and miR-130. This proves that TRIM family proteins are capable of interacting with RNA species directly. TRIM22 may bind to PR mRNA and helps to stabilize it. Furthermore, RING domain but not its E3 ubiquitin ligase activity had been demonstrated to be important for TRIM22's effect on PR, suggesting the TRIM22 binds to PR mRNA via its RING finger domain.

Additional experiments need to be designed to show that TRIM22 indeed binds to PR mRNA and it is also important for us to see if TRIM22 exerts similar effect on endogenous PR, how its regulation of PR activity may affect breast cancer progression. It has been challenging to define the role of progesterone receptor in breast cancer as its

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expression and activity are regulated by and intertwined in a complex network involving many other signaling molecules, such as estrogen and growth factors (Ankrapp et al., 1998; Hewitt and Korach, 2000). The loss of PR expression in ER-positive/PR-negative breast cancer does not necessarily imply the inactivation of ER but it may also be caused by hyperactive growth factor signaling (Arpino et al., 2005). Here we provide evidence for the presence of TRIM22 as a new factor regulating PR expression and activity. The lower level of TRIM22 in breast tumor tissues may serve as an additional mechanism leading to the downregulation of PR activity during breast tumorigenesis.

Conclusions and future studies

CHAPTER 5

CONCLUSIONS AND FUTURE STUDIES

Conclusions and future studies

5.1 Conclusions

5.1.1 PRMT6 methylates ER and it also upregulates ER ligand-independent activity by competitive binding with chaperones

There are 11 putative protein arginine methyltransferases in humans and 8 of them have shown methyltransferase activity (Wolf, 2009). To date, PRMT1 is the only arginine methyltransferase that has been reported to transiently methylate ER (Le Romancer et al., 2008). Methylation of ER by PRMT1 at R260 of the DNA binding domain mediates non-genomic function of ER. This study reports for the first time that PRMT6 interacts with and methylates sex steroid hormone receptors ER, PR and AR, but not GR.

PRMT6 was identified as a new methyltransferase that can bind and methylate ER, PR and AR directly. The interaction between PRMT6 and the SHRs is ligand-independent and the site of interaction is receptor specific. PRMT6 binds to C terminus domain (LBD-region F) of ER but binds to N-terminus domains (VR-DBD) of PR. It also exerts differential effect on these receptors' ligand-independent activities. PRMT6 enhances ER ligand-independent activity but inhibits PR Δ LBD ligand-independent activity, suggesting that PRMT6 may methylate non-conserved arginine sites in each receptor to regulate their activities differently.

The regulation of ER ligand-independent activity does not require enzymatically active PRMT6. The specific binding between PRMT6 and non-chaperone bound ER, and the observation that both PRMT6 and hsp90 binds to C-terminus LBD region of ER, suggests that PRMT6 competes with hsp90 for binding at LBD, leading to the dissociation of ER

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from chaperone complex and enables ER activation. This enhancement of the ligand-independent activity of ER by PRMT6 showed that PRMT6 can promote the crosstalk with growth factor signaling and hormone independent ER activation, which is an important cause of tamoxifen resistance and hormone-independent tumor growth, and may explain for the association between PRMT6 overexpression and worse prognosis in ER+ breast cancers (Dowhan et al., 2012).

In addition, two possible arginine targets have been identified for PRMT6 in this study. LC-MS analysis on full length ER identified multiple methylated arginine residues in ER and R234 in ER DBD was demonstrated to be a possible target of PRMT6. R234K unmethylated mimic leads to increase in both ligand-dependent and ligand-independent activities whereas R234F methylation mimic abolishes ligand-independent activity but still enhance ligand-dependent activity of ER, demonstrating that increase bulkiness at this site specifically blocks the ligand-independent activation of ER. R260 in conserved PRMT recognition motif (RGG) was also shown to be methylated by PRMT6 as the mutation of R260 to lysine lead to reduced ER methylation signal by PRMT6. This suggests that PRMT1 and PRMT6 may share common arginine targets and have overlapping functions in the regulation of ER activity.

Overall, these results confirmed that PRMT6 is a novel regulator of ER, PR and AR activities methylating receptors directly. It enhances ER ligand-independent activity by competitive binding with chaperone proteins such hsp70 and hsp90, which may enable

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ER activation. A model for the possible action of PRMT6 on ER activity is shown in Figure 5.1.

Figure 5.1 Model of the involvement of PRMT6 in ER ligand-independent signaling
In the presence of high PRMT6 level, it competes with chaperone proteins such as hsp70 and 90 for ER binding at the LBD, causing the dissociation of ER from chaperone proteins. This facilitates the phosphorylation of S167 by either S6K1 or IKK, and recruitment of coactivators, resulting in ligand-independent activation of ER.

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5.1.2 TRIM22 may serve as a prognostic factor in breast cancer and regulates PR signaling

As the role of PR in breast cancer development is still controversial, understanding the function of its downstream target may help to elucidate the effect of progesterone and PR in breast cancer. TRIM22 was identified as one of the most highly upregulated gene by progesterone in breast cancer cells and may be important in PR signaling. In this study, the association of TRIM22 with breast cancer was demonstrated by the consistent downregulation of TRIM22 expression in both breast cancer cell lines and tumor tissues. The induction of TRIM22 by IFN and p53 signaling was also examined and it was also found that TRIM22 enhances ligand-dependent but inhibits ligand-independent PR activities.

TRIM22 protein level is high in normal breast tissues and correlates positively with p53 expression. Its level decreases specifically in breast tumor tissues, making it a potentially useful prognostic marker in breast cancer. Putative CpG island in the TRIM22 promoter region is heavily methylated and TRIM22 expression can be induced by DNMT inhibitor, suggesting that TRIM22 expression is regulated by DNA methylation.

Being both an important antiviral factor and downstream target of p53, TRIM22 induction by IFN is independent of p53 status in breast cancer cells. Upregulation of TRIM22 expression by p53 activation is only observed in non-malignant cells but absent in cancer cells, indicating that the pathway enabling TRIM22 induction by p53 is disrupted in cancer cells.

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Furthermore, the possible involvement of TRIM22 in breast cancer development is further demonstrated by its ability to enhance both the expression and hormone induced activity of exogenous PR, without increasing PR protein stability. This enhancement requires the RING domain of TRIM22 but is independent of its E3 ubiquitin ligase activity. Together with the reported growth inhibitory role of TRIM22 in leukemic cells, this result suggests that TRIM22 may be involved in the growth inhibition of breast cancer cells by progesterone, but further validation on the effect of TRIM22 on endogenous PR is necessary.

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5.2 Future studies

5.2.1 Regulation of ER activity by PRMT6 methylation and the association with breast cancer progression

This study characterized the interaction between SHR and PRMT6 and the methylation of SHRs by PRMT6. However, it remains unknown how the arginine methylation is regulated and contribute to the functional regulation of SHRs. Especially in the case of ER where multiple sites are methylated, how the methylation of these sites is coordinated and if there is cross-talk between different methylated sites.

Since PRMT6 methylates multiple arginines in ER, it is important to find out how each methylation contributes to the overall effect of PRMT6 on ER's activity, which sites are methylated constitutively and which are ligand induced. Mapping of arginine sites methylated by PRMT6 in ER may be achieved by using LC-MS analysis with sample digested by multiple enzymes to increase sequence coverage. As trypsin cuts after lysine and arginine, arginine recognition by this enzyme may be affected when it is methylated. Use of other endoproteinases such as GLuC and AspN will help obtain additional sequence information for regions not digested by trypsin. Similar technique can also be used to identify methylated residues in other SHRs.

More direct evidence is also needed to demonstrate that increase in PRMT6 suppresses the interaction between ER and hsp90; PRMT6 may facilitate the recruitment of coactivators to ER and activate ER independent of E2 treatment. Although it has been reported that PRMT6 is overexpressed in breast cancer, it is still unclear how it may

Conclusions and future studies

contribute to breast cancer progression (Yoshimatsu et al., 2010). Further understanding of PRMT6's enhancement of ER's ligand-independent activity may be able to provide an answer for its functional role in breast cancer. Moreover, it would be useful to see if PRMT6 overexpression in breast tumor tissues is associated with high growth factor signaling and high level of cross-talk between ER and growth factor signaling, both of which contribute to higher risk of tamoxifen resistance and poor prognosis in ER+ breast cancers.

5.2.2 Regulation of TRIM22 expression and its functional significance in breast cancer

In the present study, the expression pattern of TRIM22 in normal and tumor breast tissues was analyzed, but the mechanism downregulating TRIM22 expression in breast cancer needs to be further investigated. As it has been shown *in vitro* that TRIM22 overexpression resulted in reduced growth of leukemia cells, reactivation of TRIM22 expression in cancer may have inhibitory effect on cancer growth (Obad et al., 2004). In addition, it is necessary to evaluate the association of TRIM22 expression with different stages/types of breast cancer to see if there is any indication of better survival with higher TRIM22 expression.

It will also be important to validate the absence of TRIM22 induction by p53 activation in other cancer cell lines and explore the possible reason responsible for it. This will provide useful information on the deregulation of p53 signaling during tumorigenesis.

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The involvement of TRIM22 in PR signaling shed new light on the function of TRIM22 in breast cancer. The possible binding between TRIM22 protein and PR mRNA can be studied using chromatin immunoprecipitation. It is critical to translate the effect of TRIM22 into an *in vivo* system to show that it indeed regulates PR activity under physiological conditions.

References

REFERENCES

- (1992). Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. Early Breast Cancer Trialists' Collaborative Group. *Lancet* 339, 1-15.
- (2011). Singapore Cancer Registry Interim Annual Registry Report Trend in Cancer Incidence in Singapore 2005-2009. http://www.nrdo.gov.sg/uploadedFiles/NRDO/Cancer_Trends_Report%20_05-09.pdf.
- (2012). American Cancer Society. Cancer Facts & Figures 2012. <http://www.cancer.org/acs/groups/content/@epidemiologysurveillance/documents/document/acspc-031941.pdf>.
- Abdel-Hafiz, H., Dudevior, M.L., and Horwitz, K.B. (2009). Mechanisms underlying the control of progesterone receptor transcriptional activity by SUMOylation. *J Biol Chem* 284, 9099-9108.
- Acin, S., Li, Z., Mejia, O., Roop, D.R., El-Naggar, A.K., and Caulin, C. (2011). Gain-of-function mutant p53 but not p53 deletion promotes head and neck cancer progression in response to oncogenic K-ras. *J Pathol* 225, 479-489.
- Aguirre, J.I., Plotkin, L.I., Gortazar, A.R., Millan, M.M., O'Brien, C.A., Manolagas, S.C., and Bellido, T. (2007). A novel ligand-independent function of the estrogen receptor is essential for osteocyte and osteoblast mechanotransduction. *J Biol Chem* 282, 25501-25508.
- Ali, S., and Coombes, R.C. (2002). Endocrine-responsive breast cancer and strategies for combating resistance. *Nature reviews Cancer* 2, 101-112.
- Allton, K., Jain, A.K., Herz, H.M., Tsai, W.W., Jung, S.Y., Qin, J., Bergmann, A., Johnson, R.L., and Barton, M.C. (2009). Trim24 targets endogenous p53 for degradation. *Proc Natl Acad Sci U S A* 106, 11612-11616.
- Althuis, M.D., Fergenbaum, J.H., Garcia-Closas, M., Brinton, L.A., Madigan, M.P., and Sherman, M.E. (2004). Etiology of hormone receptor-defined breast cancer: a systematic review of the literature. *Cancer Epidemiol Biomarkers Prev* 13, 1558-1568.
- Anderson, J., Gordon, A., McManus, A., Shipley, J., and Pritchard-Jones, K. (1999). Disruption of imprinted genes at chromosome region 11p15.5 in paediatric rhabdomyosarcoma. *Neoplasia* 1, 340-348.
- Ankrapp, D.P., Bennett, J.M., and Haslam, S.Z. (1998). Role of epidermal growth factor in the acquisition of ovarian steroid hormone responsiveness in the normal mouse mammary gland. *J Cell Physiol* 174, 251-260.

References

- Aranda, A., and Pascual, A. (2001). Nuclear hormone receptors and gene expression. *Physiol Rev* 81, 1269-1304.
- Arnold, S.F., Obourn, J.D., Jaffe, H., and Notides, A.C. (1994). Serine 167 is the major estradiol-induced phosphorylation site on the human estrogen receptor. *Mol Endocrinol* 8, 1208-1214.
- Arnold, S.F., Vorojeikina, D.P., and Notides, A.C. (1995). Phosphorylation of tyrosine 537 on the human estrogen receptor is required for binding to an estrogen response element. *J Biol Chem* 270, 30205-30212.
- Arpino, G., Weiss, H., Lee, A.V., Schiff, R., De Placido, S., Osborne, C.K., and Elledge, R.M. (2005). Estrogen receptor-positive, progesterone receptor-negative breast cancer: association with growth factor receptor expression and tamoxifen resistance. *J Natl Cancer Inst* 97, 1254-1261.
- Arpino, G., Wiechmann, L., Osborne, C.K., and Schiff, R. (2008). Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance. *Endo Reviews* 29, 217-233.
- Atsriku, C., Britton, D.J., Held, J.M., Schilling, B., Scott, G.K., Gibson, B.W., Benz, C.C., and Baldwin, M.A. (2009). Systematic mapping of posttranslational modifications in human estrogen receptor-alpha with emphasis on novel phosphorylation sites. *Mol Cell Proteomics* 8, 467-480.
- Atwood, C.S., Hovey, R.C., Glover, J.P., Chepko, G., Ginsburg, E., Robison, W.G., and Vonderhaar, B.K. (2000). Progesterone induces side-branching of the ductal epithelium in the mammary glands of peripubertal mice. *J Endocrinol* 167, 39-52.
- Aumais, J.P., Lee, H.S., DeGannes, C., Horsford, J., and White, J.H. (1996). Function of directly repeated half-sites as response elements for steroid hormone receptors. *J Biol Chem* 271, 12568-12577.
- Aumais, J.P., Lee, H.S., Lin, R., and White, J.H. (1997). Selective interaction of hsp90 with an estrogen receptor ligand-binding domain containing a point mutation. *J Biol Chem* 272, 12229-12235.
- Auricchio, F., Migliaccio, A., and Castoria, G. (2008). Sex-steroid hormones and EGF signalling in breast and prostate cancer cells: targeting the association of Src with steroid receptors. *Steroids* 73, 880-884.
- Azizi, G., Hansen, A., and Prestwood, K.M. (2003). Effect of micronized progesterone on bone turnover in postmenopausal women on estrogen replacement therapy. *Endocr Res* 29, 133-140.
- Bachand, F. (2007). Protein arginine methyltransferases: from unicellular eukaryotes to humans. *Eukaryot Cell* 6, 889-898.

References

- Bachand, F., and Silver, P.A. (2004). PRMT3 is a ribosomal protein methyltransferase that affects the cellular levels of ribosomal subunits. *EMBO J* 23, 2641-2650.
- Baffa, R., Negrini, M., Mandes, B., Rugge, M., Ranzani, G.N., Hirohashi, S., and Croce, C.M. (1996). Loss of heterozygosity for chromosome 11 in adenocarcinoma of the stomach. *Cancer research* 56, 268-272.
- Ballestas, M.E., Chatis, P.A., and Kaye, K.M. (1999). Efficient persistence of extrachromosomal KSHV DNA mediated by latency-associated nuclear antigen. *Science* 284, 641-644.
- Bandeale, O.J., Wang, X., Campbell, M.R., Pittman, G.S., and Bell, D.A. (2011). Human single-nucleotide polymorphisms alter p53 sequence-specific binding at gene regulatory elements. *Nucleic acids research* 39, 178-189.
- Bardou, V.J., Arpino, G., Elledge, R.M., Osborne, C.K., and Clark, G.M. (2003). Progesterone receptor status significantly improves outcome prediction over estrogen receptor status alone for adjuvant endocrine therapy in two large breast cancer databases. *J Clin Oncol* 21, 1973-1979.
- Barr, S.D., Smiley, J.R., and Bushman, F.D. (2008). The interferon response inhibits HIV particle production by induction of TRIM22. *PLoS Pathog* 4, e1000007.
- Bauer, U.M., Daujat, S., Nielsen, S.J., Nightingale, K., and Kouzarides, T. (2002). Methylation at arginine 17 of histone H3 is linked to gene activation. *EMBO Rep* 3, 39-44.
- Baum, M., Buzdar, A., Cuzick, J., Forbes, J., Houghton, J., Howell, A., and Sahmoud, T. (2003). Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early-stage breast cancer: results of the ATAC (Arimidex, Tamoxifen Alone or in Combination) trial efficacy and safety update analyses. *Cancer* 98, 1802-1810.
- Bedford, M.T. (2007). Arginine methylation at a glance. *J Cell Sci* 120, 4243-4246.
- Belardelli, F., Ferrantini, M., Proietti, E., and Kirkwood, J.M. (2002). Interferon-alpha in tumor immunity and immunotherapy. *Cytokine Growth Factor Rev* 13, 119-134.
- Belikov, S., Astrand, C., and Wrangé, O. (2009). FoxA1 binding directs chromatin structure and the functional response of a glucocorticoid receptor-regulated promoter. *Mol Cell Biol* 29, 5413-5425.
- Belloni, E., Trubia, M., Gasparini, P., Micucci, C., Tapinassi, C., Confalonieri, S., Nuciforo, P., Martino, B., Lo-Coco, F., Pelicci, P.G., *et al.* (2005). 8p11 myeloproliferative syndrome with a novel t(7;8) translocation leading to fusion of the FGFR1 and TIF1 genes. *Genes Chromosomes Cancer* 42, 320-325.
- Benchimol, S. (2001). p53-dependent pathways of apoptosis. *Cell Death Differ* 8, 1049-1051.

References

- Benecke, A., Chambon, P., and Gronemeyer, H. (2000). Synergy between estrogen receptor alpha activation functions AF1 and AF2 mediated by transcription intermediary factor TIF2. *EMBO Rep* 1, 151-157.
- Benoit, G., Cooney, A., Giguere, V., Ingraham, H., Lazar, M., Muscat, G., Perlmann, T., Renaud, J.P., Schwabe, J., Sladek, F., *et al.* (2006). International Union of Pharmacology. LXVI. Orphan nuclear receptors. *Pharmacol Rev* 58, 798-836.
- Beral, V. (2003). Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet* 362, 419-427.
- Bernardi, R., and Pandolfi, P.P. (2007). Structure, dynamics and functions of promyelocytic leukaemia nuclear bodies. *Nat Rev Mol Cell Biol* 8, 1006-1016.
- Bernardi, R., Papa, A., and Pandolfi, P.P. (2008). Regulation of apoptosis by PML and the PML-NBs. *Oncogene* 27, 6299-6312.
- Bevan, C.L., Hoare, S., Claessens, F., Heery, D.M., and Parker, M.G. (1999). The AF1 and AF2 domains of the androgen receptor interact with distinct regions of SRC1. *Mol Cell Biol* 19, 8383-8392.
- Billaut-Mulot, O., Cocude, C., Kolesnitchenko, V., Truong, M.J., Chan, E.K., Hachula, E., de la Tribonniere, X., Capron, A., and Bahr, G.M. (2001). SS-56, a novel cellular target of autoantibody responses in Sjogren syndrome and systemic lupus erythematosus. *J Clin Invest* 108, 861-869.
- Bledsoe, R.K., Montana, V.G., Stanley, T.B., Delves, C.J., Apolito, C.J., McKee, D.D., Consler, T.G., Parks, D.J., Stewart, E.L., Willson, T.M., *et al.* (2002). Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. *Cell* 110, 93-105.
- Bonaccorsi, L., Marchiani, S., Muratori, M., Forti, G., and Baldi, E. (2004). Gefitinib ('IRESSA', ZD1839) inhibits EGF-induced invasion in prostate cancer cells by suppressing PI3 K/AKT activation. *J Cancer Res Clin Oncol* 130, 604-614.
- Boonyaratanakornkit, V., Scott, M.P., Ribon, V., Sherman, L., Anderson, S.M., Maller, J.L., Miller, W.T., and Edwards, D.P. (2001). Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases. *Mol Cell* 8, 269-280.
- Borden, K.L., Martin, S.R., O'Reilly, N.J., Lally, J.M., Reddy, B.A., Etkin, L.D., and Freemont, P.S. (1993). Characterisation of a novel cysteine/histidine-rich metal binding domain from *Xenopus* nuclear factor XNF7. *FEBS Lett* 335, 255-260.
- Bossi, G., Lapi, E., Strano, S., Rinaldo, C., Blandino, G., and Sacchi, A. (2006). Mutant p53 gain of function: reduction of tumor malignancy of human cancer cell lines through abrogation of mutant p53 expression. *Oncogene* 25, 304-309.

References

- Bouazzaoui, A., Kreutz, M., Eisert, V., Dinauer, N., Heinzelmann, A., Hallenberger, S., Strayle, J., Walker, R., Rubsamen-Waigmann, H., Andreesen, R., *et al.* (2006). Stimulated trans-acting factor of 50 kDa (Staf50) inhibits HIV-1 replication in human monocyte-derived macrophages. *Virology* 356, 79-94.
- Boulanger, M.C., Liang, C., Russell, R.S., Lin, R., Bedford, M.T., Wainberg, M.A., and Richard, S. (2005). Methylation of Tat by PRMT6 regulates human immunodeficiency virus type 1 gene expression. *J Virol* 79, 124-131.
- Boulanger, M.C., Miranda, T.B., Clarke, S., Di Fruscio, M., Suter, B., Lasko, P., and Richard, S. (2004). Characterization of the Drosophila protein arginine methyltransferases DART1 and DART4. *Biochem J* 379, 283-289.
- Boulay, J.L., Stiefel, U., Taylor, E., Dolder, B., Merlo, A., and Hirth, F. (2009). Loss of heterozygosity of TRIM3 in malignant gliomas. *BMC cancer* 9, 71.
- Branscombe, T.L., Frankel, A., Lee, J.H., Cook, J.R., Yang, Z., Pestka, S., and Clarke, S. (2001). PRMT5 (Janus kinase-binding protein 1) catalyzes the formation of symmetric dimethylarginine residues in proteins. *J Biol Chem* 276, 32971-32976.
- Britton, D.J., Scott, G.K., Schilling, B., Atsriku, C., Held, J.M., Gibson, B.W., Benz, C.C., and Baldwin, M.A. (2008). A novel serine phosphorylation site detected in the N-terminal domain of estrogen receptor isolated from human breast cancer cells. *J Am Soc Mass Spectrom* 19, 729-740.
- Bromberg, J.F., Horvath, C.M., Wen, Z., Schreiber, R.D., and Darnell, J.E., Jr. (1996). Transcriptionally active Stat1 is required for the antiproliferative effects of both interferon alpha and interferon gamma. *Proc Natl Acad Sci U S A* 93, 7673-7678.
- Brown, R.S. (2005). Zinc finger proteins: getting a grip on RNA. *Curr Opin Struct Biol* 15, 94-98.
- Brzozowski, A.M., Pike, A.C., Dauter, Z., Hubbard, R.E., Bonn, T., Engstrom, O., Ohman, L., Greene, G.L., Gustafsson, J.A., and Carlquist, M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* 389, 753-758.
- Budhram-Mahadeo, V.S., Bowen, S., Lee, S., Perez-Sanchez, C., Ensor, E., Morris, P.J., and Latchman, D.S. (2006). Brn-3b enhances the pro-apoptotic effects of p53 but not its induction of cell cycle arrest by cooperating in trans-activation of bax expression. *Nucleic Acids Res* 34, 6640-6652.
- Bunone, G., Briand, P.A., Miksicek, R.J., and Picard, D. (1996). Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *The EMBO journal* 15, 2174-2183.
- Burns, J.E., Baird, M.C., Clark, L.J., Burns, P.A., Edington, K., Chapman, C., Mitchell, R., Robertson, G., Soutar, D., and Parkinson, E.K. (1993). Gene mutations and increased

References

levels of p53 protein in human squamous cell carcinomas and their cell lines. *Br J Cancer* 67, 1274-1284.

Caffo, O., Doglioni, C., Veronese, S., Bonzanini, M., Marchetti, A., Buttitta, F., Fina, P., Leek, R., Morelli, L., Palma, P.D., *et al.* (1996). Prognostic value of p21(WAF1) and p53 expression in breast carcinoma: an immunohistochemical study in 261 patients with long-term follow-up. *Clin Cancer Res* 2, 1591-1599.

Campbell, R.A., Bhat-Nakshatri, P., Patel, N.M., Constantinidou, D., Ali, S., and Nakshatri, H. (2001). Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. *The Journal of biological chemistry* 276, 9817-9824.

Carascossa, S., Dudek, P., Cenni, B., Briand, P.A., and Picard, D. (2010). CARM1 mediates the ligand-independent and tamoxifen-resistant activation of the estrogen receptor alpha by cAMP. *Genes Dev* 24, 708-719.

Carroll, J.S., Liu, X.S., Brodsky, A.S., Li, W., Meyer, C.A., Szary, A.J., Eeckhoutte, J., Shao, W., Hestermann, E.V., Geistlinger, T.R., *et al.* (2005). Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. *Cell* 122, 33-43.

Castano, E., Vorojeikina, D.P., and Notides, A.C. (1997). Phosphorylation of serine-167 on the human oestrogen receptor is important for oestrogen response element binding and transcriptional activation. *Biochem J* 326 (Pt 1), 149-157.

Cha, B., Kim, W., Kim, Y.K., Hwang, B.N., Park, S.Y., Yoon, J.W., Park, W.S., Cho, J.W., Bedford, M.T., and Jho, E.H. (2011). Methylation by protein arginine methyltransferase 1 increases stability of Axin, a negative regulator of Wnt signaling. *Oncogene* 30, 2379-2389.

Chambraud, B., Berry, M., Redeuilh, G., Chambon, P., and Baulieu, E.E. (1990). Several regions of human estrogen receptor are involved in the formation of receptor-heat shock protein 90 complexes. *J Biol Chem* 265, 20686-20691.

Chan, J.Y., Hsieh, T.Y., Liu, S.T., Chou, W.Y., Chung, M.H., and Huang, S.M. (2009). Physical and functional interactions between hnRNP K and PRMT family proteins. *FEBS letters* 583, 281-286.

Chauchereau, A., Amazit, L., Quesne, M., Guiochon-Mantel, A., and Milgrom, E. (2003). Sumoylation of the progesterone receptor and of the steroid receptor coactivator SRC-1. *J Biol Chem* 278, 12335-12343.

Chaudhri, R.A., Olivares-Navarrete, R., Cuenca, N., Hadadi, A., Boyan, B.D., and Schwartz, Z. (2012). Membrane estrogen signaling enhances tumorigenesis and metastatic potential of breast cancer cells via estrogen receptor-alpha36 (ERalpha36). *J Biol Chem* 287, 7169-7181.

References

Chen, C.C., Hardy, D.B., and Mendelson, C.R. (2011). Progesterone receptor inhibits proliferation of human breast cancer cells via induction of MAPK phosphatase 1 (MKP-1/DUSP1). *J Biol Chem* 286, 43091-43102.

Chen, C.L., Ip, S.M., Cheng, D., Wong, L.C., and Ngan, H.Y. (2000a). Loss of imprinting of the IGF-II and H19 genes in epithelial ovarian cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 6, 474-479.

Chen, D., Huang, S.M., and Stallcup, M.R. (2000b). Synergistic, p160 coactivator-dependent enhancement of estrogen receptor function by CARM1 and p300. *The Journal of biological chemistry* 275, 40810-40816.

Chen, D., Ma, H., Hong, H., Koh, S.S., Huang, S.M., Schurter, B.T., Aswad, D.W., and Stallcup, M.R. (1999a). Regulation of transcription by a protein methyltransferase. *Science* 284, 2174-2177.

Chen, D., Pace, P.E., Coombes, R.C., and Ali, S. (1999b). Phosphorylation of human estrogen receptor alpha by protein kinase A regulates dimerization. *Mol Cell Biol* 19, 1002-1015.

Chen, D., Riedl, T., Washbrook, E., Pace, P.E., Coombes, R.C., Egly, J.M., and Ali, S. (2000c). Activation of estrogen receptor alpha by S118 phosphorylation involves a ligand-dependent interaction with TFIIH and participation of CDK7. *Mol Cell* 6, 127-137.

Chen, Z., Yuhanna, I.S., Galcheva-Gargova, Z., Karas, R.H., Mendelsohn, M.E., and Shaul, P.W. (1999c). Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. *J Clin Invest* 103, 401-406.

Cheng, D., Cote, J., Shaaban, S., and Bedford, M.T. (2007). The arginine methyltransferase CARM1 regulates the coupling of transcription and mRNA processing. *Mol Cell* 25, 71-83.

Chevillard-Briet, M., Trouche, D., and Vandel, L. (2002). Control of CBP co-activating activity by arginine methylation. *The EMBO journal* 21, 5457-5466.

Cho, S.J., and Pyo, S. (2010). Interferon-gamma enhances the apoptosis of macrophages under trophic stress through activation of p53 and the JAK1 pathway. *Arch Pharm Res* 33, 285-291.

Christiaens, V., Bevan, C.L., Callewaert, L., Haelens, A., Verrijdt, G., Rombauts, W., and Claessens, F. (2002). Characterization of the two coactivator-interacting surfaces of the androgen receptor and their relative role in transcriptional control. *The Journal of biological chemistry* 277, 49230-49237.

Chrousos, G.P. (2009). Stress and disorders of the stress system. *Nat Rev Endocrinol* 5, 374-381.

References

- Chu, Y., and Yang, X. (2011). SUMO E3 ligase activity of TRIM proteins. *Oncogene* 30, 1108-1116.
- Chung, W.Y., Yuan, L., Feng, L., Hensle, T., and Tycko, B. (1996). Chromosome 11p15.5 regional imprinting: comparative analysis of KIP2 and H19 in human tissues and Wilms' tumors. *Hum Mol Genet* 5, 1101-1108.
- Cirillo, L.A., Lin, F.R., Cuesta, I., Friedman, D., Jarnik, M., and Zaret, K.S. (2002). Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. *Mol Cell* 9, 279-289.
- Cirillo, L.A., and Zaret, K.S. (1999). An early developmental transcription factor complex that is more stable on nucleosome core particles than on free DNA. *Mol Cell* 4, 961-969.
- Conneely, O.M., Mulac-Jericevic, B., Lydon, J.P., and De Mayo, F.J. (2001). Reproductive functions of the progesterone receptor isoforms: lessons from knock-out mice. *Mol Cell Endocrinol* 179, 97-103.
- Conway, K., Parrish, E., Edmiston, S.N., Tolbert, D., Tse, C.K., Geradts, J., Livasy, C.A., Singh, H., Newman, B., and Millikan, R.C. (2005). The estrogen receptor-alpha A908G (K303R) mutation occurs at a low frequency in invasive breast tumors: results from a population-based study. *Breast Cancer Res* 7, R871-880.
- Cook, J.R., Lee, J.H., Yang, Z.H., Krause, C.D., Herth, N., Hoffmann, R., and Pestka, S. (2006). FBXO11/PRMT9, a new protein arginine methyltransferase, symmetrically dimethylates arginine residues. *Biochem Biophys Res Commun* 342, 472-481.
- Coppes, M.J., Bonetta, L., Huang, A., Hoban, P., Chilton-MacNeill, S., Campbell, C.E., Weksberg, R., Yeger, H., Reeve, A.E., and Williams, B.R. (1992). Loss of heterozygosity mapping in Wilms tumor indicates the involvement of three distinct regions and a limited role for nondisjunction or mitotic recombination. *Genes Chromosomes Cancer* 5, 326-334.
- Cotter, M.A., 2nd, and Robertson, E.S. (1999). The latency-associated nuclear antigen tethers the Kaposi's sarcoma-associated herpesvirus genome to host chromosomes in body cavity-based lymphoma cells. *Virology* 264, 254-264.
- Couse, J.E., Mahato, D., Eddy, E.M., and Korach, K.S. (2001). Molecular mechanism of estrogen action in the male: insights from the estrogen receptor null mice. *Reprod Fertil Dev* 13, 211-219.
- Couse, J.F., and Korach, K.S. (1999). Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev* 20, 358-417.
- Couse, J.F., Lindzey, J., Grandien, K., Gustafsson, J.A., and Korach, K.S. (1997). Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. *Endocrinology* 138, 4613-4621.

References

- Craft, N., Shostak, Y., Carey, M., and Sawyers, C.L. (1999). A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. *Nat Med* 5, 280-285.
- Cripps, K.J., Purdie, C.A., Carder, P.J., White, S., Komine, K., Bird, C.C., and Wyllie, A.H. (1994). A study of stabilisation of p53 protein versus point mutation in colorectal carcinoma. *Oncogene* 9, 2739-2743.
- Cui, Y., Zhang, M., Pestell, R., Curran, E.M., Welshons, W.V., and Fuqua, S.A. (2004). Phosphorylation of estrogen receptor alpha blocks its acetylation and regulates estrogen sensitivity. *Cancer Res* 64, 9199-9208.
- Curtis, S.W., Washburn, T., Sewall, C., DiAugustine, R., Lindzey, J., Couse, J.F., and Korach, K.S. (1996). Physiological coupling of growth factor and steroid receptor signaling pathways: estrogen receptor knockout mice lack estrogen-like response to epidermal growth factor. *Proc Natl Acad Sci U S A* 93, 12626-12630.
- Dahlman-Wright, K., Cavailles, V., Fuqua, S.A., Jordan, V.C., Katzenellenbogen, J.A., Korach, K.S., Maggi, A., Muramatsu, M., Parker, M.G., and Gustafsson, J.A. (2006). International Union of Pharmacology. LXIV. Estrogen receptors. *Pharmacol Rev* 58, 773-781.
- Dai, C., and Gu, W. (2010). p53 post-translational modification: deregulated in tumorigenesis. *Trends Mol Med* 16, 528-536.
- Daniel, A.R., Faivre, E.J., and Lange, C.A. (2007a). Phosphorylation-dependent antagonism of sumoylation derepresses progesterone receptor action in breast cancer cells. *Mol Endocrinol* 21, 2890-2906.
- Daniel, A.R., Gaviglio, A.L., Czaplicki, L.M., Hillard, C.J., Housa, D., and Lange, C.A. (2010). The progesterone receptor hinge region regulates the kinetics of transcriptional responses through acetylation, phosphorylation, and nuclear retention. *Mol Endocrinol* 24, 2126-2138.
- Daniel, A.R., Qiu, M., Faivre, E.J., Ostrander, J.H., Skildum, A., and Lange, C.A. (2007b). Linkage of progestin and epidermal growth factor signaling: Phosphorylation of progesterone receptors mediates transcriptional hypersensitivity and increased ligand-independent breast cancer cell growth. *Steroids* 72, 188-201.
- Daniel, A.R., Qiu, M., and Lange, C.A. (2005). Progesterone Receptor Ser 294 phosphorylation by the MAPK pathway regulates PR localization, transcription and turnover. *Steroids* 70, 473-474.
- Daniel, B., Rangarajan, A., Mukherjee, G., Vallikad, E., and Krishna, S. (1997). The link between integration and expression of human papillomavirus type 16 genomes and cellular changes in the evolution of cervical intraepithelial neoplastic lesions. *J Gen Virol* 78 (Pt 5), 1095-1101.

References

- Danielian, P.S., White, R., Lees, J.A., and Parker, M.G. (1992). Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. *EMBO J* 11, 1025-1033.
- Das, S., Raj, L., Zhao, B., Kimura, Y., Bernstein, A., Aaronson, S.A., and Lee, S.W. (2007). Hzf Determines cell survival upon genotoxic stress by modulating p53 transactivation. *Cell* 130, 624-637.
- Daujat, S., Bauer, U.M., Shah, V., Turner, B., Berger, S., and Kouzarides, T. (2002). Crosstalk between CARM1 methylation and CBP acetylation on histone H3. *Curr Biol* 12, 2090-2097.
- Dawson, D.W., Pearce, S.F., Zhong, R., Silverstein, R.L., Frazier, W.A., and Bouck, N.P. (1997). CD36 mediates the In vitro inhibitory effects of thrombospondin-1 on endothelial cells. *J Cell Biol* 138, 707-717.
- Dawson, D.W., Volpert, O.V., Pearce, S.F., Schneider, A.J., Silverstein, R.L., Henkin, J., and Bouck, N.P. (1999). Three distinct D-amino acid substitutions confer potent antiangiogenic activity on an inactive peptide derived from a thrombospondin-1 type 1 repeat. *Mol Pharmacol* 55, 332-338.
- De Preter, K., Vandesompele, J., Heimann, P., Yigit, N., Beckman, S., Schramm, A., Eggert, A., Stallings, R.L., Benoit, Y., Renard, M., *et al.* (2006). Human fetal neuroblast and neuroblastoma transcriptome analysis confirms neuroblast origin and highlights neuroblastoma candidate genes. *Genome Biol* 7, R84.
- de The, H., Lavau, C., Marchio, A., Chomienne, C., Degos, L., and Dejean, A. (1991). The PML-RAR alpha fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. *Cell* 66, 675-684.
- Dergham, S.T., Dugan, M.C., Joshi, U.S., Chen, Y.C., Du, W., Smith, D.W., Arlauskas, P., Crissman, J.D., Vaitkevicius, V.K., and Sarkar, F.H. (1997). The clinical significance of p21(WAF1/CIP-1) and p53 expression in pancreatic adenocarcinoma. *Cancer* 80, 372-381.
- Dighe, A.S., Richards, E., Old, L.J., and Schreiber, R.D. (1994). Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. *Immunity* 1, 447-456.
- Dillon, S.C., Zhang, X., Trievel, R.C., and Cheng, X. (2005). The SET-domain protein superfamily: protein lysine methyltransferases. *Genome Biol* 6, 227.
- Dowhan, D.H., Harrison, M.J., Eriksson, N.A., Bailey, P., Pearen, M.A., Fuller, P.J., Funder, J.W., Simpson, E.R., Leedman, P.J., Tilley, W.D., *et al.* (2012). Protein arginine methyltransferase 6-dependent gene expression and splicing: association with breast cancer outcomes. *Endocr Relat Cancer* 19, 509-526.
- Duan, Z., Gao, B., Xu, W., and Xiong, S. (2008). Identification of TRIM22 as a RING finger E3 ubiquitin ligase. *Biochem Biophys Res Commun* 374, 502-506.

References

- Dunn, G.P., Koebel, C.M., and Schreiber, R.D. (2006). Interferons, immunity and cancer immunoediting. *Nat Rev Immunol* 6, 836-848.
- Duplessis, T.T., Williams, C.C., Hill, S.M., and Rowan, B.G. (2011). Phosphorylation of Estrogen Receptor alpha at serine 118 directs recruitment of promoter complexes and gene-specific transcription. *Endocrinology* 152, 2517-2526.
- Dupont, S., Krust, A., Gansmuller, A., Dierich, A., Chambon, P., and Mark, M. (2000). Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development* 127, 4277-4291.
- Eckhoute, J., Carroll, J.S., Geistlinger, T.R., Torres-Arzayus, M.I., and Brown, M. (2006). A cell-type-specific transcriptional network required for estrogen regulation of cyclin D1 and cell cycle progression in breast cancer. *Genes Dev* 20, 2513-2526.
- Eickelberg, O., Roth, M., Lox, R., Bruce, V., Rudiger, J., Johnson, M., and Block, L.H. (1999). Ligand-independent activation of the glucocorticoid receptor by beta2-adrenergic receptor agonists in primary human lung fibroblasts and vascular smooth muscle cells. *J Biol Chem* 274, 1005-1010.
- El-Andaloussi, N., Valovka, T., Touelle, M., Steinacher, R., Focke, F., Gehrig, P., Covic, M., Hassa, P.O., Schar, P., Hubscher, U., *et al.* (2006). Arginine methylation regulates DNA polymerase beta. *Mol Cell* 22, 51-62.
- El-Husseini, A.E., Fretier, P., and Vincent, S.R. (2001). Cloning and characterization of a gene (RNF22) encoding a novel brain expressed ring finger protein (BERP) that maps to human chromosome 11p15.5. *Genomics* 71, 363-367.
- El Messaoudi, S., Fabbriozio, E., Rodriguez, C., Chuchana, P., Fauquier, L., Cheng, D., Theillet, C., Vandell, L., Bedford, M.T., and Sardet, C. (2006). Coactivator-associated arginine methyltransferase 1 (CARM1) is a positive regulator of the Cyclin E1 gene. *Proc Natl Acad Sci U S A* 103, 13351-13356.
- Eldin, P., Papon, L., Oteiza, A., Brocchi, E., Lawson, T.G., and Mechti, N. (2009). TRIM22 E3 ubiquitin ligase activity is required to mediate antiviral activity against encephalomyocarditis virus. *J Gen Virol* 90, 536-545.
- Emmen, J.M., Couse, J.F., Elmore, S.A., Yates, M.M., Kissling, G.E., and Korach, K.S. (2005). In vitro growth and ovulation of follicles from ovaries of estrogen receptor (ER){alpha} and ER{beta} null mice indicate a role for ER{beta} in follicular maturation. *Endocrinology* 146, 2817-2826.
- Endoh, H., Maruyama, K., Masuhiro, Y., Kobayashi, Y., Goto, M., Tai, H., Yanagisawa, J., Metzger, D., Hashimoto, S., and Kato, S. (1999). Purification and identification of p68 RNA helicase acting as a transcriptional coactivator specific for the activation function 1 of human estrogen receptor alpha. *Mol Cell Biol* 19, 5363-5372.

References

- Fagan, D.H., and Yee, D. (2008). Crosstalk between IGF1R and estrogen receptor signaling in breast cancer. *J Mammary Gland Biol Neoplasia* 13, 423-429.
- Fagioli, M., Alcalay, M., Tomassoni, L., Ferrucci, P.F., Mencarelli, A., Riganelli, D., Grignani, F., Pozzan, T., Nicoletti, I., and Pelicci, P.G. (1998). Cooperation between the RING + B1-B2 and coiled-coil domains of PML is necessary for its effects on cell survival. *Oncogene* 16, 2905-2913.
- Fairall, L., Schwabe, J.W., Chapman, L., Finch, J.T., and Rhodes, D. (1993). The crystal structure of a two zinc-finger peptide reveals an extension to the rules for zinc-finger/DNA recognition. *Nature* 366, 483-487.
- Faivre, E.J., Daniel, A.R., Hillard, C.J., and Lange, C.A. (2008). Progesterone receptor rapid signaling mediates serine 345 phosphorylation and tethering to specificity protein 1 transcription factors. *Mol Endocrinol* 22, 823-837.
- Feng, Q., Yi, P., Wong, J., and O'Malley, B.W. (2006). Signaling within a coactivator complex: methylation of SRC-3/AIB1 is a molecular switch for complex disassembly. *Mol Cell Biol* 26, 7846-7857.
- Ferrentino, R., Bassi, M.T., Chitayat, D., Tabolacci, E., and Meroni, G. (2007). MID1 mutation screening in a large cohort of Opitz G/BBB syndrome patients: twenty-nine novel mutations identified. *Hum Mutat* 28, 206-207.
- Frank, M.B., Itoh, K., Fujisaku, A., Pontarotti, P., Mattei, M.G., and Neas, B.R. (1993). The mapping of the human 52-kD Ro/SSA autoantigen gene to human chromosome 11, and its polymorphisms. *Am J Hum Genet* 52, 183-191.
- Frankel, A., Yadav, N., Lee, J., Branscombe, T.L., Clarke, S., and Bedford, M.T. (2002). The novel human protein arginine N-methyltransferase PRMT6 is a nuclear enzyme displaying unique substrate specificity. *J Biol Chem* 277, 3537-3543.
- Freemont, P.S. (2000). RING for destruction? *Curr Biol* 10, R84-87.
- Frietze, S., Lupien, M., Silver, P.A., and Brown, M. (2008). CARM1 regulates estrogen-stimulated breast cancer growth through up-regulation of E2F1. *Cancer Res* 68, 301-306.
- Gaben, A.M., Sabbah, M., Redeuilh, G., Bedin, M., and Mester, J. (2012). Ligand-free estrogen receptor activity complements IGF1R to induce the proliferation of the MCF-7 breast cancer cells. *BMC Cancer* 12, 291.
- Gack, M.U., Shin, Y.C., Joo, C.H., Urano, T., Liang, C., Sun, L., Takeuchi, O., Akira, S., Chen, Z., Inoue, S., *et al.* (2007). TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature* 446, 916-920.
- Gallagher, R.E., Willman, C.L., Slack, J.L., Andersen, J.W., Li, Y.P., Viswanatha, D., Bloomfield, C.D., Appelbaum, F.R., Schiffer, C.A., Tallman, M.S., *et al.* (1997). Association of PML-RAR alpha fusion mRNA type with pretreatment hematologic

References

characteristics but not treatment outcome in acute promyelocytic leukemia: an intergroup molecular study. *Blood* 90, 1656-1663.

Gallagher-Beckley, A.J., Williams, J.G., and Cidlowski, J.A. (2011). Ligand-independent phosphorylation of the glucocorticoid receptor integrates cellular stress pathways with nuclear receptor signaling. *Mol Cell Biol* 31, 4663-4675.

Gao, B., Duan, Z., Xu, W., and Xiong, S. (2009). Tripartite motif-containing 22 inhibits the activity of hepatitis B virus core promoter, which is dependent on nuclear-located RING domain. *Hepatology* 50, 424-433.

Gao, B., Wang, Y., Xu, W., Duan, Z., and Xiong, S. (2010a). A 5' extended IFN-stimulating response element is crucial for IFN-gamma-induced tripartite motif 22 expression via interaction with IFN regulatory factor-1. *J Immunol* 185, 2314-2323.

Gao, J., Senthil, M., Ren, B., Yan, J., Xing, Q., Yu, J., Zhang, L., and Yim, J.H. (2010b). IRF-1 transcriptionally upregulates PUMA, which mediates the mitochondrial apoptotic pathway in IRF-1-induced apoptosis in cancer cells. *Cell Death Differ* 17, 699-709.

Gasco, M., Shami, S., and Crook, T. (2002). The p53 pathway in breast cancer. *Breast Cancer Res* 4, 70-76.

Gaston, V., Le Bouc, Y., Soupre, V., Burglen, L., Donadieu, J., Oro, H., Audry, G., Vazquez, M.P., and Gicquel, C. (2001). Analysis of the methylation status of the KCNQ1OT and H19 genes in leukocyte DNA for the diagnosis and prognosis of Beckwith-Wiedemann syndrome. *Eur J Hum Genet* 9, 409-418.

Gaughan, L., Stockley, J., Wang, N., McCracken, S.R., Treumann, A., Armstrong, K., Shaheen, F., Watt, K., McEwan, I.J., Wang, C., *et al.* (2010). Regulation of the androgen receptor by SET9-mediated methylation. *Nucleic Acids Res* 39, 1266-1279.

Generali, D., Buffa, F.M., Berruti, A., Brizzi, M.P., Campo, L., Bonardi, S., Bersiga, A., Allevi, G., Milani, M., Aguggini, S., *et al.* (2009). Phosphorylated ERalpha, HIF-1alpha, and MAPK signaling as predictors of primary endocrine treatment response and resistance in patients with breast cancer. *J Clin Oncol* 27, 227-234.

Giaccia, A.J., and Kastan, M.B. (1998). The complexity of p53 modulation: emerging patterns from divergent signals. *Gene Dev* 12, 2973-2983.

Giono, L.E., and Manfredi, J.J. (2006). The p53 tumor suppressor participates in multiple cell cycle checkpoints. *J Cell Physiol* 209, 13-20.

Giri, D.D., Dundas, S.A., Nottingham, J.F., and Underwood, J.C. (1989). Oestrogen receptors in benign epithelial lesions and intraduct carcinomas of the breast: an immunohistological study. *Histopathology* 15, 575-584.

References

- Gongora, C., Tissot, C., Cerdan, C., and Mechti, N. (2000). The interferon-inducible Staf50 gene is downregulated during T cell costimulation by CD2 and CD28. *J Interferon Cytokine Res* 20, 955-961.
- Gottlicher, M., Heck, S., and Herrlich, P. (1998). Transcriptional cross-talk, the second mode of steroid hormone receptor action. *J Mol Med (Berl)* 76, 480-489.
- Gougelet, A., Bouclier, C., Marsaud, V., Maillard, S., Mueller, S.O., Korach, K.S., and Renoir, J.M. (2005). Estrogen receptor alpha and beta subtype expression and transactivation capacity are differentially affected by receptor-, hsp90- and immunophilin-ligands in human breast cancer cells. *J Steroid Biochem Mol Biol* 94, 71-81.
- Goulet, I., Gauvin, G., Boisvenue, S., and Cote, J. (2007). Alternative splicing yields protein arginine methyltransferase 1 isoforms with distinct activity, substrate specificity, and subcellular localization. *J Biol Chem* 282, 33009-33021.
- Gras, E., Pons, C., Machin, P., Matias-Guiu, X., and Prat, J. (2001). Loss of heterozygosity at the RB-1 locus and pRB immunostaining in epithelial ovarian tumors: a molecular, immunohistochemical, and clinicopathologic study. *Int J Gynecol Pathol* 20, 335-340.
- Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J.M., Argos, P., and Chambon, P. (1986). Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature* 320, 134-139.
- Greene, G.L., Gilna, P., Waterfield, M., Baker, A., Hort, Y., and Shine, J. (1986). Sequence and expression of human estrogen receptor complementary DNA. *Science* 231, 1150-1154.
- Gresser, I. (2007). The antitumor effects of interferon: a personal history. *Biochimie* 89, 723-728.
- Groshong, S.D., Owen, G.I., Grimison, B., Schauer, I.E., Todd, M.C., Langan, T.A., Sclafani, R.A., Lange, C.A., and Horwitz, K.B. (1997). Biphasic regulation of breast cancer cell growth by progesterone: role of the cyclin-dependent kinase inhibitors, p21 and p27(Kip1). *Mol Endocrinol* 11, 1593-1607.
- Gruber, D.M., and Huber, J.C. (1999). Conjugated estrogens--the natural SERMs. *Gynecol Endocrinol* 13 Suppl 6, 9-12.
- Guccione, E., Bassi, C., Casadio, F., Martinato, F., Cesaroni, M., Schuchlautz, H., Luscher, B., and Amati, B. (2007). Methylation of histone H3R2 by PRMT6 and H3K4 by an MLL complex are mutually exclusive. *Nature* 449, 933-937.
- Guo, J.P., Shu, S.K., Esposito, N.N., Coppola, D., Koomen, J.M., and Cheng, J.Q. (2009). IKKepsilon phosphorylation of estrogen receptor alpha Ser-167 and contribution to tamoxifen resistance in breast cancer. *J Biol Chem* 285, 3676-3684.

References

- Gutterman, J.U. (1994). Cytokine therapeutics: lessons from interferon alpha. *Proc Natl Acad Sci U S A* 91, 1198-1205.
- Hafezi-Moghadam, A., Simoncini, T., Yang, Z., Limbourg, F.P., Plumier, J.C., Rebsamen, M.C., Hsieh, C.M., Chui, D.S., Thomas, K.L., Prorock, A.J., *et al.* (2002). Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. *Nat Med* 8, 473-479.
- Hagan, C.R., Daniel, A.R., Dressing, G.E., and Lange, C.A. (2011a). Role of phosphorylation in progesterone receptor signaling and specificity. *Mol Cell Endocrinol.*
- Hagan, C.R., Regan, T.M., Dressing, G.E., and Lange, C.A. (2011b). ck2-Dependent Phosphorylation of Progesterone Receptors (PR) on Ser81 Regulates PR-B Isoform-Specific Target Gene Expression in Breast Cancer Cells. *Mol Cell Biol* 31, 2439-2452.
- Hall, T.M. (2005). Multiple modes of RNA recognition by zinc finger proteins. *Curr Opin Struct Biol* 15, 367-373.
- Hardy, D.B., Janowski, B.A., Chen, C.C., and Mendelson, C.R. (2008). Progesterone receptor inhibits aromatase and inflammatory response pathways in breast cancer cells via ligand-dependent and ligand-independent mechanisms. *Mol Endocrinol* 22, 1812-1824.
- Harrison, M.J., Tang, Y.H., and Dowhan, D.H. (2010). Protein arginine methyltransferase 6 regulates multiple aspects of gene expression. *Nucleic Acids Res* 38, 2201-2216.
- Hatakeyama, S. (2011). TRIM proteins and cancer. *Nat Rev Cancer* 11, 792-804.
- He, L.M., Zhang, C.G., Zhou, Z., and Xu, T. (2003). Rapid inhibitory effects of corticosterone on calcium influx in rat dorsal root ganglion neurons. *Neuroscience* 116, 325-333.
- Helguero, L.A., Faulds, M.H., Gustafsson, J.A., and Haldosen, L.A. (2005). Estrogen receptors alfa (ERalpha) and beta (ERbeta) differentially regulate proliferation and apoptosis of the normal murine mammary epithelial cell line HC11. *Oncogene* 24, 6605-6616.
- Hellman, A., and Chess, A. (2010). Extensive sequence-influenced DNA methylation polymorphism in the human genome. *Epigenetics Chromatin* 3, 11.
- Hewitt, S.C., and Korach, K.S. (2000). Progesterone action and responses in the alphaERKO mouse. *Steroids* 65, 551-557.
- Hobisch, A., Eder, I.E., Putz, T., Horninger, W., Bartsch, G., Klocker, H., and Culig, Z. (1998). Interleukin-6 regulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor. *Cancer Res* 58, 4640-4645.
- Holm, C., Kok, M., Michalides, R., Fles, R., Koornstra, R.H., Wesseling, J., Hauptmann, M., Neeffjes, J., Peterse, J.L., Stal, O., *et al.* (2009). Phosphorylation of the oestrogen

References

- receptor alpha at serine 305 and prediction of tamoxifen resistance in breast cancer. *J Pathol* 217, 372-379.
- Hovland, A.R., Powell, R.L., Takimoto, G.S., Tung, L., and Horwitz, K.B. (1998). An N-terminal inhibitory function, IF, suppresses transcription by the A-isoform but not the B-isoform of human progesterone receptors. *J Biol Chem* 273, 5455-5460.
- Huang, S., Litt, M., and Felsenfeld, G. (2005). Methylation of histone H4 by arginine methyltransferase PRMT1 is essential in vivo for many subsequent histone modifications. *Genes Dev* 19, 1885-1893.
- Hung, C.M., and Li, C. (2004). Identification and phylogenetic analyses of the protein arginine methyltransferase gene family in fish and ascidians. *Gene* 340, 179-187.
- Hurtado, A., Holmes, K.A., Ross-Innes, C.S., Schmidt, D., and Carroll, J.S. (2010). FOXA1 is a key determinant of estrogen receptor function and endocrine response. *Nat Genet* 43, 27-33.
- Huse, B., Verca, S.B., Matthey, P., and Rusconi, S. (1998). Definition of a negative modulation domain in the human progesterone receptor. *Mol Endocrinol* 12, 1334-1342.
- Hyllus, D., Stein, C., Schnabel, K., Schiltz, E., Imhof, A., Dou, Y., Hsieh, J., and Bauer, U.M. (2007). PRMT6-mediated methylation of R2 in histone H3 antagonizes H3 K4 trimethylation. *Genes Dev* 21, 3369-3380.
- Iberg, A.N., Espejo, A., Cheng, D., Kim, D., Michaud-Levesque, J., Richard, S., and Bedford, M.T. (2008). Arginine methylation of the histone H3 tail impedes effector binding. *J Biol Chem* 283, 3006-3010.
- Ikonen, T., Palvimo, J.J., and Janne, O.A. (1997). Interaction between the amino- and carboxyl-terminal regions of the rat androgen receptor modulates transcriptional activity and is influenced by nuclear receptor coactivators. *The Journal of biological chemistry* 272, 29821-29828.
- Invernizzi, C.F., Xie, B., Frankel, F.A., Feldhammer, M., Roy, B.B., Richard, S., and Wainberg, M.A. (2007). Arginine methylation of the HIV-1 nucleocapsid protein results in its diminished function. *AIDS* 21, 795-805.
- Invernizzi, C.F., Xie, B., Richard, S., and Wainberg, M.A. (2006). PRMT6 diminishes HIV-1 Rev binding to and export of viral RNA. *Retrovirology* 3, 93.
- Issaeva, N., Bozko, P., Enge, M., Protopopova, M., Verhoef, L.G., Masucci, M., Pramanik, A., and Selivanova, G. (2004). Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. *Nat Med* 10, 1321-1328.
- Jacobsen, B.M., Schittone, S.A., Richer, J.K., and Horwitz, K.B. (2005). Progesterone-independent effects of human progesterone receptors (PRs) in estrogen receptor-positive

References

breast cancer: PR isoform-specific gene regulation and tumor biology. *Mol Endocrinol* 19, 574-587.

Javanbakht, H., Yuan, W., Yeung, D.F., Song, B., Diaz-Griffero, F., Li, Y., Li, X., Stremlau, M., and Sodroski, J. (2006). Characterization of TRIM5alpha trimerization and its contribution to human immunodeficiency virus capsid binding. *Virology* 353, 234-246.

Jayne, S., Rothgiesser, K.M., and Hottiger, M.O. (2009). CARM1 but not Its Enzymatic Activity Is Required for Transcriptional Coactivation of NF- κ B-Dependent Gene Expression. *Journal of Molecular Biology* 394, 485-495.

Jeng, M.H., Parker, C.J., and Jordan, V.C. (1992). Estrogenic potential of progestins in oral contraceptives to stimulate human breast cancer cell proliferation. *Cancer Res* 52, 6539-6546.

Jenster, G. (2000). Ligand-independent activation of the androgen receptor in prostate cancer by growth factors and cytokines. *J Pathol* 191, 227-228.

Jiang, J., Sarwar, N., Peston, D., Kulinskaya, E., Shousha, S., Coombes, R.C., and Ali, S. (2007). Phosphorylation of estrogen receptor-alpha at Ser167 is indicative of longer disease-free and overall survival in breast cancer patients. *Clin Cancer Res* 13, 5769-5776.

Jobert, L., Argentini, M., and Tora, L. (2009). PRMT1 mediated methylation of TAF15 is required for its positive gene regulatory function. *Exp Cell Res* 315, 1273-1286.

Joel, P.B., Smith, J., Sturgill, T.W., Fisher, T.L., Blenis, J., and Lannigan, D.A. (1998). pp90rsk1 regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. *Mol Cell Biol* 18, 1978-1984.

Joel, P.B., Traish, A.M., and Lannigan, D.A. (1995). Estradiol and phorbol ester cause phosphorylation of serine 118 in the human estrogen receptor. *Mol Endocrinol* 9, 1041-1052.

Johnston, S.R., and Dowsett, M. (2003). Aromatase inhibitors for breast cancer: lessons from the laboratory. *Nature reviews Cancer* 3, 821-831.

Joo, H.M., Kim, J.Y., Jeong, J.B., Seong, K.M., Nam, S.Y., Yang, K.H., Kim, C.S., Kim, H.S., Jeong, M., An, S., *et al.* (2011). Ret finger protein 2 enhances ionizing radiation-induced apoptosis via degradation of AKT and MDM2. *Eur J Cell Biol* 90, 420-431.

Kaesler, M.D., Pebernard, S., and Iggo, R.D. (2004). Regulation of p53 stability and function in HCT116 colon cancer cells. *J Biol Chem* 279, 7598-7605.

Kagan, R.M., and Clarke, S. (1994). Widespread occurrence of three sequence motifs in diverse S-adenosylmethionine-dependent methyltransferases suggests a common structure for these enzymes. *Arch Biochem Biophys* 310, 417-427.

References

- Kagawa, S., Gu, J., Honda, T., McDonnell, T.J., Swisher, S.G., Roth, J.A., and Fang, B. (2001). Deficiency of caspase-3 in MCF7 cells blocks Bax-mediated nuclear fragmentation but not cell death. *Clinical cancer research : an official journal of the American Association for Cancer Research* 7, 1474-1480.
- Kajaste-Rudnitski, A., Marelli, S.S., Pultrone, C., Pertel, T., Uchil, P.D., Mechti, N., Mothes, W., Poli, G., Luban, J., and Vicenzi, E. (2011). TRIM22 inhibits HIV-1 transcription independently of its E3 ubiquitin ligase activity, Tat, and NF-kappaB-responsive long terminal repeat elements. *J Virol* 85, 5183-5196.
- Kakizuka, A., Miller, W.H., Jr., Umesono, K., Warrell, R.P., Jr., Frankel, S.R., Murty, V.V., Dmitrovsky, E., and Evans, R.M. (1991). Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML. *Cell* 66, 663-674.
- Kaneshiro, K., Tsutsumi, S., Tsuji, S., Shirahige, K., and Aburatani, H. (2007). An integrated map of p53-binding sites and histone modification in the human ENCODE regions. *Genomics* 89, 178-188.
- Kaplan, D.H., Shankaran, V., Dighe, A.S., Stockert, E., Aguet, M., Old, L.J., and Schreiber, R.D. (1998). Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc Natl Acad Sci U S A* 95, 7556-7561.
- Karnik, P., Paris, M., Williams, B.R., Casey, G., Crowe, J., and Chen, P. (1998). Two distinct tumor suppressor loci within chromosome 11p15 implicated in breast cancer progression and metastasis. *Hum Mol Genet* 7, 895-903.
- Kaspar, F., Klocker, H., Denninger, A., and Cato, A.C. (1993). A mutant androgen receptor from patients with Reifenstein syndrome: identification of the function of a conserved alanine residue in the D box of steroid receptors. *Mol Cell Biol* 13, 7850-7858.
- Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H., and Chambon, P. (1990). Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. *EMBO J* 9, 1603-1614.
- Katz, J.E., Dlakic, M., and Clarke, S. (2003). Automated identification of putative methyltransferases from genomic open reading frames. *Mol Cell Proteomics* 2, 525-540.
- Kee, H.J., Kim, J.R., Joung, H., Choe, N., Lee, S.E., Eom, G.H., Kim, J.C., Geyer, S.H., Jijiwa, M., Kato, T., *et al.* (2012). Ret finger protein inhibits muscle differentiation by modulating serum response factor and enhancer of polycomb1. *Cell Death Differ* 19, 121-131.
- Khan, S.H., Ling, J., and Kumar, R. (2011). TBP binding-induced folding of the glucocorticoid receptor AF1 domain facilitates its interaction with steroid receptor coactivator-1. *PLoS One* 6, e21939.

References

- Kim, H.J., and Lee, W.J. (2009). Ligand-independent activation of the androgen receptor by insulin-like growth factor-I and the role of the MAPK pathway in skeletal muscle cells. *Mol Cells* 28, 589-593.
- Kim, J.D., Kako, K., Kakiuchi, M., Park, G.G., and Fukamizu, A. (2008). EWS is a substrate of type I protein arginine methyltransferase, PRMT8. *Int J Mol Med* 22, 309-315.
- Kim, K.S., Kang, K.W., Seu, Y.B., Baek, S.H., and Kim, J.R. (2009). Interferon-gamma induces cellular senescence through p53-dependent DNA damage signaling in human endothelial cells. *Mech Ageing Dev* 130, 179-188.
- Kim, S., Merrill, B.M., Rajpurohit, R., Kumar, A., Stone, K.L., Papov, V.V., Schneiders, J.M., Szer, W., Wilson, S.H., Paik, W.K., *et al.* (1997). Identification of N(G)-methylarginine residues in human heterogeneous RNP protein A1: Phe/Gly-Gly-Gly-Arg-Gly-Gly-Gly/Phe is a preferred recognition motif. *Biochemistry* 36, 5185-5192.
- Kim, Y.R., Lee, B.K., Park, R.Y., Nguyen, N.T., Bae, J.A., Kwon, D.D., and Jung, C. (2010). Differential CARM1 expression in prostate and colorectal cancers. *BMC Cancer* 10, 197.
- Kimmins, S., and MacRae, T.H. (2000). Maturation of steroid receptors: an example of functional cooperation among molecular chaperones and their associated proteins. *Cell Stress Chaperon* 5, 76-86.
- Klug, A. (1999). Zinc finger peptides for the regulation of gene expression. *Journal of molecular biology* 293, 215-218.
- Klugbauer, S., and Rabes, H.M. (1999). The transcription coactivator HTIF1 and a related protein are fused to the RET receptor tyrosine kinase in childhood papillary thyroid carcinomas. *Oncogene* 18, 4388-4393.
- Ko, S., Ahn, J., Song, C.S., Kim, S., Knapczyk-Stwora, K., and Chatterjee, B. (2011). Lysine methylation and functional modulation of androgen receptor by Set9 methyltransferase. *Mol Endocrinol* 25, 433-444.
- Koc, A., Wheeler, L.J., Mathews, C.K., and Merrill, G.F. (2004). Hydroxyurea arrests DNA replication by a mechanism that preserves basal dNTP pools. *J Biol Chem* 279, 223-230.
- Koeffler, H.P., Miller, C., Nicolson, M.A., Ranyard, J., and Bosselman, R.A. (1986). Increased expression of p53 protein in human leukemia cells. *Proc Natl Acad Sci U S A* 83, 4035-4039.
- Koide, A., Zhao, C., Naganuma, M., Abrams, J., Deighton-Collins, S., Skafar, D.F., and Koide, S. (2007). Identification of regions within the F domain of the human estrogen receptor alpha that are important for modulating transactivation and protein-protein interactions. *Mol Endocrinol* 21, 829-842.

References

- Kok, M., Zwart, W., Holm, C., Fles, R., Hauptmann, M., Van't Veer, L.J., Wessels, L.F., Neefjes, J., Stal, O., Linn, S.C., *et al.* (2011). PKA-induced phosphorylation of ERalpha at serine 305 and high PAK1 levels is associated with sensitivity to tamoxifen in ER-positive breast cancer. *Breast Cancer Res Treat* 125, 1-12.
- Komiya, T., Hosono, Y., Hirashima, T., Masuda, N., Yasumitsu, T., Nakagawa, K., Kikui, M., Ohno, A., Fukuoka, M., and Kawase, I. (1997). p21 expression as a predictor for favorable prognosis in squamous cell carcinoma of the lung. *Clin Cancer Res* 3, 1831-1835.
- Kraus, W.L., McInerney, E.M., and Katzenellenbogen, B.S. (1995). Ligand-dependent, transcriptionally productive association of the amino- and carboxyl-terminal regions of a steroid hormone nuclear receptor. *Proc Natl Acad Sci U S A* 92, 12314-12318.
- Krege, J.H., Hodgin, J.B., Couse, J.F., Enmark, E., Warner, M., Mahler, J.F., Sar, M., Korach, K.S., Gustafsson, J.A., and Smithies, O. (1998). Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proc Natl Acad Sci U S A* 95, 15677-15682.
- Krum, S.A., and Brown, M. (2008). Unraveling estrogen action in osteoporosis. *Cell Cycle* 7, 1348-1352.
- Krum, S.A., Miranda-Carboni, G.A., Lupien, M., Eeckhoutte, J., Carroll, J.S., and Brown, M. (2008). Unique ERalpha cisomes control cell type-specific gene regulation. *Mol Endocrinol* 22, 2393-2406.
- Kuiper, G.G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., and Gustafsson, J.A. (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* 138, 863-870.
- Kuiper, G.G., Enmark, E., Peltto-Huikko, M., Nilsson, S., and Gustafsson, J.A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci U S A* 93, 5925-5930.
- Kumar, R., and Litwack, G. (2009). Structural and functional relationships of the steroid hormone receptors' N-terminal transactivation domain. *Steroids* 74, 877-883.
- Kumar, R., and Thompson, E.B. (2003). Transactivation functions of the N-terminal domains of nuclear hormone receptors: protein folding and coactivator interactions. *Mol Endocrinol* 17, 1-10.
- Kurash, J.K., Lei, H., Shen, Q., Marston, W.L., Granda, B.W., Fan, H., Wall, D., Li, E., and Gaudet, F. (2008). Methylation of p53 by Set7/9 mediates p53 acetylation and activity in vivo. *Mol Cell* 29, 392-400.
- Lakowski, T.M., and Frankel, A. (2009). Kinetic analysis of human protein arginine N-methyltransferase 2: formation of monomethyl- and asymmetric dimethyl-arginine residues on histone H4. *Biochem J* 421, 253-261.

References

- Lam, C.T., Tang, C.M., Lau, K.W., and Lung, M.L. (2002). Loss of heterozygosity on chromosome 11 in esophageal squamous cell carcinomas. *Cancer Lett* 178, 75-81.
- Lane, D.P. (1992). Cancer. p53, guardian of the genome. *Nature* 358, 15-16.
- Lange, C.A. (2004). Making sense of cross-talk between steroid hormone receptors and intracellular signaling pathways: who will have the last word? *Mol Endocrinol* 18, 269-278.
- Lange, C.A., Shen, T.J., and Horwitz, K.B. (2000). Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. *Proc Natl Acad Sci U S A* 97, 1032-1037.
- Lavery, D.N., and McEwan, I.J. (2005). Structure and function of steroid receptor AF1 transactivation domains: induction of active conformations. *Biochem J* 391, 449-464.
- Lazar, M.A. (2003). Nuclear receptor corepressors. *Nucl Recept Signal* 1, e001.
- Lazennec, G., Bresson, D., Lucas, A., Chauveau, C., and Vignon, F. (2001). ER beta inhibits proliferation and invasion of breast cancer cells. *Endocrinology* 142, 4120-4130.
- Le Douarin, B., Zechel, C., Garnier, J.M., Lutz, Y., Tora, L., Pierrat, P., Heery, D., Gronemeyer, H., Chambon, P., and Losson, R. (1995). The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein T18. *EMBO J* 14, 2020-2033.
- Le Mellay, V., Grosse, B., and Lieberherr, M. (1997). Phospholipase C beta and membrane action of calcitriol and estradiol. *J Biol Chem* 272, 11902-11907.
- Le Romancer, M., Treilleux, I., Leconte, N., Robin-Lespinasse, Y., Sentis, S., Bouchekioua-Bouzaghoul, K., Goddard, S., Gobert-Gosse, S., and Corbo, L. (2008). Regulation of estrogen rapid signaling through arginine methylation by PRMT1. *Mol Cell* 31, 212-221.
- Lee, J., Sayegh, J., Daniel, J., Clarke, S., and Bedford, M.T. (2005a). PRMT8, a new membrane-bound tissue-specific member of the protein arginine methyltransferase family. *J Biol Chem* 280, 32890-32896.
- Lee, J.H., Cook, J.R., Yang, Z.H., Mirochnitchenko, O., Gunderson, S.I., Felix, A.M., Herth, N., Hoffmann, R., and Pestka, S. (2005b). PRMT7, a new protein arginine methyltransferase that synthesizes symmetric dimethylarginine. *J Biol Chem* 280, 3656-3664.
- Leo, J.C., Guo, C., Woon, C.T., Aw, S.E., and Lin, V.C. (2004). Glucocorticoid and mineralocorticoid cross-talk with progesterone receptor to induce focal adhesion and growth inhibition in breast cancer cells. *Endocrinology* 145, 1314-1321.

References

- Li, L.C., and Dahiya, R. (2002). MethPrimer: designing primers for methylation PCRs. *Bioinformatics* 18, 1427-1431.
- Li, X., Song, B., Xiang, S.H., and Sodroski, J. (2007). Functional interplay between the B-box 2 and the B30.2(SPRY) domains of TRIM5alpha. *Virology* 366, 234-244.
- Lichy, J.H., Zavar, M., Tsai, M.M., O'Leary, T.J., and Taubenberger, J.K. (1998). Loss of heterozygosity on chromosome 11p15 during histological progression in microdissected ductal carcinoma of the breast. *The American journal of pathology* 153, 271-278.
- Lienlaf, M., Hayashi, F., Di Nunzio, F., Tochio, N., Kigawa, T., Yokoyama, S., and Diaz-Griffero, F. (2011). Contribution of E3-ubiquitin ligase activity to HIV-1 restriction by TRIM5alpha(rh): structure of the RING domain of TRIM5alpha. *J Virol* 85, 8725-8737.
- Lin, W.J., Gary, J.D., Yang, M.C., Clarke, S., and Herschman, H.R. (1996). The mammalian immediate-early TIS21 protein and the leukemia-associated BTG1 protein interact with a protein-arginine N-methyltransferase. *J Biol Chem* 271, 15034-15044.
- Linke, S.P., Clarkin, K.C., Di Leonardo, A., Tsou, A., and Wahl, G.M. (1996). A reversible, p53-dependent G0/G1 cell cycle arrest induced by ribonucleotide depletion in the absence of detectable DNA damage. *Genes Dev* 10, 934-947.
- Liu, D.P., Song, H., and Xu, Y. (2010). A common gain of function of p53 cancer mutants in inducing genetic instability. *Oncogene* 29, 949-956.
- Lo, H.S., Wang, Z., Hu, Y., Yang, H.H., Gere, S., Buetow, K.H., and Lee, M.P. (2003). Allelic variation in gene expression is common in the human genome. *Genome Res* 13, 1855-1862.
- Lu, N.Z., Wardell, S.E., Burnstein, K.L., Defranco, D., Fuller, P.J., Giguere, V., Hochberg, R.B., McKay, L., Renoir, J.M., Weigel, N.L., *et al.* (2006). International Union of Pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily: glucocorticoid, mineralocorticoid, progesterone, and androgen receptors. *Pharmacol Rev* 58, 782-797.
- Lubahn, D.B., Moyer, J.S., Golding, T.S., Couse, J.F., Korach, K.S., and Smithies, O. (1993). Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc Natl Acad Sci U S A* 90, 11162-11166.
- Luisi, B.F., Xu, W.X., Otwinowski, Z., Freedman, L.P., Yamamoto, K.R., and Sigler, P.B. (1991). Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature* 352, 497-505.
- Lupas, A. (1996). Coiled coils: new structures and new functions. *Trends Biochem Sci* 21, 375-382.

References

- Lupien, M., and Brown, M. (2009). Cistromics of hormone-dependent cancer. *Endocr Relat Cancer* 16, 381-389.
- Lupien, M., Eeckhoute, J., Meyer, C.A., Krum, S.A., Rhodes, D.R., Liu, X.S., and Brown, M. (2009). Coactivator function defines the active estrogen receptor alpha cistrome. *Mol Cell Biol* 29, 3413-3423.
- Lynch, H.T., Silva, E., Snyder, C., and Lynch, J.F. (2008). Hereditary breast cancer: part I. Diagnosing hereditary breast cancer syndromes. *Breast J* 14, 3-13.
- Lyons, L.S., Rao, S., Balkan, W., Faysal, J., Maiorino, C.A., and Burnstein, K.L. (2008). Ligand-independent activation of androgen receptors by Rho GTPase signaling in prostate cancer. *Mol Endocrinol* 22, 597-608.
- MacGregor, J.I., and Jordan, V.C. (1998). Basic guide to the mechanisms of antiestrogen action. *Pharmacol Rev* 50, 151-196.
- Mader, S., Kumar, V., de Verneuil, H., and Chambon, P. (1989). Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid-responsive element. *Nature* 338, 271-274.
- Majumder, S., Liu, Y., Ford, O.H., 3rd, Mohler, J.L., and Whang, Y.E. (2006). Involvement of arginine methyltransferase CARM1 in androgen receptor function and prostate cancer cell viability. *Prostate* 66, 1292-1301.
- Maor, S., Mayer, D., Yarden, R.I., Lee, A.V., Sarfstein, R., Werner, H., and Papa, M.Z. (2006). Estrogen receptor regulates insulin-like growth factor-I receptor gene expression in breast tumor cells: involvement of transcription factor Sp1. *J Endocrinol* 191, 605-612.
- Martin, H.M., Filipe, M.I., Morris, R.W., Lane, D.P., and Silvestre, F. (1992). p53 expression and prognosis in gastric carcinoma. *Int J Cancer* 50, 859-862.
- Maruani, D.M., Spiegel, T.N., Harris, E.N., Shachter, A.S., Unger, H.A., Herrero-Gonzalez, S., and Holz, M.K. (2012). Estrogenic regulation of S6K1 expression creates a positive regulatory loop in control of breast cancer cell proliferation. *Oncogene*, doi: 10.1038/onc.2011.1657.
- Marusyk, A., Wheeler, L.J., Mathews, C.K., and DeGregori, J. (2007). p53 mediates senescence-like arrest induced by chronic replicational stress. *Mol Cell Biol* 27, 5336-5351.
- Masuihiro, Y., Mezaki, Y., Sakari, M., Takeyama, K., Yoshida, T., Inoue, K., Yanagisawa, J., Hanazawa, S., O'Malley B, W., and Kato, S. (2005). Splicing potentiation by growth factor signals via estrogen receptor phosphorylation. *Proc Natl Acad Sci U S A* 102, 8126-8131.

References

- Mathioudaki, K., Papadokostopoulou, A., Scorilas, A., Xynopoulos, D., Agnanti, N., and Talieri, M. (2008). The PRMT1 gene expression pattern in colon cancer. *Br J Cancer* *99*, 2094-2099.
- Mathioudaki, K., Scorilas, A., Ardavanis, A., Lymberi, P., Tsiambas, E., Devetzi, M., Apostolaki, A., and Talieri, M. (2011). Clinical evaluation of PRMT1 gene expression in breast cancer. *Tumour Biol* *32*, 575-582.
- Matthews, J.M., and Sunde, M. (2002). Zinc fingers--folds for many occasions. *IUBMB Life* *54*, 351-355.
- McEwan, I.J. (2004). Molecular mechanisms of androgen receptor-mediated gene regulation: structure-function analysis of the AF-1 domain. *Endocrine-related cancer* *11*, 281-293.
- Medunjanin, S., Hermani, A., De Servi, B., Grisouard, J., Rincke, G., and Mayer, D. (2005). Glycogen synthase kinase-3 interacts with and phosphorylates estrogen receptor alpha and is involved in the regulation of receptor activity. *The Journal of biological chemistry* *280*, 33006-33014.
- Meister, G., Eggert, C., Buhler, D., Brahms, H., Kambach, C., and Fischer, U. (2001). Methylation of Sm proteins by a complex containing PRMT5 and the putative U snRNP assembly factor pICln. *Curr Biol* *11*, 1990-1994.
- Melnick, A., and Licht, J.D. (1999). Deconstructing a disease: RARalpha, its fusion partners, and their roles in the pathogenesis of acute promyelocytic leukemia. *Blood* *93*, 3167-3215.
- Metivier, R., Penot, G., Flouriot, G., and Pakdel, F. (2001). Synergism between ERalpha transactivation function 1 (AF-1) and AF-2 mediated by steroid receptor coactivator protein-1: requirement for the AF-1 alpha-helical core and for a direct interaction between the N- and C-terminal domains. *Mol Endocrinol* *15*, 1953-1970.
- Meyer, R., Wolf, S.S., and Obendorf, M. (2007). PRMT2, a member of the protein arginine methyltransferase family, is a coactivator of the androgen receptor. *J Steroid Biochem Mol Biol* *107*, 1-14.
- Michalides, R., Griekspoor, A., Balkenende, A., Verwoerd, D., Janssen, L., Jalink, K., Floore, A., Velds, A., van't Veer, L., and Neeffjes, J. (2004). Tamoxifen resistance by a conformational arrest of the estrogen receptor alpha after PKA activation in breast cancer. *Cancer cell* *5*, 597-605.
- Michaud-Levesque, J., and Richard, S. (2009). Thrombospondin-1 is a transcriptional repression target of PRMT6. *J Biol Chem* *284*, 21338-21346.
- Min, J., Feng, Q., Li, Z., Zhang, Y., and Xu, R.M. (2003). Structure of the catalytic domain of human DOT1L, a non-SET domain nucleosomal histone methyltransferase. *Cell* *112*, 711-723.

References

Miranda-Carboni, G.A., Guemes, M., Bailey, S., Anaya, E., Corselli, M., Peault, B., and Krum, S.A. (2011). GATA4 regulates estrogen receptor-alpha-mediated osteoblast transcription. *Mol Endocrinol* 25, 1126-1136.

Mostaqul Huq, M.D., Gupta, P., Tsai, N.P., White, R., Parker, M.G., and Wei, L.N. (2006). Suppression of receptor interacting protein 140 repressive activity by protein arginine methylation. *EMBO J* 25, 5094-5104.

Mulac-Jericevic, B., Lydon, J.P., DeMayo, F.J., and Conneely, O.M. (2003). Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform. *Proc Natl Acad Sci U S A* 100, 9744-9749.

Murphy, L., Cherlet, T., Adeyinka, A., Niu, Y., Snell, L., and Watson, P. (2004). Phospho-serine-118 estrogen receptor-alpha detection in human breast tumors in vivo. *Clinical cancer research : an official journal of the American Association for Cancer Research* 10, 1354-1359.

Musgrove, E.A., and Sutherland, R.L. (2009). Biological determinants of endocrine resistance in breast cancer. *Nature reviews Cancer* 9, 631-643.

Naeem, H., Cheng, D., Zhao, Q., Underhill, C., Tini, M., Bedford, M.T., and Torchia, J. (2007a). The activity and stability of the transcriptional coactivator p/CIP/SRC-3 are regulated by CARM1-dependent methylation. *Mol Cell Biol* 27, 120-134.

Naeem, H., Cheng, D., Zhao, Q., Underhill, C., Tini, M., Bedford, M.T., and Torchia, J. (2007b). The activity and stability of the transcriptional coactivator p/CIP/SRC-3 are regulated by CARM1-dependent methylation. *Mol Cell Biol* 27, 120-134.

Nisole, S., Stoye, J.P., and Saib, A. (2005). TRIM family proteins: retroviral restriction and antiviral defence. *Nat Rev Microbiol* 3, 799-808.

O'Briant, K.C., and Bepler, G. (1997). Delineation of the centromeric and telomeric chromosome segment 11p15.5 lung cancer suppressor regions LOH11A and LOH11B. *Genes Chromosomes Cancer* 18, 111-114.

Obad, S., Brunnstrom, H., Vallon-Christersson, J., Borg, A., Drott, K., and Gullberg, U. (2004). Staf50 is a novel p53 target gene conferring reduced clonogenic growth of leukemic U-937 cells. *Oncogene* 23, 4050-4059.

Obad, S., Olofsson, T., Mechti, N., Gullberg, U., and Drott, K. (2007). Expression of the IFN-inducible p53-target gene TRIM22 is down-regulated during erythroid differentiation of human bone marrow. *Leuk Res* 31, 995-1001.

Oda, K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T., Nishimori, H., Tamai, K., Tokino, T., Nakamura, Y., *et al.* (2000). p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell* 102, 849-862.

References

- Ogawa, M., Onoda, N., Maeda, K., Kato, Y., Nakata, B., Kang, S.M., Sowa, M., and Hirakawa, K. (2001). A combination analysis of p53 and p21 in gastric carcinoma as a strong indicator for prognosis. *Int J Mol Med* 7, 479-483.
- Okada, S., Tokunaga, E., Kitao, H., Akiyoshi, S., Yamashita, N., Saeki, H., Oki, E., Morita, M., Kakeji, Y., and Maehara, Y. (2012). Loss of heterozygosity at BRCA1 locus is significantly associated with aggressiveness and poor prognosis in breast cancer. *Ann Surg Oncol* 19, 1499-1507.
- Oonate, S.A., Boonyaratanakornkit, V., Spencer, T.E., Tsai, S.Y., Tsai, M.J., Edwards, D.P., and O'Malley, B.W. (1998). The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 domains of steroid receptors. *J Biol Chem* 273, 12101-12108.
- Oonate, S.A., Tsai, S.Y., Tsai, M.J., and O'Malley, B.W. (1995). Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* 270, 1354-1357.
- Oren, M., and Rotter, V. (2010). Mutant p53 gain-of-function in cancer. *Cold Spring Harb Perspect Biol* 2, a001107.
- Ozato, K., Shin, D.M., Chang, T.H., and Morse, H.C., 3rd (2008). TRIM family proteins and their emerging roles in innate immunity. *Nat Rev Immunol* 8, 849-860.
- Pak, M.L., Lakowski, T.M., Thomas, D., Vhuiyan, M.I., Husecken, K., and Frankel, A. (2011). A protein arginine N-methyltransferase 1 (PRMT1) and 2 heteromeric interaction increases PRMT1 enzymatic activity. *Biochemistry* 50, 8226-8240.
- Pallis, L., Wilking, N., Cedermark, B., Rutqvist, L.E., and Skoog, L. (1992). Receptors for estrogen and progesterone in breast carcinoma in situ. *Anticancer Res* 12, 2113-2115.
- Pang, D., Kocherginsky, M., Krausz, T., Kim, S.Y., and Conzen, S.D. (2006). Dexamethasone decreases xenograft response to Paclitaxel through inhibition of tumor cell apoptosis. *Cancer Biol Ther* 5, 933-940.
- Park, B.W., Kim, K.S., Heo, M.K., Ko, S.S., Hong, S.W., Yang, W.I., Kim, J.H., Kim, G.E., and Lee, K.S. (2003). Expression of estrogen receptor-beta in normal mammary and tumor tissues: is it protective in breast carcinogenesis? *Breast Cancer Res Treat* 80, 79-85.
- Park, H.Y., Kim, M.K., Moon, S.I., Cho, Y.H., and Lee, C.H. (2006). Cell cycle arrest and apoptotic induction in LNCaP cells by MCS-C2, novel cyclin-dependent kinase inhibitor, through p53/p21WAF1/CIP1 pathway. *Cancer Sci* 97, 430-436.
- Park, Y., Park, J., Lee, Y., Lim, W., Oh, B.C., Shin, C., and Kim, W. (2011). Mammalian MST2 kinase and human Salvador activate and reduce estrogen receptor alpha in the absence of ligand. *J Mol Med (Berl)* 89, 181-191.

References

- Pedram, A., Razandi, M., Sainson, R.C., Kim, J.K., Hughes, C.C., and Levin, E.R. (2007). A conserved mechanism for steroid receptor translocation to the plasma membrane. *J Biol Chem* 282, 22278-22288.
- Pellegata, N.S., Antoniono, R.J., Redpath, J.L., and Stanbridge, E.J. (1996). DNA damage and p53-mediated cell cycle arrest: a reevaluation. *Proc Natl Acad Sci U S A* 93, 15209-15214.
- Perlman, E.J., Grundy, P.E., Anderson, J.R., Jennings, L.J., Green, D.M., Dome, J.S., Shamberger, R.C., Ruteshouser, E.C., and Huff, V. (2011). WT1 mutation and 11P15 loss of heterozygosity predict relapse in very low-risk wilms tumors treated with surgery alone: a children's oncology group study. *J Clin Oncol* 29, 698-703.
- Pestka, S. (2003). A dance between interferon-alpha/beta and p53 demonstrates collaborations in tumor suppression and antiviral activities. *Cancer Cell* 4, 85-87.
- Pett, M.R., Herdman, M.T., Palmer, R.D., Yeo, G.S., Shivji, M.K., Stanley, M.A., and Coleman, N. (2006). Selection of cervical keratinocytes containing integrated HPV16 associates with episome loss and an endogenous antiviral response. *Proc Natl Acad Sci U S A* 103, 3822-3827.
- Phalke, S., Mzoughi, S., Bezzi, M., Jennifer, N., Mok, W.C., Low, D.H., Thike, A.A., Kuznetsov, V.A., Tan, P.H., Voorhoeve, P.M., *et al.* (2012). p53-Independent regulation of p21Waf1/Cip1 expression and senescence by PRMT6. *Nucleic Acids Res* 40, 9534-9542.
- Picard, F., Wanatabe, M., Schoonjans, K., Lydon, J., O'Malley, B.W., and Auwerx, J. (2002). Progesterone receptor knockout mice have an improved glucose homeostasis secondary to beta -cell proliferation. *Proc Natl Acad Sci U S A* 99, 15644-15648.
- Pierson-Mullany, L.K., and Lange, C.A. (2004a). Phosphorylation of progesterone receptor serine 400 mediates ligand-independent transcriptional activity in response to activation of cyclin-dependent protein kinase 2. *Mol Cell Biol* 24, 10542-10557.
- Pierson-Mullany, L.K., and Lange, C.A. (2004b). Phosphorylation of progesterone receptor serine 400 mediates ligand-independent transcriptional activity in response to activation of cyclin-dependent protein kinase 2. *Mol Cell Biol* 24, 10542-10557.
- Piette, J., Neel, H., and Marechal, V. (1997). Mdm2: keeping p53 under control. *Oncogene* 15, 1001-1010.
- Polyak, K., Hamilton, S.R., Vogelstein, B., and Kinzler, K.W. (1996). Early alteration of cell-cycle-regulated gene expression in colorectal neoplasia. *Am J Pathol* 149, 381-387.
- Porter, W., Saville, B., Hoivik, D., and Safe, S. (1997). Functional synergy between the transcription factor Sp1 and the estrogen receptor. *Mol Endocrinol* 11, 1569-1580.

References

- Powell, E., Wang, Y., Shapiro, D.J., and Xu, W. (2010). Differential requirements of Hsp90 and DNA for the formation of estrogen receptor homodimers and heterodimers. *J Biol Chem* 285, 16125-16134.
- Pratt, W.B. (1998). The hsp90-based chaperone system: involvement in signal transduction from a variety of hormone and growth factor receptors. *Proc Soc Exp Biol Med* 217, 420-434.
- Pratt, W.B., and Toft, D.O. (1997a). Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr Rev* 18, 306-360.
- Pratt, W.B., and Toft, D.O. (1997b). Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr Rev* 18, 306-360.
- Pratt, W.B., and Toft, D.O. (2003a). Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Exp Biol Med* (Maywood) 228, 111-133.
- Pratt, W.B., and Toft, D.O. (2003b). Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Exp Biol Med* 228, 111-133.
- Qi, C., Chang, J., Zhu, Y., Yeldandi, A.V., Rao, S.M., and Zhu, Y.J. (2002). Identification of protein arginine methyltransferase 2 as a coactivator for estrogen receptor alpha. *J Biol Chem* 277, 28624-28630.
- Radoja, N., Komine, M., Jho, S.H., Blumenberg, M., and Tomic-Canic, M. (2000). Novel mechanism of steroid action in skin through glucocorticoid receptor monomers. *Mol Cell Biol* 20, 4328-4339.
- Rajbhandari, P., Finn, G., Solodin, N.M., Singarapu, K.K., Sahu, S.C., Markley, J.L., Kadunc, K.J., Ellison-Zelski, S.J., Kariagina, A., Haslam, S.Z., *et al.* (2011). Regulation of estrogen receptor alpha N-terminus conformation and function by peptidyl prolyl isomerase Pin1. *Mol Cell Biol* 32, 445-457.
- Ravdin, P.M., Green, S., Dorr, T.M., McGuire, W.L., Fabian, C., Pugh, R.P., Carter, R.D., Rivkin, S.E., Borst, J.R., Belt, R.J., *et al.* (1992). Prognostic significance of progesterone receptor levels in estrogen receptor-positive patients with metastatic breast cancer treated with tamoxifen: results of a prospective Southwest Oncology Group study. *J Clin Oncol* 10, 1284-1291.
- Reddy, B.A., Etkin, L.D., and Freemont, P.S. (1992). A novel zinc finger coiled-coil domain in a family of nuclear proteins. *Trends Biochem Sci* 17, 344-345.
- Reik, W., and Walter, J. (2001). Genomic imprinting: parental influence on the genome. *Nat Rev Genet* 2, 21-32.

References

- Renne, R., Barry, C., Dittmer, D., Compitello, N., Brown, P.O., and Ganem, D. (2001). Modulation of cellular and viral gene expression by the latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus. *J Virol* 75, 458-468.
- Reymond, A., Meroni, G., Fantozzi, A., Merla, G., Cairo, S., Luzi, L., Riganelli, D., Zanaria, E., Messali, S., Cainarca, S., *et al.* (2001). The tripartite motif family identifies cell compartments. *EMBO J* 20, 2140-2151.
- Rho, J., Choi, S., Seong, Y.R., Cho, W.K., Kim, S.H., and Im, D.S. (2001). Prmt5, which forms distinct homo-oligomers, is a member of the protein-arginine methyltransferase family. *J Biol Chem* 276, 11393-11401.
- Robker, R.L., Russell, D.L., Espey, L.L., Lydon, J.P., O'Malley, B.W., and Richards, J.S. (2000). Progesterone-regulated genes in the ovulation process: ADAMTS-1 and cathepsin L proteases. *Proc Natl Acad Sci U S A* 97, 4689-4694.
- Rogatsky, I., Trowbridge, J.M., and Garabedian, M.J. (1999). Potentiation of human estrogen receptor alpha transcriptional activation through phosphorylation of serines 104 and 106 by the cyclin A-CDK2 complex. *The Journal of biological chemistry* 274, 22296-22302.
- Rogatsky, I., Zarembek, K.A., and Yamamoto, K.R. (2001). Factor recruitment and TIF2/GRIPI corepressor activity at a collagenase-3 response element that mediates regulation by phorbol esters and hormones. *EMBO J* 20, 6071-6083.
- Romanczuk, H., and Howley, P.M. (1992). Disruption of either the E1 or the E2 regulatory gene of human papillomavirus type 16 increases viral immortalization capacity. *Proc Natl Acad Sci U S A* 89, 3159-3163.
- Rowan, B.G., Garrison, N., Weigel, N.L., and O'Malley, B.W. (2000). 8-Bromo-cyclic AMP induces phosphorylation of two sites in SRC-1 that facilitate ligand-independent activation of the chicken progesterone receptor and are critical for functional cooperation between SRC-1 and CREB binding protein. *Mol Cell Biol* 20, 8720-8730.
- Ruike, Y., Imanaka, Y., Sato, F., Shimizu, K., and Tsujimoto, G. (2010). Genome-wide analysis of aberrant methylation in human breast cancer cells using methyl-DNA immunoprecipitation combined with high-throughput sequencing. *BMC Genomics* 11, 137.
- Sarquis, M.S., Weber, F., Shen, L., Broelsch, C.E., Jhiang, S.M., Zedenius, J., Frilling, A., and Eng, C. (2006). High frequency of loss of heterozygosity in imprinted, compared with nonimprinted, genomic regions in follicular thyroid carcinomas and atypical adenomas. *J Clin Endocrinol Metab* 91, 262-269.
- Sartorius, C.A., Melville, M.Y., Hovland, A.R., Tung, L., Takimoto, G.S., and Horwitz, K.B. (1994). A third transactivation function (AF3) of human progesterone receptors located in the unique N-terminal segment of the B-isoform. *Mol Endocrinol* 8, 1347-1360.

References

- Saurin, A.J., Borden, K.L., Boddy, M.N., and Freemont, P.S. (1996). Does this have a familiar RING? *Trends Biochem Sci* 21, 208-214.
- Savkur, R.S., and Burris, T.P. (2004). The coactivator LXXLL nuclear receptor recognition motif. *J Pept Res* 63, 207-212.
- Sawyer, S.L., Emerman, M., and Malik, H.S. (2007). Discordant evolution of the adjacent antiretroviral genes TRIM22 and TRIM5 in mammals. *PLoS Pathog* 3, e197.
- Schapira, M. (2011). Structural Chemistry of Human SET Domain Protein Methyltransferases. *Curr Chem Genomics* 5, 85-94.
- Schiebe, M., Ohneseit, P., Hoffmann, W., Meyermann, R., Rodemann, H.P., and Bamberg, M. (2001). Loss of heterozygosity at 11p15 and p53 alterations in malignant gliomas. *Journal of cancer research and clinical oncology* 127, 325-328.
- Schmitt, F.C. (1995). Multistep progression from an oestrogen-dependent growth towards an autonomous growth in breast carcinogenesis. *Eur J Cancer* 31A, 2049-2052.
- Schumacher, M., Guennoun, R., Robert, F., Carelli, C., Gago, N., Ghoumari, A., Gonzalez Deniselle, M.C., Gonzalez, S.L., Ibanez, C., Labombarda, F., *et al.* (2004). Local synthesis and dual actions of progesterone in the nervous system: neuroprotection and myelination. *Growth Horm IGF Res* 14 Suppl A, S18-33.
- Schwabe, J.W., Chapman, L., Finch, J.T., and Rhodes, D. (1993a). The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. *Cell* 75, 567-578.
- Schwabe, J.W., Chapman, L., Finch, J.T., Rhodes, D., and Neuhaus, D. (1993b). DNA recognition by the oestrogen receptor: from solution to the crystal. *Structure* 1, 187-204.
- Schwamborn, J.C., Berezikov, E., and Knoblich, J.A. (2009). The TRIM-NHL protein TRIM32 activates microRNAs and prevents self-renewal in mouse neural progenitors. *Cell* 136, 913-925.
- Schwienbacher, C., Gramantieri, L., Scelfo, R., Veronese, A., Calin, G.A., Bolondi, L., Croce, C.M., Barbanti-Brodano, G., and Negrini, M. (2000). Gain of imprinting at chromosome 11p15: A pathogenetic mechanism identified in human hepatocarcinomas. *Proc Natl Acad Sci U S A* 97, 5445-5449.
- Scorilas, A., Black, M.H., Talieri, M., and Diamandis, E.P. (2000). Genomic organization, physical mapping, and expression analysis of the human protein arginine methyltransferase 1 gene. *Biochem Biophys Res Commun* 278, 349-359.
- Sebastian, S., and Luban, J. (2005). TRIM5alpha selectively binds a restriction-sensitive retroviral capsid. *Retrovirology* 2, 40.

References

- Sheeler, C.Q., Singleton, D.W., and Khan, S.A. (2003). Mutation of serines 104, 106, and 118 inhibits dimerization of the human estrogen receptor in yeast. *Endocr Res* 29, 237-255.
- Shen, Y., and White, E. (2001). p53-dependent apoptosis pathways. *Adv Cancer Res* 82, 55-84.
- Shi, X., Kachirskaja, I., Yamaguchi, H., West, L.E., Wen, H., Wang, E.W., Dutta, S., Appella, E., and Gozani, O. (2007). Modulation of p53 function by SET8-mediated methylation at lysine 382. *Mol Cell* 27, 636-646.
- Shoker, B.S., Jarvis, C., Sibson, D.R., Walker, C., and Sloane, J.P. (1999). Oestrogen receptor expression in the normal and pre-cancerous breast. *J Pathol* 188, 237-244.
- Simental, J.A., Sar, M., Lane, M.V., French, F.S., and Wilson, E.M. (1991). Transcriptional activation and nuclear targeting signals of the human androgen receptor. *J Biol Chem* 266, 510-518.
- Simoncini, T., Hafezi-Moghadam, A., Brazil, D.P., Ley, K., Chin, W.W., and Liao, J.K. (2000). Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* 407, 538-541.
- Singh, R., Gaiha, G., Werner, L., McKim, K., Mlisana, K., Luban, J., Walker, B.D., Karim, S.S., Brass, A.L., and Ndung'u, T. (2011). Association of TRIM22 with the type 1 interferon response and viral control during primary HIV-1 infection. *J Virol* 85, 208-216.
- Singh, S., Simon, M., Meybohm, I., Jantke, I., Jonat, W., Maass, H., and Goedde, H.W. (1993). Human breast cancer: frequent p53 allele loss and protein overexpression. *Hum Genet* 90, 635-640.
- Sinkevicius, K.W., Burdette, J.E., Woloszyn, K., Hewitt, S.C., Hamilton, K., Sugg, S.L., Temple, K.A., Wondisford, F.E., Korach, K.S., Woodruff, T.K., *et al.* (2008). An estrogen receptor- α knock-in mutation provides evidence of ligand-independent signaling and allows modulation of ligand-induced pathways in vivo. *Endocrinology* 149, 2970-2979.
- Sivaramakrishnan, G., Sun, Y., Rajmohan, R., and Lin, V.C. (2009). B30.2/SPRY domain in tripartite motif-containing 22 is essential for the formation of distinct nuclear bodies. *FEBS Lett* 583, 2093-2099.
- Skafar, D.F., and Zhao, C. (2008). The multifunctional estrogen receptor- α F domain. *Endocrine* 33, 1-8.
- Skildum, A., Faivre, E., and Lange, C.A. (2005). Progesterone receptors induce cell cycle progression via activation of mitogen-activated protein kinases. *Mol Endocrinol* 19, 327-339.

References

- Skliris, G.P., Nugent, Z.J., Rowan, B.G., Penner, C.R., Watson, P.H., and Murphy, L.C. (2010). A phosphorylation code for oestrogen receptor- α predicts clinical outcome to endocrine therapy in breast cancer. *Endocr Relat Cancer* 17, 589-597.
- Smiraglia, D.J., Rush, L.J., Fruhwald, M.C., Dai, Z., Held, W.A., Costello, J.F., Lang, J.C., Eng, C., Li, B., Wright, F.A., *et al.* (2001). Excessive CpG island hypermethylation in cancer cell lines versus primary human malignancies. *Hum Mol Genet* 10, 1413-1419.
- Smith, D.F., and Toft, D.O. (1993). Steroid receptors and their associated proteins. *Mol Endocrinol* 7, 4-11.
- Soejima, H., and Wagstaff, J. (2005). Imprinting centers, chromatin structure, and disease. *J Cell Biochem* 95, 226-233.
- Solomon, H., Madar, S., and Rotter, V. (2011). Mutant p53 gain of function is interwoven into the hallmarks of cancer. *J Pathol* 225, 475-478.
- Soong, R., Robbins, P.D., Dix, B.R., Grieu, F., Lim, B., Knowles, S., Williams, K.E., Turbett, G.R., House, A.K., and Iacopetta, B.J. (1996). Concordance between p53 protein overexpression and gene mutation in a large series of common human carcinomas. *Hum Pathol* 27, 1050-1055.
- Sorlie, T., Perou, C.M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., *et al.* (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* 98, 10869-10874.
- Soussi, T., and Lozano, G. (2005). p53 mutation heterogeneity in cancer. *Biochem Biophys Res Commun* 331, 834-842.
- Spencer, T.E., Jenster, G., Burcin, M.M., Allis, C.D., Zhou, J., Mizzen, C.A., McKenna, N.J., Onate, S.A., Tsai, S.Y., Tsai, M.J., *et al.* (1997). Steroid receptor coactivator-1 is a histone acetyltransferase. *Nature* 389, 194-198.
- Street, S.E., Cretney, E., and Smyth, M.J. (2001). Perforin and interferon- γ activities independently control tumor initiation, growth, and metastasis. *Blood* 97, 192-197.
- Strehl, C., Gaber, T., Lowenberg, M., Hommes, D.W., Verhaar, A.P., Schellmann, S., Hahne, M., Fangradt, M., Wagegg, M., Hoff, P., *et al.* (2011). Origin and functional activity of the membrane-bound glucocorticoid receptor. *Arthritis Rheum* 63, 3779-3788.
- Strom, A., Hartman, J., Foster, J.S., Kietz, S., Wimalasena, J., and Gustafsson, J.A. (2004). Estrogen receptor β inhibits 17 β -estradiol-stimulated proliferation of the breast cancer cell line T47D. *Proc Natl Acad Sci U S A* 101, 1566-1571.

References

Subramanian, K., Jia, D., Kapoor-Vazirani, P., Powell, D.R., Collins, R.E., Sharma, D., Peng, J., Cheng, X., and Vertino, P.M. (2008). Regulation of estrogen receptor alpha by the SET7 lysine methyltransferase. *Mol Cell* 30, 336-347.

Sun, M., Paciga, J.E., Feldman, R.I., Yuan, Z., Coppola, D., Lu, Y.Y., Shelley, S.A., Nicosia, S.V., and Cheng, J.Q. (2001). Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor alpha (ERalpha) via interaction between ERalpha and PI3K. *Cancer Res* 61, 5985-5991.

Swiercz, R., Person, M.D., and Bedford, M.T. (2005). Ribosomal protein S2 is a substrate for mammalian PRMT3 (protein arginine methyltransferase 3). *Biochem J* 386, 85-91.

Sykes, S.M., Mellert, H.S., Holbert, M.A., Li, K., Marmorstein, R., Lane, W.S., and McMahon, S.B. (2006). Acetylation of the p53 DNA-binding domain regulates apoptosis induction. *Mol Cell* 24, 841-851.

Szyf, M. (2012). DNA methylation signatures for breast cancer classification and prognosis. *Genome Med* 4, 26.

Takahashi, M., Inaguma, Y., Hiai, H., and Hirose, F. (1988). Developmentally regulated expression of a human "finger"-containing gene encoded by the 5' half of the ret transforming gene. *Mol Cell Biol* 8, 1853-1856.

Takaoka, A., Hayakawa, S., Yanai, H., Stoiber, D., Negishi, H., Kikuchi, H., Sasaki, S., Imai, K., Shibue, T., Honda, K., *et al.* (2003). Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence. *Nature* 424, 516-523.

Takeshita, A., Cardona, G.R., Koibuchi, N., Suen, C.S., and Chin, W.W. (1997). TRAM-1, A novel 160-kDa thyroid hormone receptor activator molecule, exhibits distinct properties from steroid receptor coactivator-1. *J Biol Chem* 272, 27629-27634.

Takimoto, G.S., Tasset, D.M., Eppert, A.C., and Horwitz, K.B. (1992). Hormone-induced progesterone receptor phosphorylation consists of sequential DNA-independent and DNA-dependent stages: analysis with zinc finger mutants and the progesterone antagonist ZK98299. *Proc Natl Acad Sci U S A* 89, 3050-3054.

Takimoto, G.S., Tung, L., Abdel-Hafiz, H., Abel, M.G., Sartorius, C.A., Richer, J.K., Jacobsen, B.M., Bain, D.L., and Horwitz, K.B. (2003). Functional properties of the N-terminal region of progesterone receptors and their mechanistic relationship to structure. *The Journal of steroid biochemistry and molecular biology* 85, 209-219.

Taneda, T., Miyata, S., Kousaka, A., Inoue, K., Koyama, Y., Mori, Y., and Tohyama, M. (2007). Specific regional distribution of protein arginine methyltransferase 8 (PRMT8) in the mouse brain. *Brain Res* 1155, 1-9.

References

- Tang, J., Frankel, A., Cook, R.J., Kim, S., Paik, W.K., Williams, K.R., Clarke, S., and Herschman, H.R. (2000). PRMT1 is the predominant type I protein arginine methyltransferase in mammalian cells. *J Biol Chem* 275, 7723-7730.
- Tasker, J.G., Di, S., and Malcher-Lopes, R. (2006). Minireview: rapid glucocorticoid signaling via membrane-associated receptors. *Endocrinology* 147, 5549-5556.
- Tetel, M.J., Giangrande, P.H., Leonhardt, S.A., McDonnell, D.P., and Edwards, D.P. (1999). Hormone-dependent interaction between the amino- and carboxyl-terminal domains of progesterone receptor in vitro and in vivo. *Mol Endocrinol* 13, 910-924.
- Tezel, G.G., Uner, A., Yildiz, I., Guler, G., and Takahashi, M. (2009). RET finger protein expression in invasive breast carcinoma: relationship between RFP and ErbB2 expression. *Pathol Res Pract* 205, 403-408.
- Tharakan, R., Lepont, P., Singleton, D., Kumar, R., and Khan, S. (2008). Phosphorylation of estrogen receptor alpha, serine residue 305 enhances activity. *Mol Cell Endocrinol* 295, 70-78.
- Thomas, R.S., Sarwar, N., Phoenix, F., Coombes, R.C., and Ali, S. (2008). Phosphorylation at serines 104 and 106 by Erk1/2 MAPK is important for estrogen receptor-alpha activity. *J Mol Endocrinol* 40, 173-184.
- Thornton, J.W., Need, E., and Crews, D. (2003). Resurrecting the ancestral steroid receptor: ancient origin of estrogen signaling. *Science* 301, 1714-1717.
- Tissot, C., and Mechti, N. (1995). Molecular cloning of a new interferon-induced factor that represses human immunodeficiency virus type 1 long terminal repeat expression. *J Biol Chem* 270, 14891-14898.
- Tissot, C., Taviaux, S.A., Diriong, S., and Mechti, N. (1996). Localization of Staf50, a member of the Ring finger family, to 11p15 by fluorescence in situ hybridization. *Genomics* 34, 151-153.
- Toillon, R.A., Chopin, V., Jouy, N., Fauquette, W., Boilly, B., and Le Bourhis, X. (2002). Normal breast epithelial cells induce p53-dependent apoptosis and p53-independent cell cycle arrest of breast cancer cells. *Breast Cancer Res Treat* 71, 269-280.
- Toniato, E., Chen, X.P., Losman, J., Flati, V., Donahue, L., and Rothman, P. (2002). TRIM8/GERP RING finger protein interacts with SOCS-1. *J Biol Chem* 277, 37315-37322.
- Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E., and Chambon, P. (1989). The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell* 59, 477-487.

References

- Treeck, O., Lattrich, C., Springwald, A., and Ortmann, O. (2009). Estrogen receptor beta exerts growth-inhibitory effects on human mammary epithelial cells. *Breast Cancer Res Treat* 120, 557-565.
- Tremblay, G.B., Tremblay, A., Labrie, F., and Giguere, V. (1998). Ligand-independent activation of the estrogen receptors alpha and beta by mutations of a conserved tyrosine can be abolished by antiestrogens. *Cancer Res* 58, 877-881.
- Truss, M., Bartsch, J., Chalepakis, G., and Beato, M. (1992). Artificial steroid hormone response element generated by dam-methylation. *Nucleic Acids Res* 20, 1483-1486.
- Truss, M., Chalepakis, G., and Beato, M. (1990). Contacts between steroid hormone receptors and thymines in DNA: an interference method. *Proc Natl Acad Sci U S A* 87, 7180-7184.
- Tsai, H.W., Katzenellenbogen, J.A., Katzenellenbogen, B.S., and Shupnik, M.A. (2004). Protein kinase A activation of estrogen receptor alpha transcription does not require proteasome activity and protects the receptor from ligand-mediated degradation. *Endocrinology* 145, 2730-2738.
- Tsukamoto, H., Kato, T., Enomoto, A., Nakamura, N., Shimono, Y., Jijiwa, M., Asai, N., Murakumo, Y., Shibata, K., Kikkawa, F., *et al.* (2009). Expression of Ret finger protein correlates with outcomes in endometrial cancer. *Cancer Sci* 100, 1895-1901.
- Tung, L., Abdel-Hafiz, H., Shen, T., Harvell, D.M., Nitao, L.K., Richer, J.K., Sartorius, C.A., Takimoto, G.S., and Horwitz, K.B. (2006). Progesterone receptors (PR)-B and -A regulate transcription by different mechanisms: AF-3 exerts regulatory control over coactivator binding to PR-B. *Mol Endocrinol* 20, 2656-2670.
- Uchil, P.D., Quinlan, B.D., Chan, W.T., Luna, J.M., and Mothes, W. (2008). TRIM E3 ligases interfere with early and late stages of the retroviral life cycle. *PLoS Pathog* 4, e16.
- Ueda, T., Mawji, N.R., Bruchovsky, N., and Sadar, M.D. (2002). Ligand-independent activation of the androgen receptor by interleukin-6 and the role of steroid receptor coactivator-1 in prostate cancer cells. *J Biol Chem* 277, 38087-38094.
- Varley, J.M., Brammar, W.J., Lane, D.P., Swallow, J.E., Dolan, C., and Walker, R.A. (1991). Loss of chromosome 17p13 sequences and mutation of p53 in human breast carcinomas. *Oncogene* 6, 413-421.
- Vikhanskaya, F., Lee, M.K., Mazzeletti, M., Brogini, M., and Sabapathy, K. (2007). Cancer-derived p53 mutants suppress p53-target gene expression--potential mechanism for gain of function of mutant p53. *Nucleic Acids Res* 35, 2093-2104.
- Voegel, J.J., Heine, M.J., Zechel, C., Chambon, P., and Gronemeyer, H. (1996). TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *EMBO J* 15, 3667-3675.

References

- Waldmann, T., Izzo, A., Kamieniarz, K., Richter, F., Vogler, C., Sarg, B., Lindner, H., Young, N.L., Mittler, G., Garcia, B.A., *et al.* (2011). Methylation of H2AR29 is a novel repressive PRMT6 target. *Epigenetics Chromatin* 4, 11.
- Wang, C., Fu, M., Angeletti, R.H., Siconolfi-Baez, L., Reutens, A.T., Albanese, C., Lisanti, M.P., Katzenellenbogen, B.S., Kato, S., Hopp, T., *et al.* (2001a). Direct acetylation of the estrogen receptor alpha hinge region by p300 regulates transactivation and hormone sensitivity. *J Biol Chem* 276, 18375-18383.
- Wang, C., Ivanov, A., Chen, L., Fredericks, W.J., Seto, E., Rauscher, F.J., 3rd, and Chen, J. (2005). MDM2 interaction with nuclear corepressor KAP1 contributes to p53 inactivation. *EMBO J* 24, 3279-3290.
- Wang, H., Huang, Z.Q., Xia, L., Feng, Q., Erdjument-Bromage, H., Strahl, B.D., Briggs, S.D., Allis, C.D., Wong, J., Tempst, P., *et al.* (2001b). Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor. *Science* 293, 853-857.
- Wang, Q., Li, W., Zhang, Y., Yuan, X., Xu, K., Yu, J., Chen, Z., Beroukhi, R., Wang, H., Lupien, M., *et al.* (2009). Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer. *Cell* 138, 245-256.
- Wang, Y., Gao, B., Xu, W., and Xiong, S. (2011). BRG1 is indispensable for IFN-gamma-induced TRIM22 expression, which is dependent on the recruitment of IRF-1. *Biochem Biophys Res Commun* 410, 549-554.
- Wang, Z.G., Ruggero, D., Ronchetti, S., Zhong, S., Gaboli, M., Rivi, R., and Pandolfi, P.P. (1998). PML is essential for multiple apoptotic pathways. *Nat Genet* 20, 266-272.
- Watson, C.S. (1999). Signaling themes shared between peptide and steroid hormones at the plasma membrane. *Sci STKE* 1999, PE1.
- Webb, P., Nguyen, P., Shinsako, J., Anderson, C., Feng, W., Nguyen, M.P., Chen, D., Huang, S.M., Subramanian, S., McKinerney, E., *et al.* (1998). Estrogen receptor activation function 1 works by binding p160 coactivator proteins. *Mol Endocrinol* 12, 1605-1618.
- Wei, C.L., Wu, Q., Vega, V.B., Chiu, K.P., Ng, P., Zhang, T., Shahab, A., Yong, H.C., Fu, Y., Weng, Z., *et al.* (2006). A global map of p53 transcription-factor binding sites in the human genome. *Cell* 124, 207-219.
- Weis, K.E., Ekena, K., Thomas, J.A., Lazennec, G., and Katzenellenbogen, B.S. (1996). Constitutively active human estrogen receptors containing amino acid substitutions for tyrosine 537 in the receptor protein. *Mol Endocrinol* 10, 1388-1398.
- Wieland, S., Thimme, R., Purcell, R.H., and Chisari, F.V. (2004). Genomic analysis of the host response to hepatitis B virus infection. *Proc Natl Acad Sci U S A* 101, 6669-6674.

References

- Williams, C., Edvardsson, K., Lewandowski, S.A., Strom, A., and Gustafsson, J.A. (2008). A genome-wide study of the repressive effects of estrogen receptor beta on estrogen receptor alpha signaling in breast cancer cells. *Oncogene* 27, 1019-1032.
- Wittmann, S., Wunder, C., Zirn, B., Furtwangler, R., Wegert, J., Graf, N., and Gessler, M. (2008). New prognostic markers revealed by evaluation of genes correlated with clinical parameters in Wilms tumors. *Genes Chromosomes Cancer* 47, 386-395.
- Wolf, S.S. (2009). The protein arginine methyltransferase family: an update about function, new perspectives and the physiological role in humans. *Cell Mol Life Sci* 66, 2109-2121.
- Wozniak, R.J., Klimecki, W.T., Lau, S.S., Feinstein, Y., and Futscher, B.W. (2007). 5-Aza-2'-deoxycytidine-mediated reductions in G9A histone methyltransferase and histone H3 K9 di-methylation levels are linked to tumor suppressor gene reactivation. *Oncogene* 26, 77-90.
- Wu, S.C., and Zhang, Y. (2009). Minireview: role of protein methylation and demethylation in nuclear hormone signaling. *Mol Endocrinol* 23, 1323-1334.
- Wyckoff, M.H., Chambliss, K.L., Mineo, C., Yuhanna, I.S., Mendelsohn, M.E., Mumby, S.M., and Shaul, P.W. (2001). Plasma membrane estrogen receptors are coupled to endothelial nitric-oxide synthase through Galpha(i). *J Biol Chem* 276, 27071-27076.
- Xie, B., Invernizzi, C.F., Richard, S., and Wainberg, M.A. (2007). Arginine methylation of the human immunodeficiency virus type 1 Tat protein by PRMT6 negatively affects Tat Interactions with both cyclin T1 and the Tat transactivation region. *J Virol* 81, 4226-4234.
- Xu-Monette, Z.Y., Medeiros, L.J., Li, Y., Orlowski, R.Z., Andreeff, M., Bueso-Ramos, C.E., Greiner, T.C., McDonnell, T.J., and Young, K.H. (2012). Dysfunction of the TP53 tumor suppressor gene in lymphoid malignancies. *Blood* 119, 3668-3683.
- Xu, W., Chen, H., Du, K., Asahara, H., Tini, M., Emerson, B.M., Montminy, M., and Evans, R.M. (2001). A transcriptional switch mediated by cofactor methylation. *Science* 294, 2507-2511.
- Yadav, N., Lee, J., Kim, J., Shen, J., Hu, M.C., Aldaz, C.M., and Bedford, M.T. (2003). Specific protein methylation defects and gene expression perturbations in coactivator-associated arginine methyltransferase 1-deficient mice. *Proc Natl Acad Sci U S A* 100, 6464-6468.
- Yamagata, K., Daitoku, H., Takahashi, Y., Namiki, K., Hisatake, K., Kako, K., Mukai, H., Kasuya, Y., and Fukamizu, A. (2008). Arginine methylation of FOXO transcription factors inhibits their phosphorylation by Akt. *Mol Cell* 32, 221-231.
- Yamashita, H., Nishio, M., Kobayashi, S., Ando, Y., Sugiura, H., Zhang, Z., Hamaguchi, M., Mita, K., Fujii, Y., and Iwase, H. (2005). Phosphorylation of estrogen receptor alpha

References

serine 167 is predictive of response to endocrine therapy and increases postrelapse survival in metastatic breast cancer. *Breast Cancer Res* 7, R753-764.

Yamashita, H., Nishio, M., Toyama, T., Sugiura, H., Kondo, N., Kobayashi, S., Fujii, Y., and Iwase, H. (2008). Low phosphorylation of estrogen receptor alpha (ERalpha) serine 118 and high phosphorylation of ERalpha serine 167 improve survival in ER-positive breast cancer. *Endocrine-related cancer* 15, 755-763.

Yamnik, R.L., Digilova, A., Davis, D.C., Brodt, Z.N., Murphy, C.J., and Holz, M.K. (2009). S6 kinase 1 regulates estrogen receptor alpha in control of breast cancer cell proliferation. *J Biol Chem* 284, 6361-6369.

Yamnik, R.L., and Holz, M.K. (2010). mTOR/S6K1 and MAPK/RSK signaling pathways coordinately regulate estrogen receptor alpha serine 167 phosphorylation. *FEBS Lett* 584, 124-128.

Yang, B., O'Herrin, S.M., Wu, J., Reagan-Shaw, S., Ma, Y., Bhat, K.M., Gravekamp, C., Setaluri, V., Peters, N., Hoffmann, F.M., *et al.* (2007). MAGE-A, mMage-b, and MAGE-C proteins form complexes with KAP1 and suppress p53-dependent apoptosis in MAGE-positive cell lines. *Cancer Res* 67, 9954-9962.

Yang, Y., Li, C.C., and Weissman, A.M. (2004). Regulating the p53 system through ubiquitination. *Oncogene* 23, 2096-2106.

Yoneyama, M., and Fujita, T. (2007). RIG-I family RNA helicases: cytoplasmic sensor for antiviral innate immunity. *Cytokine Growth Factor Rev* 18, 545-551.

Yoshimatsu, M., Toyokawa, G., Hayami, S., Unoki, M., Tsunoda, T., Field, H.I., Kelly, J.D., Neal, D.E., Maehara, Y., Ponder, B.A., *et al.* (2010). Dysregulation of PRMT1 and PRMT6, Type I arginine methyltransferases, is involved in various types of human cancers. *Int J Cancer* 128, 562-573.

Yu, S., Gao, B., Duan, Z., Xu, W., and Xiong, S. (2011). Identification of tripartite motif-containing 22 (TRIM22) as a novel NF-kappaB activator. *Biochem Biophys Res Commun* 410, 247-251.

Yuan, E., Li, C.M., Yamashiro, D.J., Kandel, J., Thaker, H., Murty, V.V., and Tycko, B. (2005). Genomic profiling maps loss of heterozygosity and defines the timing and stage dependence of epigenetic and genetic events in Wilms' tumors. *Mol Cancer Res* 3, 493-502.

Yuan, X.W., Zhu, X.F., Huang, X.F., Sheng, P.Y., He, A.S., Yang, Z.B., Deng, R., Feng, G.K., and Liao, W.M. (2007). Interferon-alpha enhances sensitivity of human osteosarcoma U2OS cells to doxorubicin by p53-dependent apoptosis. *Acta Pharmacol Sin* 28, 1835-1841.

References

- Zhang, F., and Sriram, S. (2009). Identification and characterization of the interferon-beta-mediated p53 signal pathway in human peripheral blood mononuclear cells. *Immunology* 128, e905-918.
- Zhang, G., Yanamala, N., Lathrop, K.L., Zhang, L., Klein-Seetharaman, J., and Srinivas, H. (2010a). Ligand-independent antiapoptotic function of estrogen receptor-beta in lung cancer cells. *Mol Endocrinol* 24, 1737-1747.
- Zhang, J., Das, S.C., Kotalik, C., Pattnaik, A.K., and Zhang, L. (2004). The latent membrane protein 1 of Epstein-Barr virus establishes an antiviral state via induction of interferon-stimulated genes. *J Biol Chem* 279, 46335-46342.
- Zhang, J., Wang, J., Wood, C., Xu, D., and Zhang, L. (2005). Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 replication and transcription activator regulates viral and cellular genes via interferon-stimulated response elements. *J Virol* 79, 5640-5652.
- Zhang, M.F., Zhang, Z.Y., Fu, J., Yang, Y.F., and Yun, J.P. (2009). Correlation between expression of p53, p21/WAF1, and MDM2 proteins and their prognostic significance in primary hepatocellular carcinoma. *J Transl Med* 7, 110.
- Zhang, X., and Cheng, X. (2003). Structure of the predominant protein arginine methyltransferase PRMT1 and analysis of its binding to substrate peptides. *Structure* 11, 509-520.
- Zhang, X., Zhou, L., and Cheng, X. (2000). Crystal structure of the conserved core of protein arginine methyltransferase PRMT3. *EMBO J* 19, 3509-3519.
- Zhang, X.P., Liu, F., and Wang, W. (2010b). Coordination between cell cycle progression and cell fate decision by the p53 and E2F1 pathways in response to DNA damage. *J Biol Chem* 285, 31571-31580.
- Zhao, C., Lam, E.W., Sunter, A., Enmark, E., De Bella, M.T., Coombes, R.C., Gustafsson, J.A., and Dahlman-Wright, K. (2003). Expression of estrogen receptor beta isoforms in normal breast epithelial cells and breast cancer: regulation by methylation. *Oncogene* 22, 7600-7606.
- Zikan, M., Bohm, J., Pavlista, D., and Cibula, D. (2011). Comparative analysis of loss of heterozygosity and expression profile in normal tissue, DCIS and invasive breast cancer. *Clin Transl Oncol* 13, 652-655.
- Zirn, B., Hartmann, O., Samans, B., Krause, M., Wittmann, S., Mertens, F., Graf, N., Eilers, M., and Gessler, M. (2006). Expression profiling of Wilms tumors reveals new candidate genes for different clinical parameters. *Int J Cancer* 118, 1954-1962.
- Zurita-Lopez, C.I., Sandberg, T., Kelly, R., and Clarke, S.G. (2012). Human protein arginine methyltransferase 7 (PRMT7) is a type III enzyme forming omega-NG-monomethylated arginine residues. *J Biol Chem* 287, 7859-7870.