REGULATION OF RUNX3 IN GASTRIC AND BREAST CANCER

BY

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DISSERTATION

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Thesis Abstract

RUNX3 is a transcription factor that is ubiquitously expressed in different tissues and has been shown to have diverse functions in many developmental procedures. Recently it has also been acknowledged that RUNX3 is involved as a tumor suppressor in many distinct cancers in different tissues. In this thesis, we will examine the regulation of this tumor suppressor in gastric cancer and breast cancer.

Chronic infection with *cagA*-positive *Helicobacter pylori* is the strongest risk factor for the development of gastric adenocarcinoma. The *cagA* gene product CagA is injected into gastric epithelial cells and disturbs cellular functions by physically interacting with and deregulating a variety of cellular signaling molecules. RUNX3 is expressed gastric epithelial tissues, and is frequently inactivated in gastric cancer. In the first part of the thesis, we showed that *H. pylori* infection inactivates the gastric tumor suppressor RUNX3 in a CagA-dependent manner. CagA directly associates with RUNX3 through a specific recognition of the PY motif of RUNX3 by a WW domain of CagA. Deletion of the WW domains of CagA or mutation of the PY motif in RUNX3 abolishes the ability of CagA to induce the ubiquitination and degradation of RUNX3, thereby extinguishing its ability to inhibit the transcriptional activation of RUNX3. This study identify RUNX3 as a novel cellular target of *H. pylori* CagA and also reveal a mechanism by which CagA functions as an oncoprotein by blocking the activity of gastric tumor suppressor RUNX3.

RUNX3 has also been known to be inactivated in breast cancer through dual mechanism of cytoplasmic mislocalization as well as promoter hypermethylation. Recent studies in our lab have shown that RUNX3 knockout mice have an approximate 20% increased chance of

developing breast cancer compared to WT mice. At the same time, MCF7 cells expressing RUNX3 resulted in smaller tumor growth in a tumorigenicity assay compared to MCF7 cells expressing a control vector, further affirming RUNX3's importance as a tumor suppressor in breast cancer. Pin1 is an isomerase that is over-expressed in human breast cancer. Pin1 specifically isomerizes only the Ser/Thr-Pro bonds in certain proteins, which allows it to act as a molecular switch controlling protein functions. In the second part of this thesis, we discuss the findings that Pin1 interacts specifically with tumor suppressor protein RUNX3 through 4 separate phosphorylated Ser/Thr-Pro motifs on the RUNX3 protein. Through this interaction with Pin1, the ubiquitination of RUNX3 is markedly enhanced, resulting in decreased protein stability. RUNX3 is therefore targeted to the 26S proteasome for degradation in the presence of Pin1. Our data shows a novel pathway through which tumor suppressor protein RUNX3 can be inactivated and hence regulated in breast tissues by Pin1.

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Introduction -- RUNX3 in Cancer.

1.1 Brief Introduction to the RUNX family.

Over the years, the RUNX gene family which encodes for Runt domain containing transcription factors has been attracting a broad interest group because of their involvement in cell lineage determination during development and various forms of cancers. RUNX1 is associated with hematopoeisis, RUNX2 is important in bone formation. RUNX3 is ubiquitously expressed in many tissues with diverse biological functions, of which the most widely studied is its role as a tumor suppressor.

The transcription factors encoded by the RUNX mammalian family was discovered by virologists who made used of DNA and RNA tumor viruses to study cellular growth and differentiation processes. DNA and RNA tumor viruses have been involved in many landmark studies that defined numerous basic processes of carcinogenesis. p53, for example, was found to be bound to the middle T antigen of a DNA virus, *simian virus 40* [1]. One of the methods of research capitalized on the usage of embryonal carcinoma (EC) cells, which have properties identical to early embryos [2]. Under specific conditions, EC cells that are introduced into blastocysts can differentiate into normal tissues in chimeric mice [3, 4]. Virologists found that EC cell lines, such as F9 and PCC4, are resistant to infection by several viruses including polyomavirus (Py) until cells are induced to differentiate. [5, 6]. Py contains an enhancer region that determines the differentiation of stage-specific viral infections, and vigorous analysis of the Py enhancer led to the isolation of a critical cellular transcription factor [7, 8], Py enhancer binding protein 2 (PEBP2), later known as a RUNX protein [9]. The PEBP2 was identified as a developmental regulator that was only expressed when EC cells undergo differentiation.

Besides DNA and RNA tumor viruses, murine retroviruses were also found to induce tumors in various tissues. Oncogenes and proto-oncogenes were identified by their transduction into viral genomes or by viral integration into regulatory regions of cellular genes. RUNX protein was identified as a core-binding factor (CBF) that modified the regulatory elements of retrovirus enhancers, and affected the oncogenic potential of the virus [10-12]. Further studies confirmed that PEBP2 and CBP are identical proteins. Subsequently, two other genes that had been identified by other groups of researchers were also found to be related to PEBP2/CBF, eventually forming the RUNX family of proteins [13-16].

The functions of RUNX gene family were largely obtained through studies of various RUNX knockout mice. Embryonic RUNX1 mice of 12.5-13.5 days old had no or very few definitive hematopoietic progenitors in their fetal livers or yolk sac [17-19]. Further studies showed that RUNX1 is vital in the generation of hematopoietic stem cells (HSCs) during embryogenesis [20-22]. HSCs are generated from several distinct embryonic sites, examples of which include the para-aortic splanchnopleura (PAS) and the aorta-gonad-mesonephros (AGM) which is formed in later developmental stages. RUNX1 is expressed in all of these hematogenic endothelial cells [23] and lack of RUNX1 in these endothelial cells renders them incapable of producing HSCs. Hence, presenting RUNX1 as an early marker of endothelial and mesenchymal cells in the sites of HSC emergence.

In the case of adult hematopoiesis, conditional knock out of RUNX1 in adult mice did not result in the abolishment of HSC function. However, the mice did show defects in platelet

formation and megakryocyte differentiation [24, 25], which are disorders with predisposition to acute myelogenous leukemia (AML) [26]. There was also a decreased contribution to peripheral T-cell populations in *Runx1*^{-/-} mice, β chain rearrangements and /or β-selection [27]. RUNX1 knockout mice eventually developed myeloproliferative disease and T-cell lymophoma. RUNX1's role as a global regulator in hematopoiesis across multiple stages and lineages is further evident from the fact that is it one of the most frequently mutated genes in human leukemias [28, 29].

Likewise, through knockout mice experiments, RUNX2 was shown to be a key player in osteoblast proliferation and differentiation and is obligatory for regulation of skeletal genes, hypertrophic chodrocytes as well as endochondral and intramembraneous bone formation and skeletal development [30-32]. RUNX2 knockout mice suffocated to death soon after birth due to missing rib cage formation, a result of systemic lack of ossification. Supporting the data from *Runx2*^{-/-} mice, mutations in RUNX2 were also reported in the human congenital skeletal disorder cleidocranial dysplasia (CCD) [31, 32]. A better understanding of RUNX2 and its function will provide further insights into bone biology and novel therapeutics.

RUNX3 is expressed in a wider range of tissues than RUNX1 and RUNX2, the knockout phenotype of RUNX3 is observed in several different tissues. Firstly, the gastric mucosa of *Runx3*^{-/-} mice was found to exhibit hyperplasia as a result of increased cell proliferation and suppressed apoptosis in epithelial cells. Furthermore the cells were immune to growth inhibitory and apoptosis inducing effects of Transformation Growth Factor β (TGF-β), suggesting that

RUNX3 is a major growth regulator of gastric epithelial cells and is a tumor suppressor in gastric cancer. Subsequently over the next few years, RUNX3 was also discovered to be a putative tumor suppressor in many solid tumor types including breast cancer [33, 34], lung cancer [35], and colorectal cancer [36]. The tumor suppressor activities of RUNX3 in gastric cancer and breast cancer will be further discussed in the following chapters.

RUNX3 is also found to regulate the development of dorsal root ganglion (DRG) neurons and is required for the axon path finding of proprioceptive neuron in the spinal cord. It was reported that $Runx3^{-/-}$ mice displayed severe failure in motor coordination, and few DRG neurons synthesized parvalbumin, the proprioceptive neuronal marker [37, 38]. Furthermore, proprioceptive afferent axons failed to project to their targets in the spinal cord and muscles. DRG neurons project axons to central and peripheral targets according to the sensory modality, RUNX3 was found to be expressed in some subpopulations of DRG neutrons suggesting that the protein may have a function in regulating the paths of specific axons. These data suggest the importance of RUNX3 in regulating axonal projections of a specific subpopulation of DRG neurons.

Lastly, like $Runx1^{-/-}$ mice, $Runx3^{-/-}$ mice were found to have a defect in T cell development. These mice displayed reduced numbers of $CD8^+$ T cells in the thymus and in the circulating T-cell population [39]. On the other hand, there was an increased expression of CD4 in the peripheral $CD8^+$ cells [40]. RUNX3 deficient cytotoxic T cells had defective responses to antigens while the helper cells functioned normally. Briefly, T lymphocytes differentiates

through the following stages: Hematopoietic precursors lacking CD4 and CD8 receptors progress through a double positive stage (CD4$^+$CD8$^+$), and are then selected to become either a CD4$^+$ helper cell or CD8$^+$ cytotoxic T cell through the silencing of either CD8 or CD4 expression respectively. Two consensus RUNX binding site have been found in the regulatory element required for CD4 silencing during T-cell development, and different RUNX family members are required for unique functions at different stages [27]. RUNX1 binds to both sites and is required for epigenetic silencing in CD4$^-$ CD8$^-$ thymocytes, while RUNX3 is important in CD4 silencing in cytotoxic line of T cells.

1.2   RUNX3, a Transcription Factor, and its Anti-Tumor Properties

After purification of the RUNX family transcription factors from the Py enhancer, biochemical methods revealed that the three transcription factors are heterodimers: a DNA binding α-subunit, and a common non-DNA binding β-subunit known as PEBP2β/CBF-β [9, 13], which enhances the DNA binding property of the α-subunits. The α-subunits of the three members share a central Runt domain, which is well conserved and recognizes a specific DNA sequence. The Runt domain is highly conserved from *Drosophila melanogaster* segmentation *runt* gene which is found to be important for the developmental regulation of early Drosophila embryos [15, 41, 42]. The RUNX genes are able to encode a number of isoforms due to the presence of two alternative promoters, distal promoter P1 and proximal promoter P2 [43]. The functional significance of this complex organization and coding potential is still unclear. The most common isoform of RUNX proteins is the type 1 isoform that contains the pentapetide MRIPV at its N-terminus, we will be using this isoform for our study of RUNX3 in this thesis.

One of the most extensively studied functional properties of RUNX3 is its involvement in the TGF-β signaling pathway. TGF-β is a growth factor that controls proliferation, cellular differentiation and numerous other diverse biological pathways. During TGF-β signaling, TGF-β or bone morphogenetic protein (BMP) binds to their respective cognate receptors, resulting in the activation of the receptors as serine kinases. These serine kinases then phosphorylate Smads, which are signal transducers in the TGF-β signaling pathway. Smad2 and Smad3 are activated by TGF-β, while Smad1, 5 and 8 are activated by BMP. The Smads that can be activated by receptors are known as R-Smads, they associated with a common Smad4, known as Co-Smad

and translocate into the nucleus. The R-Smads and Co-Smad complexes then interact with respective transcription factors to regulate the transcription of target genes [44]. RUNX3 is known to interact with Smad3 and Smad1 through the MH2 domains in the C-terminal conserved regions of the Smad proteins while Smads are believed to interact with RUNX3 at at least 2 regions, one at the C-terminal transcription activation domain, and the other within the Runt domain in the middle of the protein [45].

Figure 1.1 Structure of human RUNX family of proteins and their phosphorylation sites, adapted from [46]. Conserved regions in RUNX proteins are indicated by dotted lines. AD stands for transactivation domain of RUNX1 (Ito, 1999), AD3 for transactivation domain of RUNX2 (Thirunayukkarasu 1998), ID for inhibitory domain, Q for glutamine tract, A for alanine tract and lastly, PST region for proline serine and threonine-rich region.

The synergistic relation between RUNX3 activity and TGF-β signaling was observed when the gastric mucosa of RUNX3 knockout mice was less sensitive to TGF-β induced cell

cycle arrest and apoptosis [47]. This observation together with the ability of RUNX3 to target Smad regulators to distinct nuclear foci on stimulation by TGF-β further strengthens the case for RUNX3 as a regulator of the TGF-β signaling pathway [48]. Given that RUNX3 is an integral part of the TGF-β signaling pathway, these observations fit nicely with earlier studies which found that TGF-β signal transduction pathway are interrupted in many types of cancers, including those of the gastrointestinal tract. It is therefore possible to extrapolate that RUNX3 might also function as a tumor suppressor in other types of cancers where mutations or deletions are often found in the TGF-β receptors or other components of the signaling pathway [49, 50].

TGF-β is known to inhibit the cell cycle by activating the cyclin/CDK inhibitors $p15^{INK4B}$, $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ [51-54]. It was observed in a study in 2006 that RUNX3 inhibited gastric epithelial cell growth by inducing $p21$ gene expression in response to TGF-β1. The study showed that increase in exogenous expression of RUNX3 increased endogenous $p21$ expression upon TGF-β1 stimulation. Moreover, siRNA suppression of RUNX3 expression decreased TGF-β induced $p21$ expression. In mouse and human epithelial sections, RUNX3 expression coincided with $p21$ expression. The presence of five RUNX3 binding sites on the p21 promoter also further indicated that $p21$ is a direct target of RUNX3 [55]. Upon TGF-β activation, R-Smads are activated they bind with RUNX3 and cooperatively stimulate the activation of $p21$ promoter to induce TGF-β-mediated cell cycle arrest. This discovery provides a first insight into RUNX3's role in the TGF-β signaling pathway in the gastric epithelial, suggesting that part of RUNX3's tumor suppressor activity is to influence the cell cycle machinery and induce cell cycle arrest, and thereby inhibiting cell growth.

Another gene that has been found to be a direct downstream target of RUNX3 is Bim, a pro-apoptotic gene. Bim was found to be one of the proteins involved in the activation of TGF-β induced apoptosis, and was up-regulated by RUNX3 in response to TGF-β stimulation [56]. Data from the report showed that Bim pathway is the major pathway that is operating in TGF-β mediated apoptosis by RUNX3 in vivo and in vitro. Therefore suggesting that besides regulating cell cycle arrest, another of RUNX3's tumor suppressor activity would be to mediate apoptosis through Bim. Subsequently, *claudin-1*, an integral membrane protein, important in maintaining the integrity of cell-cell adhesion was also found to be a direct transcriptional target of RUNX3. RUNX3 cooperates with TGF-β to regulate *claudin-1* transcription. RUNX3$^{-/-}$ derived gastric epithelial cells were attached weakly to each other as compared to RUNX3$^{+/+}$ cells, and had reduced levels of *claudin-1* [57]. *Claudin-1* is also believed to be a gastric tumor suppressor; it inhibits proliferation in gastric cancer cells and is down-regulated during epithelial-mesenchymal transition (EMT). These findings present another mechanism of RUNX3's anti-tumor activity, where it up-regulates Claudin1 expression and antagonizes the EMT process in gastric carcinogenesis.

Figure 1.2 RUNX3 cooperates with SMAD3 and SMAD4 to activate TGF-β-dependent growth inhibition and apoptosis by induction of *p21* and *Bim* respectively; RUNX3 induces *Claudin-1* (*Cldn-1*) expression, possibly to antagonize epithelial-mesenchymal transition process.

While regulating TGF-β signaling, RUNX3 is also found to be regulated by components of the TGF-β signaling pathway. TGF-β was observed to stimulate p300-dependent RUNX3 acetylation, and this acetylation counters Smurf-mediated ubiquitination and degradation of RUNX3 [58, 59]. Increase in RUNX3 acetylation increases its transcriptional activity. Since acetylation and ubiquitination both target lysine residues, it is probable that the acetylation of RUNX3 competes with its ubiquitination. Furthermore, mutations of the lysine residues in

RUNX3 abolished the transactivation activity of RUNX3 despite markedly increasing its stability suggesting that TGF-β mediated acetylation of RUNX3 is not only important in preventing the degradation of RUNX3 but is also important for its transactivation activity.

Figure 1.3 Hypothetical model for the acetylation, deacetylation and ubiquitination of RUNX3, from [46]. The cellular levels of RUNX amounts and activity are controlled by a dynamic equilibrium of RUNX acetylation and deacetylation. Under normal circumstances, low levels of RUNX3 activity will be maintained by decreased acetylation of RUNX3 through deacetylase activity, such that the RUNX3 protein is exposed to ubiquitin ligase and targeted for degradation. When a signal the likes of TGF-β or BMP is present, RUNX3 acetylation is increased by increased p300 activity, hence inhibiting ubiquitination and degradation of RUNX3.

Besides being an integral part of the TGF-β signaling pathway, RUNX3 is also found to antagonize Wnt signaling activity and down-regulate transcription of Wnt target genes such as *c-Myc* and *cyclinD1* in the epithelial cells of small and large intestines. Further studies showed that

RUNX3 exists in a complex with key Wnt effectors: TCF4 and β-catenin, and the resulting complex shows reduced DNA binding activity, and decrease transcriptional activation of Wnt target genes. Furthermore, this antagonism against aberrant Wnt signaling is APC independent, indicating that RUNX3 is a gatekeeper of Wnt signaling on its own [36]. RUNX3's involvement in both the TGF-β pathway and Wnt signaling may well provide an opportunity to orchestrate tumor suppressor activity through crosstalk between the two pathways. It is also interesting to note that both Wnt and TGF-β signaling pathways are also involved in EMT during embryonic development [60]. However, it is not clear if RUNX3 affects the ability of these two pathways to in EMT.

RUNX3 is also believed to have putative functions in other cancer signaling pathways. The interaction between RUNX3 and yes-associated protein (YAP), a transcription co-activator, is thought to enhance target promoter activation of RUNX3 [61]. RUNX3 also physically binds to Forkhead box O3a (FOXO3a) tumor suppressor protein at the promoter of *Bim*, enhancing transcription of Bim and subsequently increase cellular apoptosis [62]. It is entirely possible that RUNX3 is involved in other FOXO3a-related activities including regulation of intestinal inflammation [63], activation of ATM during DNA damage [64] and oxidative stress response [65]. DNA damage has known to promote carcinogenesis through genetic alternations of components involved in signaling pathway, cell cycle regulation, repair and apoptosis processes. Incidentally, reports have also linked RUNX3 with various DNA repair machineries; one example is RUNX3's interaction with DNA repair protein Ku70, a component of the major repair mechanism for double-stranded DNA breaks and non-homologous end-joining repair [66]. Finally, RUNX3 is also believed to be a mediator of cellular senescence. Oncogene-induced

senescence is an important method of tumor suppression, and RUNX3 is believed to mediate this mode of growth arrest through the induction of $p14^{ARF}$ in a p53 dependent manner [67, 68]. All these findings indicate the multi-functionalities of RUNX3 in different types of tumors. The anti-tumor mechanism of RUNX3 in each type of tumor has to be better studies in order to gain further insights into the biology of RUNX3 in different tissue types.

1.3: Knockout Phenotype of RUNX3: Gastric Cancer Tumor Suppressor

The identification of RUNX3 as a candidate tumor suppressor in gastric cancer was an important discovery in the field of gastric carcinoma. Gastric cancer is the fourth most common type of cancer and the second leading cause of cancer related death worldwide [69-71]. Risk factors leading to gastric carcinoma include diet, *Helicobactor pylori* (*H. pylori*) infection and genetic alterations [72, 73]. Many genetic mutations associated with gastric cancer have been described including loss of expressing of genes like TGF-β receptor [74] and p53 [75, 76] or over expression of erbB-2 [77] and c-met [78]. Loss of multiple chromosomal loci has also been linked to gastric cancer [79-81], but all of the above were only presented in limited cases where the significance of such genetic alternations was unknown. Until the discovery of RUNX3 as a tumor suppressor in gastric cancer, the underlying mechanism of gastric carcinogenesis was poorly understood.

In 2002, Li et at. described RUNX3 as being expressed in glandular stomach, with the strongest expression in chief cells and surface epithelial cells and to a lesser extend in parietal cells in the normal human adult gastric. RUNX3 null mouse gastric mucosa demonstrated signs of hyperplasia due to increased proliferation and resistance to TGF-β-induced growth inhibitory effect or apoptosis in the epithelial cells. Examination of 46 surgically resected gastric cancer specimens and cell lines showed that approximately 60% of the patient samples did not express RUNX3 due to hemizygous deletion of the gene or hypermethylation of its promoter region. The incidence of RUNX3 silencing appeared to happen at an early stage as well as during progression, from 40% of early stages of cancer to almost 90% of stage IV cancer. Data

indicated that loss of RUNX3 is causally linked to the genesis and progression of gastric cancer. A rare missense loss-of-function mutation in the Runt domain of RUNX3 (R122C) was also identified from a cancer patient. This single amino acid mutation resulted in complete abolishment of RUNX3's DNA binding ability and negated the tumor suppressor activity of RUNX3 in nude mouse assay. Moreover, experiments using stomach epithelial cell lines isolated from $Runx3^{+/+}$ and $Runx3^{-/-}$ mice with a $p53^{-/-}$ background indicated that only the $Runx3^{-/-}$ $p53^{-/-}$ cell line was tumorigenic in nude mice, confirming RUNX3 as gastric tumor suppressor [47].

In a subsequent study by Ito *et al* . in 2005, 97 gastric cancer cases were tested and it was discovered that in 44% of the cases, RUNX3 was not expressed, consistent with the findings from 2002. In the remaining 56% of samples where RUNX3 was expressed, only 18% showed nuclear localization of RUNX3, while 38% showed primarily, or exclusively retention of RUNX3 in the cytoplasm as an inactive form [82]. Transcription factors require translocation into the nucleus in order to carry out transactivation of target genes, and often the nuclear envelope functions as a line of regulation to prevent transcription factors from gaining access to target genes. Therefore proper sub-nuclear targeting of transcription factors is an important step in signaling transduction pathways. Transcription factors that are retained in the cytoplasm are believed to be in a basal and inactive state, likewise with the RUNX3 protein seen in 38% of the gastric cancer cases studied in 2005. With this report, it can be concluded that RUNX3 is inactivated in almost 90% of gastric cancers through hemizygous deletion of the gene, hypermethylation of the *Runx3* promoter or mis-localization of the RUNX3 protein to the

cytoplasm [82], further emphasizing on RUNX3's role as a candidate tumor suppressor in gastric cancer.

Interestingly, the human *Runx3* gene is on the short arm of human chromosome 1 at location 1p36 [83], a location that is thought to carry important tumor suppressor(s) in many types of cancers including gastric cancer, hepatocellular carcinoma, colon carcinoma, pancreatic cancer and neuroblastoma [84]. Indeed, as previously mentioned, there have been increasing reports of RUNX3 being possibly involved as a tumor suppressor in many other types of cancer including, but not limited to lung cancer [35], colorectal cancer [36], liver cancer [85] and breast cancer [33, 34]. The role of RUNX3 in breast cancer will be further discussed in the subsequent part of this chapter.

It is also important to note that RUNX3 that had been silenced through hypermethylation of its promoter can be reactivated by the combination of 5'-azacytidine and Trichostatin A (histone deacetylase inhibitors) treatment [47]. Also, although on average, 30% of gastric cancer cases show hemizygous deletions of RUNX3, mutations or small deletions in the remaining allele were very rare. Likewise for alleles silenced by hypermethylation of the gene promoter, mutations of the alleles were rare, implying that reactivation of the RUNX3 gene would possibly reactivating a fully functional protein. Also, exogenous expression of RUNX3 in a cell line where RUNX3 had been silenced was able to reverse the tumorigenicity of that cell line in nude mice [47]. These data suggest that need for more detailed studies of the RUNX3 and its tumor

suppressor activity in hope that these studies would one day be useful in countering tumor growth in gastric cancer.

1.4   RUNX3, a Possible Tumor Suppressor in Breast Cancer?

Breast cancer is the most common type of cancer amongst woman in developed countries, including the United States. With a mortality of approximately 20%, breast cancer is the second deathliest form of cancer after lung cancer [86]. Carcinogenesis of breast cancer is a multistep process that begins with aberrant growth of mammary epithelial cells, and ending with formation of invasive tumors. There are two types of breast cancer: cancers originating from milk ducts are known as ductal carcinomas, and cancers originating from breast lobules (that supply the ducts with milk) are known as lobular carcinomas. The initiation of breast cancer is due to many factors, including age, race and diet. However, genetic alterations such as mutations in BRCA1 and BRCA2 remain a strong link to many breast cancer cases [87]. Therefore, identification of the genetic changes involved in the multistep of breast cancer is important for early detection and prevention.

There has been strong evidence suggesting that mutations in the TGF-β signaling pathway might be one of these genetic aberrations that result in mammary tumor formation. Researchers noticed that majority of breast cancer cell lines did not respond to TGF-β-mediated cell cycle arrest although the TGF-β signaling cascade involving Smads was active in these cells [88] . Some other factor is thus suspected to be hindering TGF-β induced cell cycle arrest or apoptosis in breast cancer cells. RUNX3's involvement in the TGF-β-mediated tumor suppressor pathway, and also its TGF-β-dependent transactivation of *p21* and Bim genes in gastric cancer [55, 56] led researchers to suspect its likelihood as a candidate in the inactivation of TGF-β signaling pathway in breast cancer.

In fact, study by Lau *et al* . in 2006 discovered that 12 out of 19 breast cancer cells lines displayed low RUNX3 mRNA and protein expression due to promoter hypermethylation of the RUNX3 gene. They further studied RUNX3 expression in primary breast cancer specimens from patients in Singapore and found that 9 out of 44 specimens showed undetectable levels of RUNX3 through immunohistostaining of the RUNX3 protein. In the remaining 35 specimens that showed weak staining for RUNX3, the protein was localized to the cytoplasm. Using a nude mice tumorigenicity assay, the researchers showed that only nude mice injected with MDA-MB-231 stably expressing RUNX3 developed tumors while none of the mice injected with MDA-MB-231 cells expressing a control vector developed tumors [34].

These results were further confirmed by a separate study in China in 2008, where they reported that 50% of breast cancer specimens examined did not express RUNX3 do to hypermethylation of the gene promoter. They also observed that the survive rate of patients was linked to RUNX3 expression. Breast cancer patients with positive RUNX3 expression have an 84% 5-year survival rate as compared to 54% in patients with negative expression of RUNX3 [89].

The above data suggest a possible tumor suppressor role for RUNX3 in breast cancer, but it is not until a study published by Huang *et al.* in our lab with our collaborators that documented a mechanism by which RUNX3 carries out its tumor suppressor activity. In the report, we demonstrated that about one fifth of the *Runx3*$^{+/-}$ mice developed spontaneous ductal adenocarcinomas due to increase cell proliferation, compared to WT *Runx3*$^{+/+}$ mice, none of

which had tumor growths in their mammary glands. Immunohistochemistry of the *Runx3*^{+/-} tumors showed marked decrease in the RUNX3 expression compared to WT mammary tissues, supporting the notion that RUNX3 is a tumor suppressor in breast cancer. It was interesting to note that estrogen receptor-α expression was markedly increased in *Runx3*^{+/-} tumors as compared to WT tissues. Further studies using series of assays including proliferation assay, soft agar assay and nude mice tumorigenicity assay show that RUNX3 inhibit estrogen-dependent proliferation in a breast cancer cell line, MCF-7 cells [33].

The negative correlation between RUNX3 and ERα observed in *Runx3*^{+/-} mice described above was further proven in a cohort of 80 human breast ductal carcinoma samples. We observed that 70% of the samples with low RUNX3 expression had high expression of ERα while conversely, 65% of samples with high RUNX3 expression displayed low ERα expression. Subsequent investigation showed that RUNX3 induced proteasome degradation of ERα in a ligand independent manner resulting in the inactivation of ERα transcriptional activity [33].

Estrogen plays a critical role in mammary gland development [90], and its action is mediated through estrogen receptor α and β. ERα is able to function as a transcription factor regulating genes involving in cell cycle and apoptosis, resulting in cell proliferation and suppression of apoptosis [91]. ERα is over-expressed in 75% of breast cancers [91], and aberrant ERα expression and subsequent abnormal estrogen signaling is associated with initiation and progression of breast cancer [92]. Therefore, tight control of the level of ERα is important for normal mammary tissues development. It is possible that RUNX3 might recruit an E3 ubiquitin

ligase or function as an E3 ubiquitin ligase for the ubiquitination and degradation of ERα, acting as a gatekeeper for the level of ERα in the mammary tissues.

All of the above data points towards RUNX3 as a tumor suppressor in breast cancer. Huang *et al* . defined a mechanism for the tumor suppressor of RUNX3 in ERα-positive breast cancers. However, it should also be noted that since RUNX3 expression is also down-regulated in some ERα negative cells like MDA-MB-231, and that RUNX3 did reduce the invasiveness and tumor forming potential of those cells like previously described [34], the mechanism tumor suppressor activity of RUNX3 in ERα negative cancer is possibly different from ERα positive cancer.

As RUNX3 is frequently inactivated in gastric and breast tumors, studying the regulation of RUNX3 and restoring RUNX3 expression in those tissue types presents an attractive new therapeutic target that has potential for the treatment of gastric and breast carcinogenesis.

Chapter 2: *Helicobacter pylori* CagA targets gastric tumor suppressor RUNX3 for proteasome-mediated degradation

2.1 Introduction: *H. Pylori* CagA and Gastric Cancer

Infection with *H. pylori* is the strongest risk factor for gastric carcinoma [73]. *H. pylori* is a micro-aerophilic spiral-shaped bacterium which colonizes at least half of the world's population. It is subdivided into cytotoxin-associated gene A (*cagA*)-positive and *cagA*-negative strains. *CagA*-positive strains are much more potent in causing gastric mucosal damage [93, 94]. The CagA gene is a 120-145 kDa protein located at one end of the *cag pathogenicity island* (PAI), which is a roughly 40 Kb segment of the *H. pylori* genome that is considered to have been acquired by a process of horizontal transfer from an unknown organism. Genes located in this region mediate the pathogenicity of this bacterium [95-99]. One example is the type IV secretion system which delivers the *cagA*-encoded CagA protein into host cells [100].

Recent research has found *H. pylori* CagA to be a bacterial oncoprotein that acts in mammals. Transgenic expression of CagA alone in mice was able to induce abnormal proliferation of gastric epithelial cells and hematopoietic cells. CagA transgenic mice developed gastrointestinal carcinomas and leukemia, highlighting the oncogenic potential of CagA in gastric cancer [101]. While it is clear that CagA is probably sufficient to induce gastric cancer, the detailed mechanism is still unclear. Several mechanisms for CagA-dependent induction of gastrointestinal carcinomas have been proposed. For example, when CagA is delivered into the gastric epithelial cells, it causes the aberrant activation of SHP-2 tyrosine phosphatase which is a bona fide oncoprotein in human malignancies [72, 102]. Phosphorylation-independent interaction of CagA with PAR1, which inhibits PAR1 kinase activity and thereby causes junctional and polarity defects in gastric epithelial cells, is also suspected to be involved in the development of solid gastric tumors [103].

Figure 2.1 Adapted from [104]. Figure shows the sequential interaction of CagA with PAR1 and SHP-2. CM stands for CagA-multimerization sequence, which is important for the dimerization of CagA.

Another possibility is the relationship between CagA and the Wnt/β-catenin signaling pathway. The Wnt/β-catenin signaling pathway is crucially involved in colorectal carcinogenesis [105]. CagA is known to destabilize E-cadherin/β-catenin complex at the membrane, causing nuclear translocalization of β-catenin and subsequently the activation of Wnt/β-catenin signaling [106, 107]. CagA-deregulated β-catenin transactivates several genes which govern intestinal differentiation and thereby induces intestine-specific marker mucin 2 in gastric epithelial cells.

This indicates that perturbation of the E-cadherin/β-catenin by CagA plays an important role in the development of intestinal metaplasia, a premalignant transdifferentiation of gastric epithelial cells from which intestinal-type gastric adenocarcinoma arises. However, despite all of the above hypotheses, the exact mechanism of CagA-induced tumorigenesis remains obscure.

Figure 2.2 Adapted from [104]. Illustration of CagA interacting with E-cadherin at the gastric epithelial membrane, leading to the destabilization of E-cadherin/β-catenin complex, eliciting deregulated Wnt signaling.

In addition, emerging evidence discussed in chapter 1.3 that suggests that RUNX3 is a tumor suppressor whose inactivation is involved in the initiation and progression of gastric cancer, the trigger for RUNX3 inactivation within the cells is also largely unknown.

Here in this chapter, we demonstrate that virulence factor CagA of *H. pylori* specifically associates with RUNX3 and down-regulates its expression in gastric epithelial cells. CagA targets RUNX3 for ubiquitination and proteasome-mediated degradation. The identification of RUNX3 as a novel cellular target of CagA will help better understand the role of CagA as an oncogene and RUNX3 as a tumor suppressor in gastric carcinogenesis.

2.2 Materials and methods

Cell culture

Human AGS gastric epithelial cells, HEK293T and COS7 cells were obtained from ATCC (Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma, St Louis, MO, USA).

Expression plasmids and antibodies

The expression vectors for wild-type RUNX3 and its deletion mutants have been previously described [82]. HA-CagA or HA-TβRI(CA) expression vectors were kindly provided by Drs. Hatakeyama and Miyazono, respectively (Tokyo U., Japan). RUNX3 PY motif mutants and CagA WW domain deletion mutants were generated by Quikchange site-mutagenesis (Stratagene, San Diego, CA, USA) and confirmed by sequencing. Antibodies against CagA, RUNX3, Flag and His were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA).

Preparation of recombinant proteins: GST-CagA and His-RUNX3

Escherichia Coli BL21 cells were transformed with Glutathione-S-Transferase-CagA or 6-Histidine-tagged-RUNX3 plasmid DNA, plated on LB agar containing ampicillin and then incubated overnight at 37°C. The following day, one colony was inoculated in 40ml of LB containing ampicillin overnight at 37°C with vigorous shaking. The resultant culture was diluted 40 times and allowed to grow at 37°C with vigorous shaking to an Optical Density 600 (O.D.600) of 0.6. IPTG was then added to a final concentration of 0.5mM and incubated at room temperature for 4 hours with shaking to induce production of GST-CagA/His-RUNX3 protein in the BL21 bacterial cells. After 4 hours, the cells were pelleted by centrifugation and the pellet

was re-dissolved in GST binding buffer (100 mM NaCL, 50 mM Tris-HCL pH8.6, 1 mM $MgCl_2$, 1 mM EGTA) or His lysis buffer (50mM Tris pH8, 0.3M NaCl, 0.5% Triton X100, 20mM imidazole, 5mM b-mercaptoethanol) and sonicated on ice. Triton X-100 was added to a final concentration of 1% to the GST-CagA lysate. Both GST-CagA lysate and His-RUNX3 lysate were then centrifuged and the supernatant was decanted into a 50 ml conical tube and 50% slurry of GST-sepharose (Roche, Basel, Switzerland)/Nickel NTA (Qiagen, Venlo, Netherlands) beads was added to the supernatant and the mixture was rotated for 20 minutes at room temperature, following which the mixture was centrifuged. The supernatant was removed and the remaining beads were washed with GST binding buffer/His lysis buffer and then centrifuged as before. The process was repeated 3 times. GST-CagA beads were diluted to a 50% slurry and used for GST pull-down assays. His-RUNX3 fusion protein was eluted from the beads using 500µl His elusion buffer (50mM Tris pH8, 150mM NaCl, 0.5% Triton X100, 250mM imidazole, 5mM b-mercaptoethanol), and concentration measured using Bradford's Assay (Bio-Rad Technologies, Hercules, CA, USA).

H. pylori culture and infection

H. pylori NCTC11637 strain and its *cagA*-deficient isogenic mutant were purchased from ATCC and were cultured in bisulfite-free Brucella broth on agar media containing Ham's F-12 medium supplemented with 10% FBS and 5µg/ml vancomycin at 37°C in the presence of 10% CO_2. *H. pylori* was added to AGS cells for infection at an MOI of 50-100.

Infection of mice with *H. pylori*

INS-GAS mice were fed either WT *H. pylori* or cagA-deficient isogenic mutant *H. pylori* for 3 days. Mouse gastric mucosa tissues were harvested after the 3 days of infection. Animal

experiments were done in Vanderbilt University School of Medicine, Nashville, Tennessee, USA, by Ms. A. Lamb, with help from Ms. J. Romero-Gallo and Dr. R. M. Peek, Jr. Experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee.

Immunohistochemistry for RUNX3 in mouse gastric mucosa tissues

Staining was performed as described in [36] by Dr. Kosei Ito in University of Nagasaki, Nagasaki, Japan.

Establishment of AGS cell lines stably expressing Flag RUNX3

AGS cells were infected with retroviruses expressing vector control or Flag-RUNX3. Infected cells were selected with 2.5 μg/ml puromycin (Sigma, St Louis, MO, USA) and the cells stably expressing RUNX3 were confirmed by immunoblotting.

Transient transfection

For experiments using HEK293T and COS7 cells, the cells were transfected using calcium phosphate method. For experiments using AGS cells, the cells were transfected using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA).

Luciferase reporter assay

AGS cells were transfected with Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) with 0.2 μg of TβRE3-luc [45], 0.2 μg of TβR-I(CA), 0.1 μg of Flag-RUNX3, and the respective dosage of HA-CagA or its mutants with renilla luciferase reporter as a control. HEK293T cells were transfected using calcium phosphate transfection method. 48 hr post-transfection, firefly and renilla luciferase activities were measured with the

dual luciferase assay system from Promega. Firefly luciferase activity was normalized to renilla luciferase activity.

Whole cell lysis, immunoprecipitation and immunoblotting analysis

Whole cell lyses were performed using lysis buffer (50 mM HEPES pH 7.4, 250 mM NaCl, 1 mM EGTA, 1% NP-40, 1 mM PMSF, 1 x protease inhibitor cocktail tablet) for 20 minutes 4°C. Cell lysate was then either boiled with sodium dodecyl sulfate (SDS) loading buffer or used for pull-down or immunoprecipitation assays. For immunoprecipitation experiments, the cell lysates were incubated with anti-Flag or anti-HA antibodies-conjugated agarose beads for 2 hr at 4°C. All protein samples boiled with SDS loading buffer were analyzed by 10-13% SDS-polyacrylamide gel electrophoresis (PAGE) gels and then transferred to nitrocellulose membranes for western blotting. Western blot membranes were incubated in 10% milk in PBS-Tween, followed by incubation with appropriate primary antibody, then respective secondary antibody. Protein bands were visualized by anti-HA-horseradish peroxidase (HRP) followed by detection using an ECL detection kit.

Pulse-chase analysis

AGS or COS-7 cells were transfected with expression vectors for RUNX3 or RUNX3 together with CagA for 24 hr before being subjected to treatment with 100 μg/ml of cycloheximide (Sigma, St Louis, MO, USA) for a period of 0, 1, 2 or 4 hr. Cells were then lysed and analyzed with immunoblotting for the expression of RUNX3 with anti-RUNX3 antibodies.

GST-CagA pull-down assay

For glutathione-S-transferase (GST) pull-down assays, HEK293T cells were transfected respective plasmid DNAs for 48 hours and subjected to whole cell lysis protocol described above. 20 μl of 50% GST-CagA-conjugated glutathione-Sepharose beads slurry was added to the lysate described above and the mixture was incubated on a rotator for 2 hour at 4°C. The precipitated proteins were then analyzed by immunoblotting with the appropriate antibodies as described above.

2.3 Results

2.3.1 *H. Pylori* Infection Down-regulates RUNX3 Expression in a CagA-dependent Manner

To explore the possibility that *H. pylori* infection might lead to the inactivation of RUNX3, we first investigated the effect of *H. pylori* infection on the transcriptional activity of RUNX3. RUNX3 activates the TβRE luciferase reporter, which contains three RUNX binding sites [45], in the presence of constitutively activated TGF-β type I receptor (TβRI) in AGS cells (Figure 2.3a). However, the transcriptional activity of RUNX3 was inhibited by infection with wild-type (WT) *H. pylori* but not with its *cagA*-deficient isogenic mutant (Figure 2.3a), indicating that *H. pylori* down-regulates the transcriptional activity of RUNX3 and that CagA is essential for the inhibition.

To further test whether CagA is sufficient to down-regulate the transcriptional activity of RUNX3, we examined the effect of CagA on the transcriptional activity of RUNX3 in the same TβRE luciferase reporter assay. While co-transfection of CagA did not inhibit the basal activity induced by TβRI in AGS cells, it inhibited the transcriptional activity of RUNX3 in a dose-dependent fashion (Figure 2.3b). A similar inhibitory effect for CagA on RUNX3 was also observed in HEK293T epithelial cells, where RUNX3 displayed higher transcription activity than in AGS cells likely due to the higher transfection efficiency in these cells (Supplementary Figure 2.8 Pg 51-54). These data demonstrate that CagA alone is sufficient to inhibit the transcriptional activity of RUNX3.

a

b

Figure2.3 (a) CagA inhibits the transcriptional activity of RUNX3. AGS cells were transfected by Lipofectamine 2000 with TβRE reporter plasmid together with expression vectors for RUNX3 and constitutively-activate TβRI(CA) [45, 82] for 24 hr. Luciferase activity was measured 24 hr after the transfected cells were infected with WT or cagA-deficient *H. pylori*. Results represent the average of three independent experiments +/- SD. ***Figure2.3 (b)*** CagA is sufficient to inhibit the transcriptional activity of RUNX3. CagA, RUNX3 and TβRI(CA) were co-transfected with the TβRE reporter plasmid into AGS cells as indicated. 48 hr after transfection, luciferase activity was measured as in (a).

Interestingly, examining the cellular levels of RUNX3 in *H. pylori* infected AGS cells revealed that the expression of RUNX3 was down-regulated by the infection in a CagA-dependent manner (Figure 2.3c), indicating that CagA is involved in decreasing RUNX3 expression. To further confirm this, we examined the expression of RUNX3 in *H. pylori*-infected MKN-45 cells, which express endogenous RUNX3. Infection of MKN-45 cells with WT but not the *cagA*-deficient *H. pylori* also decreased the expression of RUNX3 in MKN-45 cells (Figure 2.3d). More importantly, when we investigated the expression of RUNX3 in *H. pylori* infected mice, we found that expression of RUNX3 in gastric mucosa was significantly reduced 48 h after infection with WT *H. pylori* (Figure 2.3e, left panel) compared to the expression either after

infection with the *cagA*-deficient *H. pylori* (Figure 2.3e, right panel) or in uninfected controls (data not shown). This time point precedes the development of gastric inflammation, further supporting a role for direct microbial effects in carcinogenesis. These *in vivo* data further support the conclusion that *H. pylori* infection reduces the expression of RUNX3 in a CagA-dependent manner. Supportively, co-expression of CagA with RUNX3 also decreased the cellular levels of RUNX3 (Figure 2.3f). The decreased expression of RUNX3 was due to the degradation of RUNX3, since treatment of the cells with proteasome inhibitor MG-132 reversed the decreased expression of RUNX3 (Figure 2.3f) and co-expression of CagA or infection with *H. pylori* did not alter the RUNX3 mRNA levels (Supplementary Figure 2.9&2.10). Collectively, these findings demonstrate that *H. pylori* CagA induces the proteolytic degradation of RUNX3.

Figure 2.3 (c) CagA is essential for the reduced expression of RUNX3 in response to *H. pylori* infection. AGS cells transiently expressing RUNX3 were infected with WT and cagA-deficient *H. pylori* for 24 hr, and levels of RUNX3, CagA and phosphorylated CagA were detected by immunoblotting whole-cell extracts with anti-RUNX3, anti-CagA, anti-phosphotyrosine, or anti-tubulin antibodies. **Figure 2.3 (d)** MKN-45 cells were infected with WT and cagA-deficient *H. pylori* for 48 hr, and levels of RUNX3, CagA and phosphorylated CagA were detected as in (c).

e

Figure 2.3 (e) RUNX3 expression is decreased within mouse gastric mucosa early after infection with *H. pylori* NCTC11637CagA positive strain. Immunohistochemistry for RUNX3 was performed on gastric mucosa harvested from INS-GAS mice infected with WT or cagA-deficient NCTC11637 *H. pylori* strains 48 h after inoculation. Representative staining of RUNX3 is shown for WT (left panel) or cagA-deficient mutant (right panel)-infected mice. The bars are equal to 100um.

Figure 2.3 (f) CagA is sufficient to induce the proteasome-mediated degradation of RUNX3. AGS cells were transfected with expression vectors for Flag-RUNX3 and CagA-HA. 24 hr post-transfection, cells were treated or not with MG-132 (10 µM) for 6 hr. Whole-cell lysates were immunoblotted for the expression of RUNX3, CagA and tubulin as indicated.

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2.3.2 RUNX3 Interacts with CagA through the PY Motif and the WW Domain Respectively.

The CagA-dependent degradation of RUNX3 by *H. pylori* infection prompted us to examine whether CagA might directly target RUNX3. Immunoprecipitation of CagA from transfected HEK293T cells co-immunoprecipitated RUNX3 (Figure 2.4a*). The interaction between CagA and RUNX3 was further confirmed with the *in vitro* GST pull-down (Supplementary Figure 2.11), indicating a direct interaction between RUNX3 and CagA. Furthermore, when the physical interaction between CagA and the endogenous RUNX3 was investigated in *H. pylori*-infected RUNX3-expressing MKN45 gastric cancer cells, RUNX3 co-immunoprecipitated CagA after *H. pylori* infection (Figure 2.4b). These data demonstrate that CagA associates with RUNX3 *in vitro* and *in vivo* in response to *H. pylori* infection.

Figure 2.4 (a) CagA interacts with RUNX3 in transfected cells. HEK293T cells were transfected with Flag-RUNX3 and HA-tagged CagA as indicated. The transfected HEK293T cell lysates were incubated with anti-HA antibodies-conjugated agarose beads. CagA-HA immunoprecipitates from whole-cell lysates were immunoblotted for Flag-tagged RUNX3. ***Figure 2.4 (b)*** CagA interacts with RUNX3 after *H. pylori* infection. MKN45 gastric cancer cells were infected with *H. pylori* for 0 or 24 hr. Endogenous RUNX3 was immunoprecipitated from *H. pylori*-infected MKN45 cells with either anti-IgG or anti-RUNX3 antibodies and immunoblotted for associated CagA with anti-CagA antibodies.

In order to define the region of RUNX3 responsible for its interaction with CagA, we performed an *in vitro* GST pull-down assay using cell lysates containing various deletion mutants of RUNX3 (Figure 2.4c). Full-length RUNX3 as well as two of its C-terminal deletion mutants (deleted to amino acid 325) were able to associate with CagA (Figure 2.4c). Further deletion to amino acid 283 abolished RUNX3's interaction with CagA (Figure 2.4c), indicating that the region from amino acids 283 to 325 of RUNX3 is critical for its interaction with CagA.

C

Figure 2.4(c) The region of RUNX3 from amino acids 283 to 325 is important for its interaction with CagA. Left: Schematic showing the domain structure of RUNX3 and the position of the PY motif. Right: GST-CagA was incubated with cell lysates containing Flag-tagged RUNX3 or its various deletion mutants as indicated and precipitated with glutathione agarose beads. The recovered materials were immunoblotted with anti-Flag antibodies.

Examining the sequence from amino acids 283 to 325 revealed that this region contains a PPxY sequence (known as the PY motif), which can be recognized by a WW domain within an interacting partner protein [108]. The WW domain is defined by a conserved tryptophan (W) residue and an invariant proline (P) residue placed approximately 20-22 amino acid residues apart [108]. Interestingly, in examining the sequence of CagA, we identified two WW domain-like sequences within the N terminal region of CagA (designated as WW1 and WW2, respectively) (Figure 2.4e). We then explored the possibility that the PY motif of RUNX3 and the WW domains of CagA might be involved in their interaction. We generated various mutants of RUNX3 or CagA with mutation or deletion of the PY motif or the WW domains and examined their interaction. When the first proline (P) of the PPPY sequence was mutated to alanine (A) (designated as RUNX3-P1A) or the PPPY sequence was deleted (designated as RUNX3-ΔPY), the interaction of CagA with RUNX3 was significantly impaired (Figure 2.4d), indicating that the PY motif is critical for the interaction of RUNX3 with CagA.

Figure 2.4 (d) PY motif of RUNX3 is essential for its interaction with CagA. HEK293T cells were transfected with HA-tagged CagA and WT Flag-RUNX3 or its PY motif mutants as indicated. CagA-HA immunoprecipitates were immunoblotted for the associated RUNX3 WT or PY motif mutants.

As for the CagA WW domain deletion mutants, deletion of WW1 of CagA barely affected its interaction with RUNX3 (Figure 2.4e). However, deletion of WW2 domain alone or together with WW1 completely abolished its interaction with RUNX3, confirming that the interaction between RUNX3 and CagA is through the specific recognition of the PY motif by the WW domain.

Figure 2.4 (e) WW domain 2 of CagA is critical for its interaction with RUNX3. Top: Schematic showing the relative location of the two WW domains of CagA and the sequence alignment of the WW domains of CagA with other known WW domains. Bottom: HEK293T cells were transfected with HA-tagged CagA or its WW domain deletion mutants and Flag-RUNX3 as indicated. CagA-HA immunoprecipitates were immunoblotted for the associated RUNX3.

2.3.3 Interaction between RUNX3 and CagA Reduces RUNX3 Stability.

Having identified that CagA interacts with RUNX3, we next investigated whether the recognition of the PY motif by the WW domain of CagA was required for the down-regulation of the transcriptional activity of RUNX3. When the effect of CagA on the activity of RUNX3 WT or PY motif mutants was examined, we found that RUNX3-P1A and RUNX3-ΔPY displayed resistance to CagA-induced inhibition (Figure 2.5a). Furthermore, when the capabilities of the CagA WW domain deletion mutants to down-regulate RUNX3 were tested in the TβRE reporter assay, CagA with the deletion of WW1, WW2, or both barely inhibited the activity of RUNX3 (Figure 2.5b). These results demonstrate that the interaction with CagA is critical for the inactivation of the transcriptional activity of RUNX3.

Figure 2.5 (a) RUNX3 with PY motif mutations is resistant to CagA-mediated down-regulation of its activity. WT RUNX3 or its PY motif mutants, CagA and TβRI(CA) were co-transfected with the TβRE reporter plasmid into HEK293T cells as indicated. Luciferase activity was measured as in Figure 2.3.1b. ***Figure 2.5 (b)*** WW domains of CagA are essential for the down-regulation of the transcriptional activity of RUNX3. WT or WW domain deletion mutants of CagA, RUNX3 and TβRI(CA) were co-transfected with the TβRE reporter plasmid into HEK293T cells as indicated. Luciferase activity was measured as in (a).

Next, we determined whether the interaction between RUNX3 and CagA is essential for the degradation of RUNX3. We first compared the effect of WT CagA on the stability of RUNX3 by measuring the half-life of WT RUNX3 or its PY motif mutants. Consistent with the notion that CagA promotes the degradation of RUNX3, the half-life of RUNX3 was reduced from greater than 4 hr to about 1.5 hr when CagA was co-expressed with RUNX3 (Figure 2.5c). Conversely, RUNX3 PY motif mutants were more resistant to CagA-induced degradation, with much longer half-lives (greater than 3 hr for RUNX3-P1A and greater than 4 hr for RUNX3-ΔPY) (Figure 2.5c), indicating that the PY motif is important for the CagA-induced degradation of RUNX3.

c

Figure 2.5 (c) The PY motif is important for the CagA-induced degradation of RUNX3. COS-7 cells were transfected using calcium phosphate with WT RUNX3 with or without CagA, or with RUNX3-P1A or RUNX3-ΔPY and CagA as indicated. 24 hr after transfection, cells were treated with 100 μg/ml of cycloheximide (CHX) for the indicated time points, and immunoblotted for the expression of Flag-RUNX3. A representative result from three independent experiments is shown in the left panels. Quantification of the results is shown in the right panel. Data represent the average of three independent experiments +/- SD.

Additionally, when the half-life of RUNX3 was measured with CagA WW domain deletion mutants, we found that the half-life of RUNX3 was moderately reduced when co-transfected with either WW1 or WW2 deletion mutants (Figure 2.5d). CagA with the deletion of both WW1 and WW2 domains failed to affect the half-life of RUNX3 (Figure 2.5d). Collectively, these data support the idea that CagA reduces the stability of RUNX3 via the specific recognition of the PY motif by the WW domain of CagA.

Figure 2.5 (d) WW domains of CagA are critical for CagA-induced degradation of RUNX3. COS-7 cells were transfected with RUNX3 together with WT CagA or its WW domain deletion mutants as indicated. 24 hr after transfection, cells were treated with CHX for indicated time points, immunoblotted for the expression of Flag-RUNX3, and levels were quantified as described in (c).

2.3.4 CagA stimulates the ubiquitination of RUNX3 *in vivo.*

Since CagA stimulates the degradation of RUNX3 (Figure 2.3), we next investigated whether CagA promoted the ubiquitination of RUNX3, an event that is required for proteasome-mediated degradation. When we examined the ubiquitination of endogenous RUNX3 in MKN-45 cells in response to *H. pylori* infection, we found that WT but not *cagA*-deficient *H. pylori* induced the ubiquitination of endogenous RUNX3 (Figure 2.6a), suggesting that CagA is

essential for the *H. pylori*-induced ubiquitination of RUNX3. When RUNX3 was co-transfected with ubiquitin and the ubiquitination of RUNX3 was measured, we found that co-expression of CagA significantly enhanced the ubiquitination of RUNX3 (Figure 2.6b), indicating that CagA is sufficient to promote the ubiquitination of RUNX3

Figure 2.6 (a) *H. pylori* infection induces the ubiquitination of RUNX3 in a CagA-dependent manner. MKN-45 cells were infected with WT *H. pylori* NCTC11637 and its cagA⁻ isogenic mutant. 24 h after infection, cells were treated with MG-132 (10μM) for another 24 h. Endogenous RUNX3 immunoprecipitates were immunoblotted for ubiquitination with anti-Ub antibodies (upper panel). Levels of CagA and RUNX3 are shown in the lower two panels. ***Figure 2.6 (b)*** CagA enhances ubiquitination of RUNX3 in vivo. HEK293T cells were transfected with the indicated combination of plasmids expressing Flag-RUNX3, His-ubiquitin, and CagA-HA. RUNX3 immunoprecipitates were immunoblotted for ubiquitination with anti-His antibodies (upper panel). Levels of RUNX3 and CagA are shown in the lower two panels.

We next assessed roles for the PY motif of RUNX3 and the WW domains of CagA in the CagA-induced ubiquitination of RUNX3. First, we examined the ubiquitination of RUNX3 PY motif mutants. Compared to WT RUNX3, the CagA-induced ubiquitination of RUNX3-P1A and RUNX3-ΔPY was significantly impaired, with RUNX3-ΔPY showing greater reduction in the

levels of ubiquitination (Figure 2.6c). These data are consistent with the findings that the PY motif is required for CagA-mediated degradation of RUNX3 (Figure 2.6d). When the WW domain mutants of CagA were examined for their abilities to induce the ubiquitination of RUNX3, we observed that deletion of WW1 moderately reduced CagA-induced ubiquitination of RUNX3 (Figure 2.6d). All together, these data further support the conclusion that the mutual interaction between CagA and RUNX3 is essential for the CagA-induced ubiquitination and subsequent degradation of RUNX3.

Figure 2.6 (d) WW domains of CagA are important for enhanced ubiquitination of RUNX3 by CagA. HEK293T cells were transfected with the indicated combination of plasmids expressing Flag-RUNX3, His-ubiquitin, and CagA-HA or its WW domain deletion mutants. Ubiquitination of RUNX3 was detected as in (a).

2.4 Discussion

Loss of RUNX3 expression is associated with gastric carcinogenesis [47], and hypermethylation of the *RUNX3* promoter or mis-localization of the RUNX3 protein largely attributes to the inactivation of RUNX3 in gastric cancer cells and gastric tumors [47, 82]. *H. pylori* infection has been shown to be an independent risk factor for the methylation of the promoter of *RUNX3* [109, 110]. Nevertheless, CagA appears to not be important for this epigenetic regulation [110]. In our current study, we demonstrate a CagA-dependent direct inactivation of RUNX3 in epithelial cells. CagA-induced degradation of RUNX3 in gastric epithelial cells provides another mechanism for the inactivation of RUNX3 and might account for the initiation of gastric cancer. While *H. pylori*-induced degradation of RUNX3, which is CagA dependent, might represent an immediate cellular response to infection, chronic infection with *H. pylori* might eventually induce the methylation of the *RUNX3* promoter, which is CagA independent, and account for the permanent inactivation of RUNX3.

CagA induces the ubiquitination and degradation of RUNX3 in cultured cells (Figures 2.3 & 2.5). How does CagA in fact induce the ubiquitination of RUNX3? First, CagA might function as an E3 ligase that directly targets RUNX3 for ubiquitination. Like many E3 ligases, CagA contains two WW domains (Figure 2.4e). However, cysteine, which is the essential amino acid for an E3 ligase to form a thioester bond with ubiquitin [111], is not found within CagA, excluding the possibility that CagA itself is an E3 ligase. Therefore, it is likely that CagA functions as a scaffold protein to recruit an E3 ligase for the ubiquitination of RUNX3. Further

investigation is needed to determine the E3 ubiquitin ligase that is utilized by CagA for the ubiquitination and proteasome-dependent degradation of RUNX3.

There are two WW domains (WW1 and WW2) within the N-terminal region of CagA (Figure 2.4e); however, these two domains appear to function differently regarding their involvements in the down-regulation of RUNX3. WW2, not WW1, is required for the specific interaction with the PY motif since deletion of WW2 but not WW1 abolished CagA's interaction with RUNX3 (Figure 2.4e). Although not directly involved in the interaction with RUNX3, WW1 is also important for the effective down-regulation of RUNX3. Deletion of WW1 impairs CagA's ability to induce ubiquitination and degradation of RUNX3, and to inhibit its transcriptional activity (Figures 2.5b, d and 2.6d). Therefore, it is quite possible that CagA utilizes its WW2 to associate with the PY motif of RUNX3 and its WW1 to recruit the ubiquitin E3 ligase for the degradation of RUNX3.

Emerging data demonstrate that transcription factor RUNX3 functions as a gastric tumor suppressor by regulating the expression of a variety of genes. It activates p21, Bim or Claudin-1 to attenuate cell growth, induce cell apoptosis or suppress tumor growth, respectively [55, 56, 112]. In addition, RUNX3 inhibits VEGF to suppress angiogenesis and metastasis of human gastric cancer [113]. *H. pylori* infection down-regulates the transcriptional activity of RUNX3 in a CagA-dependent manner (Figure 2.3a). Specific binding of the WW domain to the PY motif of RUNX3 is critical for the down-regulation of the transcription activity of RUNX3 (Figure 2.5a &

b). Through down-regulating the transcriptional activity of RUNX3, CagA might influence many cellular responses involved in carcinogenesis including cell growth, apoptosis and angiogenesis.

Studies from *Runx3*^{-/-} mice strongly suggest its tumor suppressor function in gastric cancer. *Runx3*^{-/-} mice displayed hyperplasia in gastric epithelial cells, and these cells were insensitive to the growth-inhibiting effects of TGF-β [47]. Recent studies from Ohnishi *et al.* demonstrate that transgenic mice expressing CagA also displayed gastric epithelial hyperplasia, and some of the mice developed gastric polyps as well as adenocarcinoma of the stomach and small intestine [101], emphasizing the oncogenic role of CagA in gastric cancer. Although the oncogenic potential of CagA relies on its ability to regulate the function of multiple host proteins, immunohistostaining from mice infected with *H. pylori* (Figure 2.3e) indicates that gastric epithelial hyperplasia might partially result from the inactivation of RUNX3 by CagA in the gastric epithelial cells. It might also be interesting to determine the levels of RUNX3 in gastric epithelial cells from CagA transgenic mice.

CagA interacts with various host cellular proteins to trigger distinct signaling pathways. Most cellular proteins targeted by CagA are cytoplasmic signaling molecules. For example, CagA associates with cytoplasmic SHP-2 or PAR1, respectively, for cell morphology or epithelial apical-basal polarity changes [102, 103]. CagA also indirectly regulates the activation of a variety of transcription factors, including NF-κB and NFAT, by its association with upstream cytoplasmic signaling molecules [114]. Interestingly, in our current study, we found that CagA directly associated with the nuclear transcription factor RUNX3, via CagA's WW

domain and RUNX3's PY motif, and induced its degradation (Figure 2.3 & 2.5). The location of this CagA-induced degradation is unknown, but our preliminary data suggested that it could possibly happen in the nuclear, since infection with *H. pylori* or co-expression of CagA does not change the nuclear localization of RUNX3 (Supplementary Figure 2.14). Supporting this, CagA was also found in the nuclear fraction of *H. pylori*-infected AGS cells (Supplementary Figure 2.15). These data suggest a potential nuclear function of CagA. In addition to its ability to interact with and regulate cytoplasmic proteins, CagA might also be able to enter the nucleus and regulate the functions of nuclear proteins. The probable nuclear localization of CagA has to be further characterized and studied.

Furthermore, signaling pathways involving CagA can be tyrosine phosphorylation-dependent and -independent manners [104, 114, 115]. Down-regulation of RUNX3 by CagA seems to be phosphorylation-independent, since a phosphorylation-deficient mutant of CagA was still able to induce the ubiquitination and degradation of RUNX3 (Supplementary Figures 2.12 & 2.13).

The identification of gastric tumor suppressor RUNX3 as a novel cellular target by CagA provides a mechanism for the carcinogenesis of CagA. The specific recognition of the PY motif of RUNX3 by the WW domain of CagA defines a novel interaction of a host protein with CagA (Figure 2.7). All these studies will contribute to the better understanding of the molecular mechanism for the initiation of gastric cancer. Regulation of the interaction between RUNX3 and CagA might be a potential target for the prevention of *H. pylori*-induced gastric cancer.

Figure 2.7 Schematic model for the role of CagA in *H. pylori* infection-induced degradation of RUNX3. After injection into cells, CagA associates with the PY motif of RUNX3 via its WW2 domain. Binding of CagA to RUNX3 recruits an unidentified E3 ligase (E3 X) and initiates the ubiquitination and proteasome-mediated degradation of RUNX3, resulting in the inactivation of its tumor suppressor activity in gastric epithelial cells.

2.5 Supplementary Figures

Figure 2.8 CagA inhibits the transcriptional activity of RUNX3 in HEK293T cells. CagA, RUNX3 and TβRI(CA) were co-transfected with the TβRE reporter plasmid into HEK293T cells as indicated. 48 hr after transfection, luciferase activity was measured as in Figure 2.3.1b.

Figure 2.9 mRNA expression of RUNX3 is not affected by CagA. AGS cells were transfected with Flag-RUNX3 and CagA for 48hr before total RNA was extracted and quantitative real-time PCR performed Expression of RUNX3 mRNA was normalized with the expression of hGAPDH.

Figure 2.10 *H. pylori* infection does not alter the mRNA expression of RUNX3. AGS cells were transfected with Flag-RUNX3 for 24 hr followed by *H. pylori* infection for 24 hr before total RNA was extracted and the mRNA level was quantified as in Supplementary Figure 2.

Figure 2.11 CagA interacts with RUNX3 in vitro. GST or GST-CagA was incubated with recombinant RUNX3 and precipitated with glutathione agarose beads. The recovered materials were immunoblotted with anti-RUNX3 antibodies. CBB: Coomassie brilliant blue staining. His-RUNX3 and GST-CagA proteins were expressed and purified from *Escherichia coli* BL-21.

Figure 2.12 CagA induces the degradation of RUNX3 independent of its tyrosine phosphorylation. AGS cells were transfected with expression vectors for Flag-RUNX3, WT CagA (CagA-HA) or phosphorylation-deficient mutant of CagA (CagA-PR-HA) for 48 hr. Whole-cell lysates were immunoblotted for the expression of RUNX3, CagA or tubulin as indicated.

Figure 2.13 CagA induces the ubiquitination of RUNX3 independent of its tyrosine phosphorylation. HEK293T cells were transfected with expression vectors for Flag-RUNX3, His-ubiquitin, and CagA-HA or CagA-PR-HA. Ubiquitination of RUNX3 was detected as in Figure 2.3.4b.

Figure 2.14 *H. pylori* infection reduces the nuclear expression of RUNX3. AGS cells stably expressing RUNX3 were infected with *H. pylori* for the indicated time points, and fractionated into cytoplasmic and nuclear fractions. Levels of RUNX3 were detected by immunoblotting fractions with anti-RUNX3 antibodies. Levels of tubulin or HDAC1 as cytoplasmic or nuclear fractionation controls are also shown.

Figure 2.15 *H. pylori* CagA localizes in both nucleus and cytoplasm. AGS cells were infected with *H. pylori* at the indicated time points, and fractionated into cytoplasmic and nuclear fractions. Levels of CagA were detected by immunoblotting fractions with anti-CagA antibodies. Levels of tubulin or HDAC1 as cytoplasmic or nuclear fractionation controls are also shown.

Chapter 3:

Peptidyl-prolyl *cis/trans* **isomerase Pin1 inhibits tumor suppressor activity of RUNX3 in breast cancer.**

3.1 Introduction: The prolyl Isomerase Pin1 in Breast Development and Cancer.

Pin1, a peptidyl-prolyl isomerase (PPIase) that specifically isomerizes only phosphorylated proline (P) directed serine (S) or threonine (T) bonds (S/T-P), is found to be a key signaling molecule involved in breast development and breast cancer [116]. A study in 2001 indicated that Pin1 was over expressed in an estimated 75% of primary human breast cancer tissues. Especially in grade II and III tumors, the mean expression level of Pin1 in cancer specimens was about 10 times higher than those of normal tissue controls. Moreover, Pin1 expression level was also higher in cell lines derived from human breast cancer than in cell lines established from normal mammary epithelial cells, emphasizing on Pin1's possible link to mammary carcinogenesis [117].

The human Pin1 gene was the first PPIase to be found essential for cell division in yeast and human cells, being identified from a yeast genetic screen searching for proteins involved in mitotic regulation [118]. Pin1 is unique in that, unlike typical PPIases, Pin1 only bind to the S/T-P bond after phosphorylation of the S/T residue [118-120]. The binding is followed by catalysis of the intrinsically rather slow *cis* to *trans* isomerization of peptide bonds N terminal of the proline residues. This isomerization process affects folding or refolding of the substrate protein, inducing conformation changes to regulate the function of the protein. These conformational changes can affect phosphorylation status, protein-protein interactions, subcellular localization or protein stability [121-123].

Figure 3.1 From [124]. Shows phosphorylation dependent regulatory mechanism carried out by Pin1. Proteins are first phosphorylated by proline-directed kinases like CDKs, MAPKs, GSK-3β allowing the binding of Pin1. Step two shows subsequent isomerization of the substrate by Pin1, inducing conformational changes resulting in a different function of the target protein.

In tandem with its over-expression in breast cancer, Pin1 is found to be capable of stimulating multiple oncogenic pathways at different levels contributing to mammary carcinogenesis. For example, over-expression of Pin1 in breast cancer cells results in the degradation of promyelocytic leukemia protein (PML), a tumor suppressor protein, that regulate multiple cellular pathways including DNA damage repair, apoptosis and cell cycle regulation.

Degradation of PML by Pin1 seemed to prevent hydrogen peroxide induced death in MDA-MB-231 breast cancer cells, increasing their proliferation, contributing to the cancer phenotype [121].

Pin1 has been reported to increase cyclin D1 gene expression by activating multiple pathways, including JNK/Ras pathway, Nuclear Factor (NF)-κB signaling pathway as well as Wnt/β-catenin signaling [117, 125-127]. Cyclin D1 has a well established role in the development of breast epithelial cells, it is known regulate cell cycle G1/Synthesis phase progression and is important for cell proliferation [128]. It is also found to be over-expressed in more than 50% of human breast cancer specimens, making it a strong prognostic factor for human breast cancer [129, 130]. Interestingly, in more than 80% of the tumors over-expressing cyclin D1, Pin1 expression level was also high [117]. Furthermore, $Pin1^{-/-}$ female mice showed similar phenotypes to cyclin D1 knockout mice, with under developed mammary epithelial duct and reduced mammary gland expansion during pregnancy [131]. All these data suggest that perhaps one of the major methods through which Pin1 is involved breast development is through the regulation of cyclin D1 function.

Pin1 has also been shown to play a role in the activation of Notch1, as a transcription factor which is deregulated in many cancers including breast cancer. Pin1 increases the transformation-inducing potential of Notch1 in soft-agar assays [123]. In addition, over-expression of Pin1 is correlated to centrosome amplification, which occurs frequently in many human cancers resulting in centrosome instability, cellular mis-segregation and aberrant cell division [132].

Figure 3.2 From [133]. Figure shows the different ways in which Pin1 catalyzed prolyl isomerization can regulate protein functions and result in a spectrum of target activities.

A recent study in 2009 linked Pin1 to the TGF-β signaling pathway. Pin1 was described to inhibit TGF-β-induced transcription and gene expression by mediating the degradation of Smad2/3 proteins via Smurf2-mediated ubiquitin-proteasome degradation. RUNX3 is an important downstream regulator in the TGF-β signaling pathway, and a target substrate of Smurf2 [134]. Based on the fact that Pin1 expression is highly up-regulated in breast cancer while RUNX3 is largely inactivated in breast cancer and also due to both proteins' involvement

in the TGF-β signaling pathway, we believe that perhaps the over-expression of Pin1 plays a role in RUNX3 inactivation.

In this part of the thesis, we showed that there is a significant inverse correlation between RUNX3 and Pin1 in breast cancer specimens. Pin1 interacts specifically with RUNX3, targeting it for proteasome degradation. Identification of Pin1 as a novel regulator of RUNX3 will better our understanding of breast carcinogenesis and RUNX3's role as a tumor suppressor in breast cancer.

3.2 Materials and Methods

Cell culture

Transformed mammary epithelial cells were purchased from ATCC (Manassas, VA, USA). MCF-7, MDA-MB-157, MDA-MB-361, MDA-MB-231 and MDA-MB-468 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS) (Sigma, St Louis, MO, USA). Hs587T was maintained in DMEM supplemented with 0.01ng/ml of bovine serum (Sigma, St Louis, MO, USA). Hcc70 and T-47D were propagated in RPMI-1640 (Sigma, St Louis, MO, USA) with 10% FBS, the medium for T-47D was further supplemented with 0.2 Units/ml of Bovine insulin (Sigma, St Louis, MO, USA). BT-20 was cultured in a 1:1 mixture of DMEM and Ham's F12 medium (Sigma, St Louis, MO, USA) with 10% FBS added. Normal mammary epithelial tissues MCF-12A was maintained in a 1:1 mixture of DMEM and Ham's F12 medium, 20 ng/ml Human epidermal growth factor (Cell Signaling, Danvers, MA, USA), 100 ng/ml cholera toxin (Sigma, St Louis, MO, USA), 0.01 mg/ml bovine insulin and 500 ng/ml hydrocortisone (Sigma, St Louis, MO, USA), and 5% horse serum (Sigma, St Louis, MO, USA). HEK293T cells were maintained in DMEM supplemented with 10% FBS.

Plasmids

The expression vectors for wild-type RUNX3 and its deletion mutants have been previously described [82]. HA-TβRI(CA) expression vector was kindly provided by Dr Miyazono (Tokyo U., Japan). GST-Pin1 was kindly provided by Dr. H.-Y.Kao (Case Western Reserve University). Flag-Pin1 and Myc-Pin1 vectors were constructed from GST-Pin into pcDNA-Flag/Myc vectors.

Flag-Pin1 C115A, W34A, and RUNX3 S/T-P mutants were generated using Quikchange site-mutagenesis (Stratagene, San Diego, CA, USA) and confirmed by sequencing.

Antibodies

Antibodies against Flag, HA, Myc, ubiquitin were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Antibody against Pin1 was purchased from R&D systems (Minneapolis, MN, USA), and antibody against RUNX3 was from Abcam (Cambridge, MA, USA).

GST-Pin1-conjugated glutathione-Sepharose beads

GST-Pin1 is purified from *Escherichia Coli* BL21 cells as stated in the protocol from Chapter 2.2: Preparation of recombinant proteins: GST-CagA and His-RUNX3.

Human tissue samples and immunohistochemistry

Paraffin blocks of mastectomy specimens from a total of 80 invasive ductal carcinomas were obtained from the pathology department of the National University Hospital, Singapore after approval of ethical issues by the Domain Specific Research Board of the National Health Care Group (NHG) of Singapore (approval code B06/006). Immunostaining was performed as in [33], by Mr. C W Ong and Dr M Salto-Tellez at Cancer Science Institute, National University of Singapore.

Establishment of MCF-7 cell lines stably expressing Flag RUNX3 and Flag RUNX3-4A

MCF-7 cells were infected with retroviruses expressing vector control or Flag-RUNX3 or Flag-RUNX3-4A. Infected cells were selected with 2.5 µg/ml puromycin (Sigma, St Louis, MO, USA) and the cells stably expressing RUNX3 WT and its mutant were confirmed by immunoblotting.

SiRNA Treatment, immunoprecipitation and immunoblotting analysis

Pin1 SiRNA or negative control SiRNA (Ambion, Inc. Austin, TX, USA) was transfected into MCF-7 cells and MDA-MB-361 cells using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen, San Diego, CA, USA). Immunoprecipitation and immunoblotting analysis were performed as previously described in Chapter 2.2: Whole cell lysis, immunoprecipitation and immunoblotting analysis.

Transient transfection and the luciferase reporter assay

MCF-7 cells were transfected with Fugene HD according to the manufacturer's protocol (Roche, Basel, Switzerland) with 0.5 μg of TβRE3-luc [45], 0.5 μg of TβR-I(CA), 0.25 μg of Flag-RUNX3, and respective dosage of Flag-Pin1 WT or its mutants. HEK293T cells were transfected using calcium phosphate transfection method. 48 hr post transfection, firefly and renilla luciferase activities were measured with the dual luciferase assay system from Promega (Madison, WI, USA). When Pin1 inhibitor, PIB (Sigma, St Louis, MO, USA), was used, 2μM of the drug was added for 48 hr prior to luciferase assay.

GST pull-down Assay

293T cells were transfected with the indicated expression vectors using the calcium phosphate method for 48 hr. Cell lysates were then incubated in vitro with 20μl of agarose beads containing GST-Pin1 or GST at $4^{o}C$ for 2 hr. The precipitated proteins were subjected to SDS-PAGE analysis (Chapter 2.2: GST pull-down Assay).

Pulse Chase Analysis

HEK293T cells were transfected with the indicated combination of expression vectors for RUNX3 and Pin1 for 24 hr before being subjected to treatment with cycloheximide (Sigma, St Louis, MO, USA) to a final concentration of 100ng/ml for a period of 0, 1, 2, or 4 hr. Cells were lysed and analyzed with immunoblotting for the expression of RUNX3 with anti-RUNX3 antibodies.

Quantitative real time PCR

RUNX-MCF-7 cells were transfected with Flag-Pin1 or pcDNA3.1 empty vector for 48 hr before total RNA was extracted using RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). Complementary DNA was synthesized with Omniscript RT kit (Qiagen, Venlo, The Netherlands) and quantitative real-time PCR was performed using Qiagen SYBR green PCR kit by 7300 real-time PCR system (ABI, Foster City, CA, USA).

3.3 Results

3.3.1 RUNX3 Levels Inversely Correlates with Pin1 Levels in Human Breast Cancer Tissues

Since Pin1 is highly over-expressed in breast cancer while RUNX3 is regularly either not expressed or mis-localized to the cytoplasm in breast cancer, we investigated whether Pin1 is involved in this inactivation of RUNX3. To address this question, we first used immunohistochemistry to examine the possible pathological correlation between Pin1 and RUNX3 expression levels in breast cancer and normal patient samples. We analyzed 80 human breast ductal carcinoma specimens from Singapore and found that over 80% of samples (19 out of 23) with low or no expression of RUNX3 displayed high expression of Pin1. Conversely, approximately 77% of samples (46 out of 59) with high expression of RUNX3 had low levels of Pin1 (Figure 3.3a and b). Statistical analysis revealed an inverse correlation between the expression of RUNX3 and Pin1 in these cancer specimens with a Spearman coefficient for correlation (RUNX3 and Pin1) of -0.615 ($p < 0.01$) (Figure 3.3b).

To further explore the possible relationship between expression levels of RUNX3 and Pin1, we examined the expression of RUNX3 and Pin1 in a panel of cell lines that includes non-tumorigenic breast epithelial cells and breast cancer cell lines. Pin1 appeared to be highly expressed in the breast cancer cell lines, while not expressed in the non-tumorigenic MCF-12A cell line. Although the expression level of RUNX3 varied across cell lines, the overall expression of RUNX3 appeared to be inversely correlated with the expression of Pin1 (Figure 3.3c)

Figure 3.3 (a and b) Correlation between RUNX3 and Pin1 levels in human breast cancers and normal tissues. *(a)* Representative immunohistochemical staining of RUNX3 and Pin1 using α-RUNX3 and α-Pin1 antibodies in normal and tumor breast tissues. Boxed Regions enlarged below. Their correlation was analyzed by Spearman rank correlation test (p<0.01) *(b)*. *(c)* Comparison of Pin1 and RUNX3 levels in mammary epithelial cell lines. The same amount of total lysates prepared from either normal human mammary epithelial cell lines or carcinoma-derived cell lines were subjected immunoblotting analysis with Pin1, RUNX3 and β-tubulin antibodies.

To better examine the correlation between Pin1 levels and RUNX3 expression, we determined the effects of Pin1 inhibition on RUNX3 expression in a breast cancer cell line, MCF-7, that stability expresses RUNX3 (hereby known as RUNX3-MCF-7) and in another breast cancer cell line, MDA-MB-361, that expresses endogenous RUNX3. Both cell lines express endogenous Pin1. Inhibition of Pin1 through siRNA knockdown showed increased exogenous RUNX3 expression in the stable line RUNX3-MCF-7, as compared to the control (Figure 3.3d). Likewise, siRNA knockdown of Pin1 in MDA-MB-361 resulted in an increase in endogenous RUNX3 expression (Figure 3.3e), indicating that RUNX3 expression level is dependent on Pin1 expression. These results suggest that Pin1 might inactivate RUNX3 through suppression of its expression level.

Figure 3.3 (d and e) RUNX3 expression level is rescued by Pin1 down-regulation. RUNX3-MCF7 cell line stably expression RUNX3 in *(d)* and MDA-MB-361 cell line endogenously expressing RUNX3 and Pin1 in *(e)* were transfected with Pin1 siRNA or control. Whole cell lysates were immunoblotted with Pin1, RUNX3 and β-tubulin antibodies.

3.3.2 Pin1 interacts with RUNX3 in a phosphorylation-dependent manner.

To explore whether Pin1 down-regulates RUNX3 expression directly, we investigated if RUNX3 is a direct substrate of Pin1. Flag-Pin1 and Myc-RUNX3 were co-expressed exogenously in HEK293T cells, and immunoprecipitation using anti-Flag antibody-conjugated beads was performed on the whole cell lysates. Pin1 was found to interact with RUNX3 *in vivo* (Figure 3.4a). The physical interaction between RUNX3 and Pin1 was also investigated using MDA-MB-157 cells that endogenously express RUNX3 and Pin1. RUNX3 was co-immunoprecipitated with Pin1 and not IgG, indicating that RUNX3 and Pin1 does interact endogenously (Figure 3.4b). To further confirm this interaction, RUNX3 was over-expressed in HEK293T cells and the cell lysates were harvested for a pull-down assay with either GST or GST-Pin-conjugated beads. RUNX3 was only pulled down together with GST-Pin1 beads, displaying the *in vitro* interaction of RUNX3 and Pin1 (Figure 3.4c).

Figure 3.4 (a) Exogenously expressed RUNX3 and Pin1 interacts *in vivo*. HEK293T cells were transfected with Myc-RUNX3 and Flag-Pin1 expression vectors. Cell lysates were immunoprecipitated with M2-Flag agarose and immunoblotted with Myc and Flag antibodies. *Figure 3.4* (b) RUNX3 and Pin1 interact endogenously. MDA-MB-157 cell lysates were immunoprecipitated with Pin1 or control IgG antibody and subjected to western blotting with RUNX3-5G4 and Pin1 antibody.

C

RUNX3 → | IB:α-Flag
GST-Pin1 → | IB:α-GST
GST → |
Input → | IB:α-Flag
1   2

Figure 3.4 (c) RUNX3 and Pin1 interacts *in vitro*. HEK293T cell lysates over-expressing RUNX3 protein were subjected to GST-Pin1 fusion protein pull-down assay. The precipitates from the pull-down were then immunoblotted with Flag and GST antibodies.

Since Pin1 is known to bind selectively to motifs containing a phosphorylated serine or threonine residue preceding a proline residue as previously discussed, we tested whether the interaction between Pin1 and RUNX3 is also phosphorylation dependent. HEK293T cells were transfected with Flag-RUNX3 plasmids and the whole cell lysates were treated with a calf intestinal alkaline phosphatase (CIP) prior to a GST pull-down assay and tested for Pin1 binding ability. Expectedly, treatment with phosphatase almost completely abolished the interaction between RUNX3 and Pin1, hinting at a phosphorylation dependent interaction between the two proteins (Figure 3.4d). In addition, to assess the possibility that this phosphorylation dependent interaction could be due to pS/T-P motifs in RUNX3, we used a mitotic phosphospecific antibody MPM2, which specifically recognizes phosphorylation events in these motifs [119, 135]. As shown in Figure 3.3.2e, RUNX3 was co-precipitated with the MPM2 antibody, indicating the presence of phosphorylated S/T-P motifs in RUNX3.

D

Figure 3.4 (d) RUNX3 and Pin1 interact *in vitro* in a phosphorylation dependent manner. HEK293T cell lysates over-expressing RUNX3 protein were treated with CIP prior to GST pull-down- assay as in (c). The precipitates from the pull-down were immunoblotted with Flag and GST antibodies.

E

Figure 3.4 (e) RUNX3 protein contains pS/T-P motif. Cell lysates from RUNX3-MCF7 stable line was immunoprecipitated with control IgG antibody or anti-MPM2 antibody and immunoblotted with Flag antibody.

3.3.3 RUNX3 interacts with Pin1 through 4 unique S/T-P motifs

To further understand the mechanism of RUNX targeting by Pin1, we went on to map the interaction sites on Pin1 and RUNX3. Pin1 is known to consist of an N-terminal WW domain and a C-terminal catalytic PPIase domain. The WW domain forms a binding pocket, binding specifically to Pin1 substrates using side chains of residues Serine $(S)^{16}$, Arginine $(R)^{17}$, Tyrosine $(Y)^{23}$ and Tryptophan $(W)^{34}$ [136]. Point mutations of any of one the substrates (mutants S16A, R17A, W34A Figure 3.5a) have been shown to interfere with Pin1 substrate binding ability. The PPIase domain on the other hand contains a cysteine (C) residue which is important for the catalysis of the cis-trans isomerization of the peptide bond in pS/T-P motifs of Pin1 substrates. Mutation of the cysteine (mutant C115A Figure 3.5a and b) in the PPIase domain to an alanine has been shown to abolish the catalytic activity of Pin1 without interfering with the Pin1's interaction with its substrate protein [136].

To investigate if RUNX3 binds to the WW domain of Pin1, we performed a GST pull-down assay as previously described using either wild-type (WT) GST-Pin1 or GST1-Pin1 with the respective point mutations. Expectedly, RUNX3 interacted with WT GST-Pin1, but failed to show interaction with both S16A and W34A (Figure 3.5a and b). Weak binding was only observed in RUNX3's interaction with GST-Pin1-R17A mutant (Figure 3.5a), while mutation of the PPIase cysteine did not abolish RUNX3 and Pin1 binding (Figure 3.5a). These results suggest that RUNX3 does bind to Pin1 through WW domain of Pin1.

Figure 3.5 (a and b) Specific binding of RUNX3 to the WW domain in Pin1. HEK293T cells over-expressing RUNX3 protein is subjected to GST pull-down assay with various GST-Pin1 mutants ***(a)***. In ***(b)***, HEK293T cells were co-transfected with Myc-Pin1 WT or W34A mutant and Flag-RUNX3. Cell lysates were immunoprecipitated with M2-Flag agarose and immunoblotted.

As mentioned in Chapter 1.2, Figure 1.1, RUNX3 has a PST (proline, serine, threonine) rich region after the Runt domain from amino 185 to 415. To identify the specific S/T-P motif/s in RUNX3 that is/are interacting with the WW-domain of Pin1, we first tried to identify the region of RUNX3 that is involved in the interaction. HEK293T cells were transfected with either full length RUNX3 (1-415) or c-terminal deletion mutants of Flag-RUNX3 (Figure 3.5c left), cell lysates were then subjected to GST-pull-down assay with WT-GST-Pin1. Full-length RUNX3 and deletion mutants, 1-373, 1-325 and 1-234 all showed significant degrees of interactions with GST-Pin1, interaction with Pin1 was only abolished with the 1-187 mutant (Figure 3.5c right). This *in vitro* interaction data indicates that Pin1 binds largely to RUNX3 through amino acids 187 to 234 of RUNX3.

Figure 3.5 (c) Region of RUNX3 from amino acid 187 to 234 is important for interaction with Pin1. Left: shows schematic representation of RUNX3 domains and the position of amino acids 187 to 234. Right: GST-Pin1 was incubated with HEK293T cell lysates containing Flag-tagged RUNX3 or its various deletion mutants as indicated and precipitated with glutathione agarose beads. The recovered materials were immunoblotted for RUNX3 with anti-RUNX3 antibodies. CBB= Coomasie blue blot.

Detailed analysis of RUNX3 amino acids 187 to 234 revealed 4 unique S/T-P motifs at locations 209, 212, 214 and 231 (Figure 3.5d top). Point mutations were made on these 4 S/T-P motifs, specifically, T209A, T212A, S214A and T231A. A final mutant containing all point mutations on all 4 S/T-P motifs listed above was also generated and named RUNX3-4A. GST-pull-down assay was done using WT-RUNX3 and the above 5 S/T-P mutants with WT-GST-Pin1. Interestingly, we noticed that GST-Pin1 was able to interact with all 4 single point mutation mutants, T209A, T212A and S214A and T231A (Figure 3.5d). Only when all 4 S/T-P motifs were mutated did we see an abolishment of Pin1 binding to RUNX3 (Figure 3.5d). This

data showed redundancy in the S/T-P binding sites, suggesting that perhaps all four S/T-P motifs identified between regions 187 to 234 of RUNX3 were able to bind to Pin1. An *in vivo* co-immunoprecipitation assay further confirmed that mutation of all 4 S/T-P binding site disrupted RUNX3's interaction with Pin1 (Figure 3.5e), indication that Pin1 interacts with RUNX3 through 4 unique S/T-P motifs on RUNX3.

Figure 3.5 (d and e) 4 unique S/T-P motifs in RUNX3 are important for binding to Pin1. ***(d)*** Top: Positions of the 4 S/T-P motifs in RUNX3 region 187 to 234. Bottom: HEK293T cell lysates over-expressing the various RUNX3 S/T-P point mutations were subjected to GST-Pin1 pull down assay and immunoblotted as in (c). ***(e)*** HEK293T cells transfected with Flag-RUNX3 WT or mutant containing point mutations of all four S/T-P motif and Myc-Pin1 is immunoprecipitated with anti-Flag antibody and immunoblotted as in (b).

3.3.4 Pin1 disrupts the function of RUNX3

To further study the physiological significance of Pin1 targeting of RUNX3, we studied if Pin1 inhibits the transcriptional activity of RUNX3. Using HEK293T cells, RUNX3 was found to activate the TβRE luciferase reporter in the presence of constitutively active TGF-β type 1 receptor (TβRI) [45]. However, this transcriptional activity was inhibited in a dose dependent manner in the presence of increasing dosage of WT Pin1, while remaining relatively unchanged with increasing dosage of the enzymatic deficient Pin1 mutant, C115A, indicating that Pin1 down-regulates the transcriptional activity of RUNX3 in an enzymatic dependent manner (Figure 3.6a). The W34A mutant which has reduced interaction with RUNX3 was only able to inhibit the transcriptional activity of RUNX3 to a small extend (Figure 3.6a). Likewise, WT Pin1 was unable to reduce the transcriptional activity of RUNX3-4A mutant that does not interact with Pin1 (Figure 3.6b). It is also interesting to note that RUNX3-4A has a reduced transactivational ability compared to WT-RUNX3 (Figure 3.6b).

The ability of Pin1 to disrupt the transcriptional activity of RUNX3 is further confirmed through luciferase assay in a physiologically relevant breast cell line, MCF-7. The activation of TβRE luciferase reporter in the presence of TβRI by RUNX3 was inhibited by Pin1 but not Pin1-C115A mutant in MCF-7 (Figure 3.6c). The addition of PiB, an inhibitor of the isomerase activity of Pin1 (Figure 3.6c), reversed the inhibition of RUNX3 activity by Pin1, supporting the notion Pin1 abolishes the function of RUNX3 in an enzymatic dependent manner.

A

| Flag-RUNX3 | − | − | + | + | + | + | + | + | + |
| Flag-Pin1 | − | − | − | WT | | C115A | | W34A | |

B

HA-TβRI(CA)	−	+	+	+	+	+
Flag-Pin1	−	−	−	+	−	+
Flag-RUNX3	−	−	+	+	+	+
			WT		4A	

Figure 3.6 (a) Pin1 inhibits the transcriptional activity of RUNX3. HEK293T cells were transfected with TβRE reporter plasmid together with expression vectors for RUNX3, constitutively activated TβRI(CA) [45, 82], and increasing doses of Pin1 WT and its different mutants as indicated. In each experiment, cells were also co-transfected with Renilla luciferase reporter plasmid which was used as an internal control. Luciferase activity was measured 48 hr after transfection and results represent the average of three independent experiments +/- SD. ***(b)*** Pin1 inhibits the transcriptional activity of RUNX3 but not RUNX3-4A. HEK293T cells were transfected with TβRE reporter plasmid, expression vectors for RUNX3 WT or mutant 4A, constitutively activated TβRI(CA), and Pin1 WT. Luciferase assay was carried out as in (a).

C

HA-TβRI(CA)	−	+	+	+	+	+
Flag-RUNX3	−	−	+	+	+	+
Flag-Pin1	−	−	−	WT	C115A	WT+PiB

Figure 3.6 (c) Pin1 inhibits the transcriptional activity of RUNX3 in MCF7 cells. MCF7 cells were transfected and luciferase assay performed as in (a). Cells were also treated with PiB (2.0μM) for 48 hours prior to luciferase assay.

RUNX3 as a transcription factor is known to recruit co-activators like p300 and synergistically transactivate downstream genes [58]. RUNX3 activated the pGL3-12-RXE luciferase promoter, which contains 12 copies of RUNX3 binding sites [58] in the presence of p300. This activation was again inhibited by WT Pin1 but not the enzymatic inactive mutant Pin1-C115A (Figure 3.6 c). Pin1 was unable to inhibit the transcriptional activity of the Pin1 binding deficient mutant, RUNX3-4A, once again confirming the notion that Pin1 inhibits the transcriptional activity of RUNX3 in an enzymatic and binding dependent manner.

Figure 3.6 (d) Pin1 inhibits the cooperation between co-activator p300 and RUNX3. HEK293T cells were transfected with pGL3-12-RXE Luc [58] together with expression vectors for RUNX3, p300 and increasing doses of Pin1 WT and enzymatic deficient mutant. Luciferase assay performed as in (a). *Figure 3.6 (e)* Pin1 and RUNX3 interaction is important to inactivate the co-activation of p300 and RUNX3. HEK293T cells were transfected with pGL3-12-RXE Luc and expression vectors for RUNX3 WT or 4A mutant, p300 and Pin1 WT. Luciferase assay performed as in (a).

3.3.5 RUNX3 stability is reduced in the presence of Pin1

The inverse correlation between RUNX3 and Pin1, as well as the ability of Pin1 to directly disrupt the function of RUNX3 prompted us to investigate whether Pin1 might regulate the stability of RUNX3. We over-expressed WT Pin1 and the enzymatic deficient mutant Pin1 C115A in RUNX3-MCF-7 stable lines and examined the effect of Pin1 on the expression of RUNX3 by immunoblotting. As expected, the expression of RUNX3 was down-regulated in the presence of WT Pin1 but not enzymatic deficient Pin1 C115A (Figure 3.7 a). This decreased in RUNX3 expression in the presence of Pin1 was reversed by treatment with increasing dosage of proteasome inhibitor MG-132 (Figure 3.7b). Moreover, quantification of RUNX3 mRNA level through real-time PCR showed that RUNX3 mRNA levels were not affected by the presence of Pin1 in the cells (Figure 3.7c), suggesting that Pin1 reduces the expression of RUNX3 at the protein level rather than at the mRNA level and that Pin1 does regulate the stability of RUNX3 protein.

Figure 3.7 (a) Pin1 reduces the expression of RUNX3. RUNX3-MCF-7 cells were transiently transfected with either Pin1 WT plasmid or C115A enzymatic deficient mutant. Levels of RUNX3 were detected by immunoblotting the whole-cell extracts with anti-Flag antibodies. *Figure 3.7 (b)* Pin1 induces proteasome-mediated degradation of RUNX3. RUNX3-MCF-7 stable line was transfected with Pin1 expression vector. 24h post transfection, cells were treated with increasing dosage of MG132 as indicated for 10h. Cell lysates were then immunoblotted for RUNX3, Flag-Pin1 and tubulin.

Figure 3.7 (c) Pin1 does not alter the mRNA expression of RUNX3. RUNX3-MCF-7 cells were transfected with Flag-Pin1 for 48 hours before total RNA was extracted. Complementary DNA was synthesized and quantitative real-time PCR was performed. Expression of RUNX3 mRNA was normalized with the expression of hGAPDH.

Subsequently, we also looked at whether interaction between Pin1 and RUNX3 is necessary to reduce stability of RUNX3. We co-expressed either WT RUNX3 or RUNX3-4A mutant in MCF-7 cells in the presence of Pin1 and immunoblotted for the expression of RUNX3. Not surprisingly, Pin1 was only able to down-regulate the expression of WT RUNX3 but not RUNX3-4A (Figure 3.7d), indicating that interaction between RUNX3 and Pin1 is necessary for the degradation of RUNX3 by Pin1.

Figure 3.7 (d) Interaction between RUNX3 and Pin1 is important for degradation of RUNX3. MCF-7 cells were transiently transfected with Flag-RUNX3 WT or 4A and Pin1 WT expression vectors. Cell lysates were immunoblotted as in (a).

E

Figure 3.7 (e) Stability of RUNX3 is reduced in the presence of Pin1. HEK293T cells were transfected with WT RUNX3 with or without Pin1 WT or Pin1 C115A, or with RUNX3-4A and Pin1 WT as indicated. 24 hr after transfection, cells were treated with CHX (10μg/ml) for the indicated time points, and immunoblotted for the expression of Flag RUNX3. A representative result from three independent experiments is shown in the left panel. Quantification of the results is shown in the right panel. Data represent the average of three independent experiments.

To more quantitatively evaluate the effect of Pin1 on the stability of RUNX3, we compared the half-life of over-expressed RUNX3 in the presence of WT Pin1 and enzymatic deficient Pin1 C115 in HEK293T cells. We performed the pulse-chase experiment using cycloheximide to inhibit the synthesis of new RUNX3 protein (Figure 3.7e left). RUNX3 was found to be relatively stable in HEK293T cells with a half life of greater than 4 hours, however, in the presence of Pin1, the degradation of RUNX3 was accelerated and the half-life of RUNX3 was reduced to about 2 hours. Conversely, the enzymatic deficient Pin1-C115A protein did not increase the degradation of RUNX3 significantly, in the presence of Pin1-C115A, the half-life of RUNX3 was also estimated to be greater than 4 hours (Figure 3.7e right). We also quantified the half-life of RUNX3-4A mutant in the presence and absence WT Pin1. We observed that RUNX3-4A has a much shorter half life (2.5 hours) than WT RUNX3, but the presence of Pin1

did not shorten this half-life further (Figure 3.7e). These data demonstrate that Pin1 reduces the stability of RUNX3 in an enzymatic and interaction dependent manner, by targeting RUNX3 for proteasome degradation.

3.3.6 Pin1 induces the ubiquitination of RUNX3.

Since Pin1 mediates proteasome degradation of RUNX3, we next investigated if Pin1 also induce the ubiquitination of RUNX3. RUNX3 was co-transfected with ubiquitin and WT Pin1 or enzymatic deficient Pin1 C115A in HEK293T cells, and the ubiquitination of RUNX3 was measured. We found that RUNX3 ubiquitination was enhanced in the presence of WT Pin1 but not the enzymatic deficient mutant, indicating that Pin1 promote the ubiquitination of RUNX3 in an enzymatic dependent manner.

Figure 3.8 (a) Pin1 enhances ubiquitination of RUNX3 *in vivo*. HEK293T cells were transfected with indicated combination of plasmids expressing Flag-RUNX3, Ha-ubiquitin and Myc-Pin1 WT and C115A. RUNX3 immunoprecipitates were immunoblotted for ubiquitination with anti-Ha antibodies in the upper panel. Levels of RUNX3 and Pin1 are shown in the lower two panels.

We also looked at the importance of Pin1 and RUNX3 interaction in the Pin1 induced ubiquitination of RUNX3. We co-transfected WT RUNX3 or RUNX3-4A in the presence and absence of WT Pin1. As expected, Pin1 was only able to significantly enhance the ubiquitination of WT RUNX3 but not RUNX3-4A mutant that is unable to bind to Pin1. This indicates that interaction of RUNX3 and Pin1 is important for the Pin1-induced RUNX3 ubiquitination, and is consistent with previous findings that Pin1's binding to RUNX3 is necessary for proteasome degradation of RUNX3.

B

Figure 3.8 (b) Interaction between Pin1 and RUNX3 is important for enhancement of RUNX3 ubiquitination by Pin1. HEK293T cells were transfected with a combination of Flag-RUNX3 WT or 4A, Ha-ubiquitin and Myc-Pin1 WT as indicated. RUNX3 ubiquitination is detected as in (a)

To better examine the consequence of Pin1 on RUNX3 ubiquitination, we determined the effect of Pin1 inhibition on RUNX3 ubiquitination in 2 physiological relevant cell lines: RUNX3-MCF-7 and MDA-MB-361. SiRNA knock down of Pin1 in both RUNX3-MCF-7 cell

line and MDA-MB-361 cell line showed a marked decrease in the exogenous and endogenous ubiquitination levels of RUNX3 respectively, indicating that the level of RUNX3 ubiquitination is dependent on the expression level of Pin1 in the cells. In conclusion, these data support the notion that Pin1 binds to RUNX3, isomerizes the S/T-P bonds in RUNX3 resulting in Pin1-induced ubiquitination of RUNX3 and subsequent degradation of the RUNX3 protein.

Figure 3.8 (c and d) Down-regulation of Pin1 enhances the ubiquitination of RUNX3. RUNX3-MCF-7 cell line stably expression RUNX3 in *(c)* and MDA-MB-361 cell line endogenously expressing RUNX3 and Pin1 in *(d)* were transfected with Pin1 siRNA or control. Cells were treated with 20μM of proteasome inhibitor, MG132, for 8 h prior to immunoprecipitation experiment. RUNX3 immunoprecipitates were immunoblotted for ubiquitination using anti-ubiquitin antibodies in the upper panel. Levels of RUNX3 and Pin1 are shown in the bottom two panels.

3.4 Discussion

Pin1 has been shown to be involved in a number of oncogenic pathways [116, 124, 133], its over-expression is observed in 75% of breast cancer specimens, and high Pin1 levels also correlates with poor prognosis [117]. In this part of the thesis, we identified tumor suppressor RUNX3 as a novel substrate for Pin1. Our data showed that expression of Pin1 and RUNX3 played an inverse correlation in breast cancer samples, and over-expression of Pin1 stimulated proteasome degradation of RUNX3, thus decreasing the transcriptional activity of RUNX3. Since RUNX3 functions as a breast cancer tumor suppressor as previously defined by Huang *et al.*, our data that shows the inactivation of RUNX3 by Pin1 may well be a new mechanism by which Pin1 contributes to mammary oncogenesis.

Through the study of a large cohort breast cancer patient samples, we have identified a significant inverse correlation between the expression of Pin1 and RUNX3 (Figure 3.3a and b). It is interesting to note that this inverse correlation is most significant in the early stages (stage I and II) of breast cancer. It is possible that in early stages of breast cancer, one of the first major mechanisms for inactivation of RUNX3 is by Pin1-induced proteasome degradation of RUNX3. In the later stages of cancer, we saw a reduced inverse correlation between the two proteins because inactivation of RUNX3 at the stage is thought to be more permanent, and could be done through hypermethylation of the RUNX3 promoter. The biological significance of this inverse correlation is further confirmed through the up-regulation of RUNX3 expression after Pin1 inhibition (Figure 3.3d and e).

The interaction between Pin1 and RUNX3 was identified by both *in vitro* and *in vivo* experiments with exogenous and endogenous proteins (Figure 3.4). Since Pin1 is known to specifically bind to and isomerizes phosphorylated S/T-P bonds, we confirmed that the interaction between Pin1 and RUNX3 is phosphorylation dependent (Figure 3.4d) and that RUNX3 does contain phosphorylated S/T-P motifs (Figure 3.4e). The interaction between RUNX3 and Pin1 is mediated by the WW domain of Pin1 (Figure 3.5a and b), which is also known to bind other target substrates. The substrate binding pocket of the WW domain, in particular, is composed of side chains Serine[16], Arginine[17], Tyrosine[23] and Tryptophan[34], which are critical for the binding specificity of Pin1 [136]. In our study, we showed that a single mutation of Serine[16], Arginine[17] and Trptophan[34] to Alanine disrupts the interaction between Pin1 and RUNX3. However, while the S16A and W34A mutations seem to completely abolish binding, the R17A mutant is still able to bind minimally to RUNX3. We also identified that RUNX3 binds to Pin1 through 4 unique S/T-P motifs between the regions 187-234 (Figure 3.5c, d and e). RUNX3 and Pin1 interaction is only disrupted after mutations of all 4 S/T-P binding sites, indicating that Pin1 binds redundantly to these four binding sites. It is possible that Pin1 binds to each of the 4 different S/T-P motifs on RUNX3 to affect different downstream target activity of RUNX3.

Our data show that Pin1 abolishes the transcriptional activity of RUNX3 (Figure 3.6). As previously discussed, inactivation of RUNX3 can happen through a number of mechanisms, including hemizygous deletion of the RUNX3 gene, hypermethylation of the RUNX3 promoter and mis-localization of the RUNX3 protein to the cytoplasm [34, 47, 82]. From our study, in addition to human breast cancer specimens showing an inverse correlation between expression of

Pin1 and RUNX3, co-expression of Pin1 and RUNX3 in breast epithelial cells MCF-7 also showed a decrease in RUNX3 protein expression level (Figure 3.7a) without any changes in RUNX3 mRNA level (Figure 3.7c) This down-regulation in RUNX3 expression can be rescued by proteasome inhibitor, MG132 (Figure 3.7b), and Pin1 also induced the ubiquitination of RUNX3 protein (Figure 3.8). These data indicate that Pin1 inactivates RUNX3 by inducing its ubiquitination and targets it for proteasome degradation. It is important to note that Pin1 is the second protein we have identified after CagA to inactivate RUNX3 by causing its degradation through the 26S proteasome. Although the precise mechanism of Pin1-induced RUNX3 ubiquitination is unknown, Pin1 is known to induce the ubiquitination of Smad2/3 proteins enhancing the interaction between Smad2/3 with their E3 ligase Smurf2 [134]. Since RUNX3 is also a substrate of Smurf2 [46, 58], we have reasons to believe that Pin1 might mediate ubiquitination in RUNX3 through the recruitment of Smurf2. It is possible to immunoprecipitate Pin1 and RUNX3 complex and subject the immunoprecipitates to mass spectrometry analysis to see if Smurf2 is also a component of the complex.

Interestingly, the phosphorylation deficient RUNX3 mutant, RUNX3-4A, which does not bind to Pin1, seems to function like a positive model for Pin1 inactivation of RUNX3. Despite its inability to bind to (Figure 3.5d and e) and be modified by Pin1, RUNX3-4A has reduced transcriptional activity compared to WT RUNX3 (Figure 3.6e) and is less stable than WT RUNX3, with a much shorter half life (Figure 3.7e). Furthermore, even in the absence of Pin1, RUNX3-4A is much more ubiquitination compared WT RUNX3 (Figure 3.8b). To better explain this phenotype of the RUNX3-4A protein, we look at the ubiquitination of RUNX3 protein. It has been observed that the region of RUNX3 between amino acids 187 and 375 is important for

the stability of the RUNX3 protein [58]. Ubiquitination occurs at lysine residues within RUNX3, there are 8 such lysine sites, only 2 are between the region of amino acids 187 to 375, Lys[186] and Lys[192] [58]. These two lysine residues are surprisingly close to the 4 S/T-P motifs (from amino acids 209 to 231) that Pin1 binds to on RUNX3. Since a phosphate group is rather large in size, phosphorylation of all 4 S/T-P motifs from amino acids 209 to 231 may clutter the regions on RUNX3 surrounding the 4 S/T-P motifs, resulting in the region becoming inaccessible to post-translational modifications. As a result, Lys[186] and Lys[192] are unreachable to E3 ligases and cannot be ubiquitinated. This will subsequently result in increase protein stability of RUNX3. When Pin1 binds to RUNX3 through the 4 S/T-P motifs, it changes the conformation of RUNX3, perhaps putting it into a more accessible conformation, allowing E3 ligases to reach the lysine sites, leading to proteasome degradation of RUNX3, hence inactivating RUNX3. The RUNX3-4A mutant, because of the lack of phosphate clusters, and also the fact that alanine is smaller than serine or threonine, the protein is possibly in a more "open" confirmation than WT RUNX3, allowing ubiquitination of the lysine residues in RUNX3-4A, hence decreasing its protein stability.

To further elucidate Pin1's regulation of RUNX3, it is also necessary to identify the kinase that is involved in phosphorylating RUNX3 at the 4 S/T-P motifs. One such candidate would be the homeodomain-interacting kinase 2 (HIPK2). HIPK2 is known to be the regulator of a large number of transcription factors including p53, CtBP and RUNX1 [137-140]. HIPK2 is needed for activation of the tumor suppressor activity of p53, it phosphorylates p53 at Serine[53] to induce apoptosis during DNA damage [139]. RUNX1 is phosphorylated by HIPK2 at proline-directed serine and threonine residues in its C-terminus. Furthermore, RUNX1 proteins bearing

leukemogenic mutations could not be phosphorylated by this mechanism, indicating that HIPK2 phosphorylation is essential for the function of RUNX1 [137, 138]. Since RUNX proteins are highly conserved, it would be interesting to find out if HIPK2 too plays a role in the phosphorylation of the proline directed serine/threonine residues in RUNX3. Phosphorylation of these residues perhaps enhances the stability of RUNX3 and promotes its tumor suppressor activity.

Figure 3.9 *Schematic model for the inactivation of RUNX3 by Pin1. RUNX3 is phosphorylated by a kinase at the S/T-P motifs. In the presence of Pin1 up-regulation, Pin1 binds to RUNX3 through the WW domain and S/T-P motifs respectively, resulting in a conformational change in the structure of RUNX3. RUNX3 then undergoes Pin1-induced ubiquitination and is targeted to the 26S proteasome for degradation.*

In conclusion, our data shows that Pin1 inactivates tumor suppressor protein RUNX3 in breast carcinomas by binding 4 unique S/T-P motifs on RUNX3, inducing ubiquitination of the

protein and targeting it for degradation (Figure 3.9). At the moment, there is support for the development of Pin1-specific inhibitors as therapeutic anti-cancer drugs [116, 117, 123, 132]. Through its down-regulation of the activity of tumor suppressor RUNX3, we propose a new mechanism by which Pin1 contributes to oncogenesis and provide additional support of the usefulness in the development and evaluation of Pin1-specific inhibitors as novel anti-cancer drugs.

Chapter 4: Conclusion

RUNX3 is a transcription factor that regulates lineage-specific gene expression in developmental processes and is involved in the formation of a variety of cancers [141]. RUNX3 is most widely studied in its role as a tumor suppressor in gastric cancer. It elicits its tumor suppressor functions by controlling the expression of many genes involved in the growth, apoptosis, and differentiation of gastric epithelial cells [55, 56, 62] as well as genes involved in angiogenesis and cell junctions [57, 113]. RUNX3 is expressed in glandular stomach epithelial cells, and loss of expression of RUNX3 is causally related to genesis and progression of gastric cancer and also correlates with differentiation, metastasis, and poor prognosis of gastric cancer [47, 142, 143]. A study in 2002 found that knocking out *Runx3* in mice lead to hyperplasia of the gastric epithelium, an early developmental step in carcinogenesis [47]. Furthermore, it was found that 90% of human gastric cancer samples showed reduced levels of RUNX3 expression due to hemizygous deletion, hypermethylation of its promoter, or protein mis-localization [47, 82]. The inactivation of RUNX3 appears to occur both at an early stage as well as during progression of gastric cancer [47, 82] and its expression correlates with the stage of the cancer; fewer late-stage tumors expressed RUNX3 than early-stage tumors [47]. While emerging evidence suggests that RUNX3 is a tumor suppressor whose inactivation is involved in the initiation and progression of gastric cancer, the trigger for RUNX3 inactivation within the cells is largely unknown.

Meanwhile, RUNX3 was also found to be inactivated in breast carcinomas. RUNX3 mRNA and protein expression were found to be lowered due to hypermethylation of the *Runx3* promoter. In breast cancer specimens that still expressed RUNX3, the RUNX3 protein showed cytoplasmic re-localization. RUNX3 was also found to have tumor suppressor activity in breast cancer when the same researchers showed that injection of nude mice with stable clones of a

MDA-MB-231 breast cancer cell line expressing a control vector resulted in tumor formation whereas nude mice that were injected with MDA-MB-231 cells expressing RUNX3 did not developed tumors [34]. Subsequently, our lab also defined a role for RUNX3 as a tumor suppressor in ERα-positive breast cancer whereby RUNX3 down regulates the expression of ERα. Enhanced ERα expression is associated with increased breast cancer risk, therefore inactivation of RUNX3 results in increase ERα stability and enhances cell proliferation in response to circulating estrogens [33].

RUNX3 functions as an important gatekeeper of cell differentiation and proliferation and its inactivation is perhaps crucial to the initiation of carcinogenesis in many tissue types. Study of the regulation of RUNX3 in these tissues will give us insight into the process of carcinogenesis and presents new therapeutic targets for potential treatment of the different cancers. In this thesis, we focus on the understanding of RUNX3 inactivation in gastric cancer by *H. pylori* infection as well as the targeting of RUNX3 by Pin1 in breast carcinomas.

It is important to study gastric cancer because worldwide, gastric cancer is the fourth most diagnosed cancer and the second most common cause of cancer-related death. It is estimated that 10,570 people will die from gastric cancer in 2010 in the United States alone [144]. Intestinal-type adenocarcinomas, which occur primarily in the distal regions of the stomach, predominate in countries where gastric cancer is more common. Diffuse-type adenocarcinomas occur more frequently in younger populations [145]. Adenocarcinomas of the stomach are often not diagnosed until they have metastasized to other tissues, which makes their prognoses very

poor [146]. Mucosa-associated lymphoid tissue lymphomas comprise a small proportion of gastric cancers, and tend to respond very well to treatment. Gastric carcinogenesis is a complex, multistep and multifactorial event and like all cancers many etiological factors contribute to the development and progression of gastric cancer. These can include the activation of oncogenes, inactivation of tumor suppressors, diet, tobacco use, and infection with *Helicobacter pylori* [73] .

H. pylori is the only bacterium classified by the World Health Organization as a Type I carcinogen, with an estimated 63% of all stomach cancers caused by infection with the bacterium [73]. The gram-negative spirochete persistently colonizes the mucosa of the stomach, where it may attach directly to the epithelial cells. Over half the world's population is infected with *H. pylori*, though the wide majority of these infections are symptomless. However, significant pathologies are found in the minority, including chronic gastritis, peptic ulcer disease, and gastric cancers [147]. Infection is most commonly associated with intestinal-type gastric adenocarcinoma, though infection can also lead to the development of non-Hodgkin's or MALT lymphomas [147]. Many proposed mechanisms for its pathogenicity exist, including infection-induced cell proliferation, epithelial cell elongation and loss of polarity, and degradation of cell–cell junctions. Recent publications together with my study in Chapter 2 indicate that *H. pylori* infection is important in the inactivation of RUNX3 by both protein degradation and promoter hypermethylation [110, 148]. Therefore, inactivation of RUNX3 might be another critical element for the pathogenesis of *H. pylori*.

Ubiquitination of Runt family proteins have long been described but the physiological significance of this process is not well defined [149]. Studies indicate that ubiquitination-dependent proteolytic degradation of RUNX3 is an important regulatory mechanism for controlling its tumor suppressor activity [46]. Ubiquitination is a series of reactions mediated by three different enzymes including E1, E2 and E3. Ubiquitination is activated by ubiquitin activating enzyme E1. Activated ubiquitin is transferred to ubiquitin conjugating enzyme E2 and is further transferred to the substrates by the ubiquitin E3 ligase. Of all three enzymes, only E3 ligase confers the substrate specificity [150]. Several E3 ligases for RUNX3 ubiquitination have been identified. For example, RUNX3 is ubiquitinated by Smurfs (Smad ubiquitin regulator factor) and Smurfs-mediated ubiquitination of RUNX3 reduced the stability and activity of RUNX3 [58]. Additionally, RUNX3 has been shown to be a target of MDM2, an E3 ligase known for the tumor suppressor p53, indicating an oncogenic surveillance function of RUNX3 [151].

Our study in Chapter 2 indicates that *H. pylori* infection induces the ubiquitination and degradation of RUNX3. Infection of *H. pylori* leads to reduced cellular levels of RUNX3 in cultured gastric epithelial cells as well as in gastric epithelial cells of infected mice. The reduced expression of RUNX3 appears to be derived from *H. pylori*-induced ubiquitination and degradation of RUNX3. Interestingly, *H. pylori*-induced degradation of RUNX3 is virulence factor CagA-dependent since wild-type but not *cagA*-deficient *H. pylori* strain down-regulates the cellular levels of RUNX3 [148]

Virulence factor CagA is encoded by pathogenesis island *cag*PAI of *H. pylori*, which also encodes a type IV secretion system. The 120-140 kDa CagA is injected into host epithelial cells via type IV secretion system [73]. Within the cells, CagA interacts with different intracellular host proteins to elicit its many roles in the pathogenesis of *H. pylori*. For example, CagA associates with and activates cytoplasmic protein tyrosine phosphatase SHP-2, resulting in cytoskeletal reorganization, cell elongation and cell scattering, and the "hummingbird" phenotype [102, 152]. CagA also associates with TAK1 to activate NF-κB and modulate NF-κB-dependent inflammatory response [153]. Through its association with various host proteins, *H. pylori* CagA is actively involved in *H. pylori*-induced gastric carcinogenesis. The ability of *H. pylori* to induce degradation of RUNX3 also relies on specific interaction of CagA with RUNX3. CagA directly interacts with RUNX3 *in vivo* and *in vitro*. More importantly, the interaction is mediated by specific recognition of PPxY (Py) motif of RUNX3 by WW domain of CagA. Blocking the interaction either by mutation of Py motif or deletion of WW domain reduces the ability of CagA to induce ubiquitination and degradation of RUNX3, emphasizing on the importance of this interaction in the degradation of RUNX3 [148].

The WW domain has been found in a variety of E3 ligases and is involved in the binding of these E3 ligases to their substrates [154]. One possibility how CagA can induced ubiquitination of RUNX3 is that CagA could be an E3 containing intrinsic ligase activity. However, the cysteine residue, which is the essential amino acid for an E3 ligase to form a thioester bond with ubiquitin [111], is not found within CagA. This raises another possibility that CagA might function as a scaffold protein to recruit an E3 ubiquitin ligase for the ubiquitination and degradation of RUNX3. Supporting this, we found that CagA immunoprecipitates from

transfected cells were able to ubiquitinate RUNX3 in vitro (unpublished data). It has to be noted that two WW domains, defined as WW1 and WW2, were identified within the N-terminal region of CagA. Although both WW domains of CagA are involved in ubiquitination and degradation of RUNX3, only WW2 of CagA is essential for CagA's interaction with Py motif of RUNX3. Currently, it is not clear why CagA needs two WW domains for the ubiquitination and degradation of RUNX3 but only one WW domain for the specific interaction. It is possible that WW2 is involved in the interaction with RUNX3 while WW1 is involved in the recruitment of an E3 ligase. However, the identity of the E3 ligase remains to be further characterized. Several E3 ligases, including Smurfs and MDM2, have been identified for the ubiquitination of RUNX3 (Jin *et al* ., 2004), it will be interesting to investigate whether any of them is actually involved in the CagA-induced degradation of RUNX3.

There are evidences from my study in chapter 2 that show that this CagA-induced degradation of RUNX3 may occur in the nucleus. The degradation of RUNX3 might in fact pose as a novel nuclear function for CagA, although this novel function has to be better characterized. By and large, CagA exists as a cytoplasmic protein, we also have to consider the possibility of CagA-induced degradation in the cytoplasm. It is probable that binding of CagA to RUNX3 might facilitate cytoplasmic localization of RUXN3, which may result in degradation of RUNX3 in cytoplasm. Jun-activation domain-binding protein 1 (Jab/CSN5) induces cytoplasmic localization and degradation of RUNX3 [155]. In addition, histone methyltransferase G9a has also been shown to promote nuclear export and induce degradation of RUNX3 in response to hypoxia [156]. Similarly, CagA might promote nuclear export of RUNX3 by sequestering RUNX3 in the cytoplasm where it can be ubiquitinated and degraded by the 26S proteasome.

Aberrant DNA methylation of CpG islands of promoters is one of the major mechanisms for inactivating tumor suppressor genes and is closely involved in the formation of cancer [157]. Not surprisingly, methylation of *Runx3* promoter represents a major mechanism for the inactivation of RUNX3 in gastric cancer. Hypermethylation of *Runx3* promoter is found in many gastric cancer cell lines and gastric cancer samples as well as in the non-cancerous gastric diseases including chronic gastritis, intestinal metaplasia and gastric adenoma with less frequency than cancer [157, 158]. Several factors including *H. pylori* infection, inflammation, and oxidative stress have been indicated to be involved in the epigenetic inactivation of RUNX3 [159]. *H. pylori* infection positively correlates with the methylation of *Runx3* promoter in gastric cancer as well as with gastric atrophy and intestinal metaplasia [110]. Supporting the role of *H. pylori* infection in the methylation of *Runx3* promoter, eradication of *H. pylori* increases the expression of RUNX3 in the glandular epithelial cells of the corpus [160].

It is well documented that RUNX3 functions as a tumor suppressor under the TGF-β signaling pathway through attenuation of cell growth and induction of apoptosis [161]. For example, RUNX3 suppresses gastric epithelial cell growth by inducing cell cycle regulator *p21*$^{(WAF1/Cip1)}$ expression and induces apoptosis of gastric epithelial cells by up-regulating pro-apoptotic factor Bim [55, 56]. Therefore, inactivation of RUNX3 by *H. pylori* might result in enhanced gastric epithelial cell proliferation and survival, which in turn facilitates *H. pylori* colonization [162]. Additionally, inactivation of RUNX3 by *H. pylori* might contribute to disruption of the proper architecture of the gastric epithelium. Claudin-1, a main component of the tight junction family proteins, is a direct and positive target of RUNX3 in gastric epithelial cells. *Runx3*$^{-/-}$ derived gastric epithelial cells have reduced levels of claudin-1 and increased

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tumorigenic potential when compared with $Runx3^{+/+}$ cells [57]. Disruption of the tight junction might allow *H. pylori* to invade intercellularly and further induce cell motility, elongation and result in onset of mitotic genes involved in cell proliferation.

In the first part of this conclusion, I summarize the recent findings that define the important role of *H. pylori* in the inactivation of gastric tumor suppressor RUNX3. Multiple lines of evidence demonstrate that various pathways and factors are involved in *H. pylori*-mediated inactivation of RUNX3. While the degradation of RUNX3 might represent one of the primary responses to host-pathogen interaction, providing a rapid outcome for proliferation and survival of gastric epithelial cells and the growth of *H. pylori*, the infection-mediated long-term silencing of RUNX3 at the epigenetic level might allow transdifferentiation of gastric epithelial cells to intestinal-type cells. *H. pylori* infection is a major risk factor for the development of gastric cancer and its precursor lesion [73] and *H. pylori* eradication with triple therapy has been an effective approach for prevention of gastric cancer [163, 164]. However, due to increased antibiotic-resistance and some beneficial effects from *H. pylori* infection, alternative medical approaches for *H. pylori* infection are required to be considered [165]. However, such new approaches rely on a better understanding of the molecular and cellular mechanism for *H. pylori*-induced gastric cancer. Although detailed mechanisms for the *H. pylori*-mediated inactivation of RUNX3 remain to be determined, this line of research promises to yield new insights into the pathogenesis of *H. pylori* and gastric cancer. Since inactivation of RUNX3 is closely associated with pathogenesis of *H. pylori*, specifically blocking the interaction between *H. pylori* CagA and RUNX3 in *H. pylori*-infected patients or reactivation of RUNX3 in gastric lesions and gastric

cancers by reducing methylation of *Runx3* promoter might reduce the tumorigenic potential of *H. pylori* but at the same time retain its beneficial effect.

In the second part of my thesis, I will discuss the regulation of RUNX3 in breast carcinogenesis. The role of RUNX3 as a tumor suppressor in breast cancer has been less extensively studied compared to its role in gastric cancer. However, our lab has clearly defined the involvement of RUNX3 in mammary carcinogenesis by reporting that 20% of female *Runx3*$^{+/-}$ mice spontaneously developed ductal carcinoma at an average of 14.5 months due to hyperproliferation of mammary ductal epithelial cells. Additionally, RUNX3 also inhibits the estrogen-dependent proliferation and transformation potential of ERα-positive MCF-7 breast cancer cells in liquid culture and in soft agar and suppresses the tumorigenicity of MCF-7 cells in severe combined immunodeficiency (SCID) mice [33].

Breast carcinoma is the most prevalent form of cancer amongst women in developed countries such as the United States (US). From 2001 to 2005, the incidence rate of breast cancer in the US population was approximately 125 cases per 100,000 people, and the mortality rate was approximately 25 cases per 100,000 people, making breast cancer death rates the second highest of all cancers after lung cancer [86]. Excluding cancers of the skin, breast cancer is the most frequently diagnosed form of cancer among women in the US, making up more than 1 in every 4 cancers diagnosed. Women in the US have a 1 in 8 chance of developing breast cancer in their lifetime. Women between 75 and 79 years of age have the highest risk of developing cancer,

with an incidence rate of 464.8 cases per 100,000 as compared to an incidence rate of 1.4 among women aged between 20 and 24 years [166].

Between 5 to 10% of breast cancers are thought to be hereditary, highlighting the importance of genetic factors in breast carcinogenesis [166]. Abnormalities in proto-oncogenes BRCA1 and 2 are thought to account for around 10% of breast cancers [87]. Other common genetic mutations include aberrations in DNA repair gene ATM, tumor suppressor gene p53, and PTEN, a protein phosphatase involved in many signaling pathways including the AKT signaling pathway. Up-regulation of Pin1, a peptidy-prolyl *cis-trans* isomerase (PPIase), is also believed to be genetically linked to breast oncology. Pin1 is known to play a role in multiple oncogenic signaling pathways, and Pin1 over-expression in breast cancer is also associated with poor prognosis of the breast cancer patients [116, 133].

PPIase Pin1 is composed of an N-terminal protein-protein interaction WW domain, and a C-terminal PPIase domain. The WW domain of Pin1 preferentially binds to peptides containing a phosphorylated serine or threonine residue preceding a proline residue (pS/T-P) motif, while the PPIase domain catalyzes cis-trans isomerization of the peptide bond on the amino-terminal side of the proline residue [116]. Through associations with its substrates, Pin1 has been found to affect phosphorylation status, protein-protein interactions, subcellular localization and protein stability [116, 121, 123, 133]. In the third chapter of my thesis, I report that RUNX3, a tumor suppressor in breast cancer, is found to be a new substrate of Pin1 and is inactivated by Pin1. We observed a significant inverse correlation between the expression of RUNX3 and Pin1 in human

breast cancer specimens as well as breast cancer cell lines. Inhibition of Pin1 up-regulates RUNX3 expression in a breast cancer cell line and Pin1 is also found to inhibit the transcriptional activity of RUNX3. Through further analysis of the two proteins, we found that Pin1 binds to RUNX3 in a method common to its interactions with its other substrates: through its WW domain binding pocket in the N-terminus. What is most intriguing is that RUNX3 has four distinct S/T-P motifs that interact with Pin1, it is only upon mutations of all four motifs that RUNX3 and Pin1 interaction is abolished, which prompted us to wonder if each of these four Pin1 binding sites corresponds to the disruption of distinct target functions of RUNX3? Detailed characterization of the Pin1 binding sites on RUNX3 will provide further insights into the inactivation of RUNX3 by Pin1.

We also found that Pin1 induced the ubiquitination of RUNX3 and subsequently target RUNX3 for proteasome degradation. Pin1 is known to control the degree of ubiquitination of its substrates and altering the fate of these proteins in cells [167]. For example, Pin1 is able to switch tumor suppressor, p53, from poly-ubiquitination, which triggers nuclear p53 degradation to oligo-ubiquitination, which triggers the nuclear export of p53 [168]. Pin1 has also been found to induce poly-ubiquitination of Smad2/3 proteins in the TGF-β signaling pathway by recruitment of E3 ligase Smurf2, and targeting Smad2/3 for proteasome degradation [134]. Moreover, RUNX3 is known to be ubiquitinated by Smurf 2 E3 ligase [58]. This, together with RUNX3's well documented involvement in the TGF-β signaling pathway suggest that perhaps the E3 ligase that mediate Pin1-induced RUNX3 ubiquitination might also be Smurf2 or a member of the Smurf E3 ligase family. Identification of the E3 ligase involved in the inactivation

of RUNX3 by Pin1 will provide more potential therapeutic targets in the treatment of breast carcinogenesis.

Pin1 is known to be involved in breast development and oncogenesis through a number of pathways, including activation of oncogenic HER/Neu/Erb2 and Ras signaling pathway, leading the enhancement of cyclin D1 gene via transcription factors like E2F, c-Jun.AP-1 and β-catenin/TCF [169-173]. Through the identification of inactivation of RUNX3 by Pin1, we gain further understanding of Pin1's role in breast carcinogenesis.

Mechanisms underlying the inactivation of RUNX3 in breast cancer are largely unknown. Approximately 80% of breast cancer specimen is known to inactivate RUNX3 through cytoplasmic sequestration of RUNX3 [34]. Nuclear exclusion of RUNX3 can occur through a number of methods, including microtubule-dependent nuclear import, reducing nuclear retention by loss of association with chromatin-related macromolecular complexes and inhibiting nuclear import [174, 175]. TGF-β signaling pathway is also thought to trigger nuclear import of RUNX3 in certain cell types [82], but due to the pathway being impaired in many breast cancer cells, this could be the reason why RUNX3 is mis-localized to the cytoplasm in breast cancer. However, it is unknown how TGF-β elicits nuclear translocation of RUNX3, or what kind of perturbations happen in cells to induce nuclear exclusion or inhibit nuclear import of RUNX3. RUNX3 is also functionally inactivated in breast cancer through hypermethylation of the *Runx3* promoter [34]. However, like cytoplasmic mis-localization, it is still largely unclear what causes the aberrant methylation of RUNX3. Identification of Pin1-induced RUNX3 degradation helps us establish

one of the mechanisms behind RUNX3's functional inactivation, perhaps one of the initial responses in the mammary carcinogenesis process. Perhaps like gastric cancer, with the inactivation of RUNX3 by Pin1 in mammary tissues, it provides an initial prompt for cell proliferation and transformation leading to oncogenesis. With a known mechanism, it will aid in the discovery of therapeutic targets for breast cancer.

RUNX3's anti-tumor activities in breast cancer are also unclear. Huang *et al*. in our lab recently identified RUNX3 mediation of ERα degradation as an anti-tumor mechanism of RUNX3 in ERα breast cancer [33]. ERα functions as a transcriptional regulator, stimulating proliferation and suppressing apoptosis through regulation of genes involved in the cell cycle and apoptosis. Abnormal estrogen signaling through ERα is associated with initiation and progression of breast cancer [91]. In ERα positive cells, RUNX3 controls the cellular amount of ERα and function as gatekeeper [33], preventing the onset of breast cancer. However, in ERα-negative MDA-MB-231 cells, RUNX3 was also found to reduce the invasiveness and tumor formation potential of these cells [34], indicating that RUNX3 also has tumor suppressor functions in ERα negative cancers. In these cancers, it is possible that RUNX3 functions under the TGF-β signaling pathway like in gastric cancer. RUNX3 may cooperate with FOXO3a or receptor-regulated Smads to induce TGF-β mediated cell cycle arrest or apoptosis. In general, further understanding is needed with regards to the tumor suppressor function of RUNX3 in breast cancer.

In conclusion, multiple lines of evidence points towards RUNX's close link to cancer pathogenesis. Frequent inactivation of RUNX3 as well as the interaction of RUNX3 with important components of various signaling pathways, for example enhancement of TGF-β-related growth inhibition and apoptosis as well as suppression of oncogenic Wnt signaling activity clearly indicate that aberrant activity of RUNX3 plays a major part in oncogenesis. Furthermore, with the increase list of proteins that has been identified to interact with RUNX3 hints at the involvement of RUNX3 in other activities such as DNA damage and repair. Therefore, a more complete understanding of RUNX3's regulation and inactivation mechanisms in respective tissues will offer important insights into the complicated steps of cancer formation, allowing exploitation during the identification of new drugs against cancer treatment.

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EDUCATION

B.S.   Biochemistry (Honors), Imperial College London, United Kingdom, 2005

Ph.D Candidate   Biochemistry, University of Illinois at Urbana-Champaign, USA, Expected in 2011

FELLOWSHIPS

2002-2005   National Science Scholarship (Undergraduate), Singapore Agency for Science, Technology and
            Research (A*STAR)

2007-2010   ASTAR Graduate Overseas Scholarship Singapore Agency for Science, Technology and Research
            (A*STAR)

RESEARCH EXPERIENCE

2007-present   Department of Biochemistry**,** University of Illinois at Urbana-Champaign
                PhD Student [Advisor: Dr. Lin-Feng Chen (UIUC) & Dr.Yoshiaki Ito (CSI-National
                University Singapore)]

- Studying the effect of *H. pylori* infection on gastric tumor suppressor gene RUNX3 using cell biology methods as well as using mouse as an animal model.
- Investigating the mechanism by which CagA, a *H. pylori* protein, down regulates the expression of RUNX3 using degradation assays, ubiquitination assays and other biochemical methods.
- Investigating the relationship between RUNX3 and Pin1, a protein up-regulated in breast cancer, using biochemical methods, so as to establish a regulation mechanism for RUNX3 in breast cancer cells.

2006–2007   Institute of Molecular and Cell Biology in Singapore
                Research Assistant (Supervisors: Dr. Yoshiaki Ito)

- Investigating RUNX3 targeting of the Wnt Signaling pathway in colorectal cancer, RUNX3   interaction with β-catenin.

2005–2006   Genome Institute of Singapore
                Research Assistant (Supervisor: Dr. Qiang Yu)

- Understanding the effect of overexpression of c-Myc (an oncogene) on histone deacetylase inhibitor (HDAC inhibitor) induced apoptosis.

2004   Johns Hopkins University at Singapore
                  Summer Research Intern (Supervisor: Dr. Qian Tao)

- Mapping the CpG islands of the promoters of oncogenes using the bisulfite sequencing method to check the methylation status of the promoters.

TEACHING EXPERIENCE

University of Illinois at Urbana-Champaign
Teaching Assistant; Biochemistry For Non-Majors (MCB 450)
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CONFERENCE PRESENTATION

Helicobacter pylori CagA targets gastric tumor suppressor RUNX3 for proteasome-mediated degradation. **Tsang YH**, Lamb A, Romero-Gallo J, Huang B, Ito K, Peek RM Jr, Ito Y, Chen LF. The 17th international RUNX workshop at Hiroshima, Japan, 2010

PUBLICATIONS

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