TYROSINE PHOSPHORYLATION OF THE RUNX1 TRANSCRIPTION FACTOR BY SRC KINASE ACTIVATES C/EBP-ALPHA IN GRANULOPOIESIS

by

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ABSTRACT

The Runx1 transcription factor is a master regulator of hematopoiesis and myeloid transformation. Its function is tightly orchestrated by diverse and appropriate signaling events to regulate cell fate decision, including granulopoiesis. Src phosphorylated Runx1 at 5 tyrosine residues; Y260 located at the transactivation domain and the C-terminal tyrosine cluster Y375, Y378, Y379 and Y386 at the repression domain. Tyrosine phosphorylated Runx1 inhibits megakaryopoiesis and thymocyte development but its effect on granulopoiesis is yet to be reported.

In luciferase reporter assay in HEK293T cells, Src tyrosine kinase synergized with wild-type (WT) Runx1 to increase its transactivation potency. Significant transactivation effect was also observed with Runx1 tyrosine (Y) to glutamate (E) or aspartate (D) phopho-mimetic mutant variants where the transactivity increased to about 2-fold compared to Runx1-WT. Conversely, tyrosine (Y) to phenylalanine (F) phospho-null mutant variants did not exhibit transactivity. Exogenous Runx1 and its C-terminal phospho-mimetic mutant variant induced the transcriptions of endogenous *Cebpa* and *PU.1* in the 32Dcl3 myeloid progenitor cell line as well as their protein productions, but not with the phospho-null Runx1.

At least two mechanisms contribute to the increased Runx1 activity upon tyrosine phosphorylation. Tyrosine phosphorylated Runx1 bound better to its consensus sequences as assessed in Gel Shift Assay, and to the endogenous *Cebpα* and *PU.1* enhancers in Chromatin Immunoprecipitation Assay. In addition, after cycloheximide treatment, high levels of Runx1-5D and 4D protein expressions could sustain up to 8 hours while the

Runx1-5F and 4F proteins were degraded between 2-4 hours. The stability of the Runx1-5D was associated with weaker interaction with ubiquitin and also its resistance to Cdh1-mediated ubiquitin proteosomal degradation.

Combining these observations, we propose that Runx1 phosphorylation by Src at the five tyrosine sites increase its transactivity, binding affinity and stability, and therefore promoting the induction of mRNA and protein expressions of *Cebpa* and *PU.1*, which are the crucial early differentiation biomarkers in myelopoiesis and granulopoiesis. Further investigations on the regulation of tyrosine phosphorylation on Runx1 will shed more insights on the molecular mechanisms underlying granulopoiesis and its implications on myeloid transformation and diseases.

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CHAPTER 1: BACKGROUND REVIEW OF RUNX1

1.1 Runx1 is a master regulator in hematopoiesis

All blood cell lineages originate from hematopoietic stem cells (HSCs). The development of lineage-committed blood cells from HSCs, and their subsequent maintenance in adulthood, is tightly coordinated to ensure the precisely timed manifestation of functionally mature blood cells in a tissue-specific and spatiotemporal manner. The biological events that regulate the hematopoiesis process include the acquisition or production of growth or differentiation cytokines or ligands, coupled with the activation of intrinsic lineage-specific transcription factors, such as the hematopoietic master regulator Runx1.

The Runx1 gene was first isolated and cloned from the leukemic cells of acute myeloid leukemia (AML) patients who were diagnosed with chromosomal translocation t(8;21) (Miyoshi H, 1991). Runx1 encodes for the DNA-binding subunit of the heterodimeric core-binding factor (CBF) transcription factor family, and it is mainly expressed in hematopoietic cells (Heller PG, 2013; North TE, 2002). The region in Runx1 gene that encodes the conserved Runt DNA binding domain (DBD) is the mammalian homolog of the *runt* gene from *Drosophila* as both of their encoded amino acid sequences are 69% identical (Meyers S, 1993). Hence, Runx1 belongs to a family of transcriptional factor known as Runx in mammalian cells - the other two members are Runx2 and Runx3. In humans, the Runx1 gene is located at the chromosome 21q22.12, spans approximately 260 kilobases, and contains 12 exons. (Levanon D, 2001).

Diversity of Runx1 is contributed by transcriptions from two different promoter sites and alternative splicing (Miyoshi H, 1995). Therefore, DNA transcription can begin either at the proximal or distal promoter, which are 160 kilobases apart, giving rise to different Runx1 isomers. Runx1a and Runx1b are transcribed from the proximal promoter, whereas Runx1c is transcribed from the distal promoter (Heller PG, 2013). In addition, alternative splicing from the exons of Runx1 yield 12 different Runx1 cDNAs and at least three isomers of Runx1. Runx1a is a truncated spliced variant (250 amino acid) from exons 3 to 7a; Runx1b (453 amino acid) starts from exons 3 to 8; Runx1c is the full-length protein (480 amino acid) that includes exons 1 and 2 (Heller PG, 2012; Miyoshi H, 1995). Thus, exon 8 encoding the transactivation domain is absent in Runx1a but is still intact in Runx1b and Runx1c.

Similar to the general structures of other transcription factors, Runx1 has two distinct domains – the DNA binding *Runt* domain (DBD), and the transactivation domain (TAD, Figure 2). The DNA binding *Runt* domain is located at the N-terminal region and facilitates its sequence-directed binding to target genes; it also mediates protein-protein interaction (Meyers S, 1993). The conformation of this binding domain is immunoglobin-like and is related to the DNA binding domain of the tumor suppressor p53 (Milton HW, 1999). The central transactivation domain of Runx1 is responsible for the regulation of target gene activation and serves as a binding site for various transcriptional co-regulators (Bae SC 1994). Located immediately adjacent to the transactivation domain is the C-terminal repression domain (REP), which overlaps partially with the negative regulatory region for heterodimerization (NRHc) (Ito Y, 1999). Another conserved domain present in Runx1 is the pentapeptide VWRPY motif at the C-terminal end of the protein, which

mediates Runx1 transcriptional repression (Nishimura M, 2004; Imai Y, 1998; Aronson BD, 1997). The amino acid sequences of all three isomers of Runx1a, Runx1b and Runx1c are more than 90% homologous (Milton HW, 1999), suggesting that they use similar mechanisms in DNA-binding and heterodimerization.

Several *in vitro* studies in transgenic mice implied that Runx1 plays crucial roles in the emergence of definitive hematopoiesis and long-term repopulating HSCs from hemogenic endothelium through the Notch signaling pathway (North TE, 2002; Li C, 2015). Conditional Runx1 excision in the vascular-endothelial-cadherin-positive mice endothelial cells revealed that Runx1 was required for the transition of endothelial cells to HSCs at E11.5, and that these emerging HSCs could engraft to adult mice (Chen MJ, 2009). Homozygous Runx1 knock-out mouse embryos failed to develop fetal liver hematopoiesis and therefore died of extensive hemorrhage at midgestation by E12.5 (Okuda T 1996), central system hemorrhages and necrosis (Wang Q, 1996). This embryo lethality in Runx1-deficient mice makes it challenging to study the function of Runx1 during embryogenesis.

After definitive hematopoiesis has occurred, Runx1 is dispensable in the maintenance of HSCs in adult hematopoiesis (Chen MJ, 2009; Ishikawa M 2004). Nonetheless, the loss of Runx1 is detrimental to the terminal maturation processes of several blood lineages. When Runx1 was conditionally deleted in the bone marrow of adult mice, granulocyte formation was perturbed while monocyte formation was increased (Guo H, 2012). Adult mice with Runx1-deficient bone marrow displayed a 30-80% decline of megakaryocyte maturation, a block of DN2 (double negative) to DN3 stage in T-lymphocytes development as well as inhibition of B-lymphocytes

differentiation. In addition, these mice showed expanded population of hematopoietic and myeloid progenitors with augmented replating capacity due to the partial block of myeloid cell differentiation (Ichikawa M, 2004). Consistent with those findings, Putz *et al.* also demonstrated that adult mice with conditional Runx1 deletions had smaller thymus with accumulation of immature DN lymphocytes, and, at longer latency, developed metastatic lymphoma, lymphocytopenia, thrombocytopenia and progressive splenomegaly (Putz G, 2006). These results revealed that the lack of Runx1 in adult mice severely impairs the structures and functions of hematopoietic progenitors and hematopoietic organs.

In order for Runx1 to function physiologically, it has to form a heterodimeric complex at the DBD region with Core Binding Factor Subunit β (CBFβ), which itself is a non-DNA binding component. (Lu J, 1995). Mice with homozygous mutation of CBFβ exhibited identical hemorrhage phenotypes as in Runx1 knock-out mouse embryos, suggesting that CBFβ is essential for Runx1 function *in vivo* (Wang Q, 1996). The association of Runx1 to CBFβ increases the DNA binding affinity of Runx1 two to three fold more strongly than Runx1 alone (Bae SC, 1994). At the same time, interaction with CBFβ also relieves the intrinsic intramolecular inhibition effect of the Runx1 molecule (Gu T-L, 2000). Once Runx1 and CBFβ dimerize, they recognize the optimal consensus sequence “PuACCPuCA” or “TGT/cGGT” on the target DNA (Kamachi, 1990; Meyers S, 1993).

The Runx1 protein is also continuously degraded via the ubiquitin-proteasome pathway in murine myeloid progenitor cell lines 32Dcl3 and U937. Runx1-CBFβ heterodimer complex protects Runx1 from proteosomal degradation in murine myeloid

progenitor and embryonic cell lines (Huang G, 2001). Therefore, association of Runx1 to CBFβ not only ensures its transcription competence but also mediates its turnover in cells.

The process of how dimerization of Runx1 and CBFβ is coordinated remains unclear although the respective locations of Runx1 and CBFβ have already been identified. Runx1 protein harbors the nuclear localization and nuclear matrix-targeting signal at its DNA binding Runt domain and therefore resides primarily in the cell nucleus (Kanno T 1998; Zeng C, 1997; Kanno T 1998; Lu J, 1995). In addition, Runx1 could be found in 2 distinct nuclear fractions, one that is tightly associated with the nuclear matrix where basal transcription complexes gather and the other is soluble in nucleoplasm (Biggs JR, 2005). When overexpressed in murine fibroblasts, CBFβ is found mainly in the cytoplasm compartment (Lu J, 1995). Further investigations of the interaction between Runx1 and CBFβ could uncover the underlying molecular mechanisms that regulate transcription in hematopoiesis.

1.2 Genetic aberrations of Runx1 contribute to leukemogenesis

The loss or dominant-negative suppression of Runx1 function due to various inactivating Runx1 somatic mutations have been commonly identified in patients afflicted with AML, spontaneous or radiation-induced myelodysplastic syndromes (MDS), myeloproliferative syndromes (MPS), familial platelet disorder (FPD) with predisposition to AML, and other hematological malignancies (Tang J-L, 2009; Zharlyganova D 2008; Fröhling S, 2005). All these diseases share a common

characteristic: that there is a combination of differentiation block and accumulation of myeloid precursors in the bone marrow.

Despite the rare incidence of heritable mutations and the remarkably smaller number of mutations occurring in the AML genome compared to adult solid carcinomas (TCGA Research Network 2013, Welch 2012), Runx1 is a frequent mutation target in the founding clones obtained from human AML patients (Ding 2012). The most prevalent form of mutation identified is the RUNX1-RUNX1T1 or t(8;21)(q22;q22) chromosomal translocation that was found in 9%-30% of patients diagnosed with AML-M2, according to the French-American-British (FAB) classification (Mrózek K, 2004; Ichikawa M, 2004; Grimwade D, 2001). Other chromosomal translocations involving Runx1 are t(16;21)(q24;q21), t(3:21)(q26;q22), and t(12;21)(p13;q22), which generate RUNX1-MTG16, RUNX1-EVI1 (in chronic myelogenous leukemia), and TEL-RUNX1 (in acute lymphoblastic leukemia) fusion proteins, respectively. These recurrent reciprocal translocations produce dominant-negative Runx1, which interferes with the function of the normal Runx1 protein encoded by the other Runx1 allele, and therefore blocks myeloid progenitor cell differentiation, and affects cell proliferation, apoptosis, self-renewal and survival, thereby contributing to leukemogenesis (Hayashi Y, 2000).

Giphart-Gassler's group reported the first case of total homozygous loss of Runx1 in humans, which is associated with AML-M0 patients (Silva FPG, 2003). Heterozygous loss-of-function mutations, however, have been commonly identified, and they consist of nonsense mutations, missense mutations, frameshifts, intragenic insertions or deletions, and amplifications (Mendler JH, 2012, Silva FPG, 2003, Roumier C, 2003; Mikhail FM, 2002). The somatic point mutations cluster in the gene region of Runx1 coding for the

binding surface of the DNA binding *Runt* domain (DBD), therefore abolishing its DNA binding ability, destabilizing its 3-dimensional structure or disrupting its heterodimerization competency to CBFβ. Sporadic Runx1 point mutations are reported in 16% of AML patients with normal karyotype (Mendler JH, 2012), 21.5% in AML-M0 subtype (Roumier C, 2003), 8% of MDS myeloblastic leukemia patients (Osato 1999), 28% of MDS/AML, and 65% of congenital neutropenia (Skokowa J, 2014). Amplifications of Runx1 were predominantly identified in children with ALL (Roumier C, 2003).

Chromosomal translocations and heterozygous point mutations or deletions of Runx1 are also underlying causes of Runx1 loss of heterozygosity (LOH) and haploinsufficency (Goyama S, 2015). The reduced dosage of Runx1 due to monoallelic germline mutation impairs the maturation processes of myeloid progenitor cells, and therefore predisposes individuals to various blood diseases, for example in MDS with propensity towards AML (MDS/AML). This observation is further enhanced by the myeloid-specific leukemia occurred in the BXH2-Runx1+/- murine model established by Ito's group that recapitulates symptoms in human MDS/AML (Yamashita 2005). In another study, lineage-negative bone marrow of Runx1-haploinsufficency mice was extensively proliferative and allowed a limited level of lineage commitment but was poorly differentiated in response to differentiation cytokine (Ng KP, 2013). The myeloproliferative characteristic could be the consequence of the upregulated Bcl-2 pro-survival gene by the normal Runx1 gene (Goyama S, 2013). Scott's group proposed that some cell types could be more sensitive and susceptible to the reduced dosage of Runx1, as the amount of Runx1 required for their normal activity varied (Michaud J, 2008).

Milton HW *et al.* pointed out that it is necessary to evaluate the functionality of the unaffected Runx1 allele so that any biochemical discrepancies between the mutant and normal Runx1 can be resolved to rescue the haploinsufficency phenotype. This approach could lead to the development of alternative therapies for AML patients (Milton HW, 1999).

Current consensus shows that single Runx1 mutation alone is insufficient or not solely responsible for leukemogenesis. Song *et al.* observed that the RUNX1-RUNX1T1 genetic alteration could be identified in bone marrow, peripheral blood and cord blood samples of healthy individuals, reinforcing this consensus (Song J, 2011). Presence of this RUNX1-RUNX1T1 translocation itself could pose a higher risk for leukemic transformation, and other second mutations during adult hematopoiesis predisposes an individual to leukemia. Hence, Runx1 has been characterized as a classic tumor suppressor gene (Silva FPG, 2003). In addition, RUNX1-RUNX1T1 fusion protein probably also contributed to the initiation of AML, as the amount of somatic mutations was higher in normal karyotype AML compared to RUNX1-RUNX1T1 in *de novo* AML patients (Kihara R, 2014).

Recent transgenic mouse models also confirmed that the expression of single Runx1 mutation is insufficient to cause AML. For example, Runx1 knock-out or RUNX1-RUNX1T1 knock-in mouse models had restricted transformation capacity and did not develop spontaneous leukemia (Ichikawa M, 2004; Rhoades KL, 2000) until cooperating mutations that altered the growth signaling pathway were introduced (Higuchi M, 2002).

These additional cooperating genetic alterations other than Runx1 are widely known to participate in the full malignant cell transformation of all types of leukemia. The expanded myeloid progenitor pool in Runx1-null bone marrow is prone to acquire these "second hits" activating mutations, in which they cooperate with the initiating Runx1 mutations to confer a myeloproliferative advantage, escalating the disease to full-blown leukemia. A recent microarray-based gene profile study revealed that RUNX1-RUNX1T1 could trigger the base excision repair pathway, which is specific in restoring point mutations (Michaud J, 2008). Therefore, abnormality in Runx1 function closely corresponds with an increased rate of mutations, generating pools of mutated progenitor cells.

Concurrent mutations of the Type III Receptor Tyrosine Kinase (RTK) genes, such as *FLT3* and c-KIT usually accompany the manifestation of leukemia in 40% of human leukemia cases (Yamashita N, 2005). N-RAS mutations were also identified in 11% of the *de novo* AML patients (Gustafson SA, 2009). AML patients who are diagnosed with exclusive RUNX1-RUNX1T1 translocations have a favorable prognostic outcome, while the presence of additional mutations of *Flt3* or cKit ameliorates their favorable prognosis (Ishikawa 2009). Concurrent and multiple genome editing in primary HSCs through the CRISPR-Cas9 method provided additional evidences that the loss of Runx1 could trigger proliferative advantage in heterozygous FLT3-ITD expressing HSCs *in vivo*, therefore resulting in a biased growth of myeloid lineage (Heckl D, 2014).

When mutation in tyrosine kinase gene is not identified in some cases of AML, there may be a possibility that mutation in protein tyrosine phosphatases or cytokine receptors could be an alternative (Welch JS, 2012). In congenital neutropenia patients

with high risk of MDS/AML, mutations in the CSF3R gene that encodes the G-CSF receptor were frequently found in combination with Runx1 mutations, implicating their cooperative participation in leukemia progression (Skokowa J, 2014).

1.3 Downstream transcriptional targets of Runx1 in granulopoiesis

Numerous findings in RUNX1-RUNX1T1 clinical cases and transgenic mice models suggest that disruption of Runx1 inhibits the maturation of granulocytes from myeloid progenitors (Ng KP, 2013; Gaidzik VI, 2011; Tokita K, 2007; Vradii D, 2005; Lam K, 2004; Pabst T, 2001; Kohzaki H, 1999). These findings imply that the downstream transcriptional program that governs granulopoiesis *in vivo* is perturbed when Runx1 activity is diminished in leukemia (Rosmarin AG, 2005).

A comprehensive microarray analysis of human bone marrow demonstrated that CCAAT/enhancer binding protein α (C/EBPα) is one of the approximately 11 thousand genes involved in the complete granulocyte differentiation from the progenitor stage (Theilgaard-Mönch K, 2005). Hong *et al*. reported that *Cebpα* transcription was down-regulated in lineage-negative marrow cells and progenitors when Runx1 was dominantly inhibited or deleted in murine bone marrow. Introduction of exogenous Runx1 induced *Cebpα* transcription, whereas the expression of exogenous C/EBPα in these Runx1-deleted marrow cells reversed the differentiation block and rescued granulocyte formation. Furthermore, *Cebpα* mRNA expression induced by Runx1 in the presence of the translational inhibitor cycloheximide was detected, which strongly indicates that C/EBPα is a direct downstream molecular target of Runx1 (Hong G, 2012). A

comprehensive review of Runx1 studies also described C/EBPα as a critical transcription factor specifically activated for the early phase of granulocyte differentiation from myeloid progenitors (Friedman AD, 2002).

Down-modulation of C/EBPα was apparent exclusively in AML patients diagnosed with CBF-disrupted AML (i.e. RUNX1-RUNX1T1 and CBFβ-SMMHC) compared to other AML subtypes (Cilloni D, 2003). In clinical samples of RUNX1-RUNX1T1 patients, the protein expression of C/EBPα was undetectable whereas the mRNA level of *Cebpa* gene was 8-fold lower (Pabst T, 2001). In addition, the expression and DNA binding ability of C/EBPα was suppressed in Cos7 cells and monocytic progenitor U937 carrying the RUNX1-RUNX1T1 chimeric gene (Tokita K, 2007; Pabst T, 2001). Reduced expressions of *Cebpa* were also detected in AML patients who had somatic Runx1 point mutations with normal karyotype (Mendler JH, 2012). Similarly, a gene expression microarray analysis in Germany demonstrated that expression of *Cebpa* was significantly downregulated in Runx1-mutated AML patients (Grossman V, 2012).

PU.1 is another molecular target of Runx1 that is necessary for normal myeloid differentiation, especially at the late maturation stage (Huang G, 2008; Friedman AD, 2002). In AML patients or Kasumi-1 cells harboring the RUNX1-RUNX1T1 chromosomal aberration, PU.1 activity was diminished, and mice with homozygous loss of PU.1 did not develop mature myeloid cells (Vangala RK, 2003). As shown in a previous study on Runx1 deficient zebrafish embryos, Runx1 regulated PU.1 via a negative feedback loop, favoring granulocyte differentiation and neutrophil production (Jin H, 2012). Conditional deletion of Runx1 in adult mice also showed a down-regulation of *Cebpa* and *PU.1* RNA level in its lineage-negative bone marrow cells and

granulocyte-monocyte progenitors, although it caused a significant skewed differentiation of monocytes compared to that of granulocytes. Furthermore, the reintroduction of exogenous Runx1 in the Runx1 deficient bone marrow cells rescued the expression of C/EBPα and granulocyte formation (Guo H, 2012). These observations were consistent with an earlier finding that PU.1 expression was down regulated in Runx1 knock-out mice, therefore inhibiting granulocytic development (Huang G, 2008).

C/EBPα is the founder member of the leucine zipper transcription factor family, whereas PU.1 belongs to the *ets* transcription factor family. They are non-redundant in their roles in myeloid cell development. Runx1 directly regulates C/EBPα and PU.1 to activate myeloid lineage maturation by interacting with their promoters and enhancers. Runx1 binds to conserved DNA sequences in the *Cebpa* promoter and the +37kb enhancer (Cooper S, 2015; Guo H, 2012). The regulation of *Pu.1* transcription by Runx1 occurs via its -14kb enhancer (Huang G, 2008).

1.4 Post-translational modifications of Runx1 facilitate recruitment, assembly and interaction with co-regulators to affect Runx1 function and myeloid development

In addition to differential transcription, alternative splicing, and the generation of different isoforms, the diverse functions of Runx1 are further expanded by its combinations of multiple post-translational modifications (PTMs) to fine-tune the precise modulation of Runx1 activities in spatiotemporal and lineage-specific conditions. Among the common types of PTM identified on Runx1 are phosphorylation, ubiquitination,

acetylation, and methylation. The amino acid residues that are being modified are conserved across the Runx proteins (Goyama 2014), implicating that the PTM mechanisms are shared among the Runx family members.

PTM facilitates the assembly of various downstream transcription factors or epigenetic components on Runx1. Multiple molecular factors usually bind to Runx1, either directly or indirectly, which subsequently affect cell fate and lineage-specific gene expressions (Prange KHM, 2014). Therefore, PTM of Runx1 could either positively or negatively impact the development of hematopoietic stem/progenitor cells depending on the types of complexes that it forms with other molecular co-regulators. For example, Runx1 promotes the progression of G1-S and S-G2/M cell cycle in myeloid progenitors through the serine/threonine phosphorylation by Cyclin-dependent kinase 1 (Cdk1)/cyclin B, Cdk6/cyclin D3 and Extracellular-Regulated Kinase (ERK) and therefore favoring cell proliferation (Zhang L, 2008). At the same time, association of cyclin D or Cdk6 to Runx1 outcompetes the heterodimerization of Runx1-CBFβ, therefore reducing the DNA binding affinity and transactivation of Runx1, subsequently down-regulating PU.1 and abrogating terminal myeloid differentiation but sustaining myeloid progenitor proliferation *in vitro* and *in vivo* (Fujimoto T, 2007; Peterson LF, 2005).

Phosphorylation may be required for the normal transcriptional process and proteosomal degradation. As mentioned earlier, Runx1 is constantly degraded in cells. Loss of serine or threonine phosphorylation at S276, S293, S303 and T300 reduced Runx1 transactivation, caused the accumulation of ubiquitinylated-Runx1, stabilized the protein and retained it at the nuclear matrix (Biggs JR, 2005). A follow-up study further revealed that the phosphorylation of these serine and threonine residues were catalyzed

by Cdk1/cyclin B and Cdk2/cyclin A, which subsequently mediated Runx1 degradation by Cdc20 and Cdh1 in the Anaphase Promoting Complex (APC) (Biggs JR, 2006). Mutation of S249 and S266 to alanine also prevented ERK-dependent phosphorylation, which led to its firm association with nuclear matrix mediated by mSin3A corepressor, thereby increasing its stability, protecting it from proteosomal-mediated degradation as well as abolishing its transactivity (Imai Y, 2004).

Similar to other transcription factors, Runx1 also modulates target gene expression through recruitment of chromatin modifiers, such as histone acetylases (HATs) or histone deacetylases (HDACs). Phosphorylation of S48, S303 and S424 by Cdk1 or Cdk6 enhanced the transactivation potential of Runx1 and triggered cell proliferation in Ba/F3 myeloid cells and lineage negative murine marrow progenitor cells, partly due to reduced interaction with HDAC1 and HDAC3 (Hong G, 2010; Zhang L, 2008). Recruitment of p300 and CREB binding protein (CBP) through homeodomain-interacting protein kinase-1/2 (HIPK1/2)-mediated phosphorylation on S249 and S276 also corresponds to increased level of Runx1 transactivation and induction of myeloid cell differentiation (Aikawa Y, 2006; Kitabayashi I, 1998). After binding to the C-terminal region of Runx1, co-activator p300 acetylates Runx1 on K24 and K43, further enhancing the DNA binding and transcriptional activity of Runx1 (Yamaguchi Y, 2004; Kitabayashi I, 1998).

Methylation is usually linked with suppression of gene expression or protein function. However, methylation of R206 and R210 by arginine methyltransferase, PRMT1, removes the association of mSin3A co-repressor to Runx1, thus enhancing Runx1 transcriptional activity (Zhao X, 2008). The suppression of Runx1 activity is

achieved by PRMT4 methylation on R223, which promotes the binding of co-repressors onto Runx1 and eventually abrogates myeloid cell differentiation (Vu LP, 2013).

1.5 Tyrosine phosphorylation of Runx1 by protein tyrosine kinases in myeloid progenitor cells

Cytokines can instruct cell fate decision and influence lineage choices. When myeloid progenitors are exposed to Granulocyte Colony Stimulating Factor (G-CSF) cytokine or other differentiation ligands, a cascade of signal transduction events is activated through the G-CSF receptor (G-CSFR) or other cognate receptors (Ward AC, 1999). Facilitated by combinations of various lineage-specific molecular transducers and transcription factors, the differentiation signals eventually steer the myeloid progenitors into the terminal granulocytic differentiation process. Tyrosine modifications on these molecular transducers, for example, phosphorylation, mediate their activation and effective signal propagation from the cell membrane into the cellular compartments.

Tyrosine phosphorylation of Runx1 by Src-family tyrosine kinases (SFK) was recently identified (Neel BG, 2012; Huang H, 2012). In murine and human megakaryoblast and T-lymphoblast cells, tyrosine phosphorylation of Runx1 impaired their commitment into mature platelets and CD8+ T-lymphocytes. Inhibition of SFK activities, on the contrary, restored the maturation processes of megakaryocyte and CD8+ T-lymphocytes. Therefore, Runx1 tyrosine phosphorylation negatively regulates megakaryopoiesis and thymocyte development. The exact members of SFK that were responsible for tyrosine phosphorylation of Runx1 were not addressed in this report, but

the subset of tyrosine residues that were modified by SFK were identified by mass-spectrometry and mutational analysis. These tyrosines are mostly located at the REP domain of Runx1 (Huang H, 2012).

In that same article, the authors also demonstrated that the non-receptor tyrosine phosphatase – Src homology region 2 domain-containing phosphatase 2 (SHP2), physically binds to Runx1 and dephosphorylates one or more Runx1 tyrosine residues, although their identity has yet to be determined (Huang H, 2012). When Ba/F3 myeloid progenitors are stimulated by G-CSF, SHP2 is phosphorylated and results in preferential activation of granulopoiesis. Inhibition of SHP2 activity reduced the formation of colony-forming-unit-granulocytes (CFU-Gs) from lineage-negative murine bone marrow mononuclear cells (Jack GD, 2009). In addition, knock-down of SHP2 expression in myeloid progenitors and in murine marrow cells also down-regulated *Cebpα* and repressed granulopoiesis; the reduction in *Cebpα* expression was restored by exogenous Runx1 (Zhang L, 2011). Besides acting on Runx1 or affecting granulocyte differentiation via C/EBPα, SHP2 also participated in the G-CSF signaling pathway by mediating the activation of Src kinase Lyn in Ba/F3 cells after Lyn binds to the G-CSFR (Futami M, 2011). Together, these findings together indicate that Runx1 is the key molecular component at the intersection of the G-CSF signaling pathway in hematopoietic progenitors cells that is regulated by SFK-SHP2 activities.

Several other protein tyrosine kinases have also been identified in myeloid progenitor cells. Among them are Hck, Lyn, Fgr, Syk and Fes, which are all major members of the SRC family kinase (SFK) (Corey-PNAS 1994; Ward-BBRC 1998). Although Hck, Lyn and Fgr are found to be highly expressed in terminally differentiated

myeloid cells, myeloid progenitors lacking these three tyrosine kinases did not affect the terminal granulocyte differentiation and showed normal neutrophil formation; conversely, the G-CSF induced proliferation and myeloid progenitor production was greatly enhanced (Mermel, 2006; Perlmutter RM, 1989). An early article revealed an opposite effect of Hck, in which the overexpression of activated Hck in 32Dcl3 myeloid progenitor interfered with granulocytic differentiation (English BK, 1996). Fes facilitated the terminal differentiation of granulocytes and monocytes in a lineage-specific manner as well as in the absence of differentiation cytokines, and concomitantly activated the *Cebpα* and *PU.1* during these differentiation processes, respectively (Kim J, 2004; Kim J, 2003).

Jak2, another cytoplasmic non-receptor tyrosine kinase known as "Just another kinase 2" promotes mIL3-induced survival of myeloid progenitor cells through the activation of ERK2 (Chaturvedi P, 1998). Activation of Jak2 is frequently linked to activation of STAT3 (Signal Transducer and Activator of Transcription 3), as both of these intracellular signaling molecules are the early responders in the G-CSF signaling cascade (Jack GD, 2009; Tian S-S, 1996). STAT3 itself is a main activation target of G-CSF receptor and is required in the steady-state homeostasis of neutrophil production and in the acute mobilization of neutrophils into circulating blood (Panopoulos A, 2006; Lee C, 2002). Tyrosine phosphorylation and activation of Jak2 and STAT3 were detected in terminally differentiated human neutrophils following GM-CSF stimulation and also in the proliferating G-CSF receptor expressing Ba/F3 myeloid progenitors following G-CSF stimulation (Al-Shami A, 1998; Avalos BR, 1997). Activating mutation of Jak2, Jak2(V617F) is implicated in myeloproliferative neoplasms, where it was identified in the

granulocytes, erythroid and myeloid precursors (Baxter EJ, 2005). Interestingly, the constitutively augmented kinase activity of Jak2(V617F) also caused the up-regulation of PU.1 expression (Irino T, 2011). Together, these reports illustrated the complex role played by Jak2 as it could contribute to both myeloid progenitor proliferation and differentiation, although its exact mechanism in these developments requires more investigation.

Despite the extensive studies published on the functions of tyrosine kinases in normal hematopoietic systems and in hematological malignancies, the ability of Src, Lyn, Syk, Hck, Fes or Jak2 to phosphorylate Runx1 to directly affect its intrinsic transactivation potential is still unknown and therefore is the focus of this study.

1.6 Objectives of study

In this study, the contribution of Runx1 tyrosine phosphorylation to the regulation of granulopoiesis and its role in the G-CSF signaling pathway is characterized. Given that SHP2 and Runx1 activate both *Cebpα* mRNA and protein expression to promote granulocyte differentiation, it was logical to speculate that tyrosine modification on Runx1 might be one of the many mechanisms to regulate C/EBPα activity.

Furthermore, SRC phosphorylates and SHP2 dephosphorylates tyrosine residues on Runx1, and SHP2 mediates the activation of SRC in the G-CSF signaling pathway. These findings imply that there is a dynamic, functional and multimeric interaction between SHP2, SRC and Runx1. This leaves open the question as to which

phosphorylated form of Runx1 mediates transcription activation and induction of C/EBPα.

We are especially interested in the biological effects of Runx1 phosphorylation during granulocyte differentiation. The inhibition of megakaryopoiesis and thymocyte development by non-phosphorylated Runx1 in adult mice bone marrow, demonstrated by Cantor's group, prompted us to **evaluate the role of Runx1 phosphorylation in myeloid progenitors and verify the sequence of events proposed in our model of the G-CSF signaling pathway in granulopoiesis** (Figure 1).

In order to achieve these aims, the design of this study is divided into 2 parts:

(1) Determine whether the tyrosine phosphorylated or the non-phosphorylated Runx1 shows transactivity potential and promote induction C/EBPα

(2) Investigate the mechanisms utilized by tyrosine phosphorylated or non-phosphorylated Runx1 to regulate its transactivity potential and activation of C/EBPα

G-CSF → SHP2 → SRC → Runx1 → C/EBPα → Granulopoiesis

Figure 1: Proposed model of G-CSF signaling pathway in granulopoiesis

CHAPTER 2: MATERIALS & METHODS

2.1 Tissue culture

Human embryonic kidney HEK293T cells (ATCC) were cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone). Lipofectamine® 2000 (Invitrogen) was used for transient transfection in HEK293T cells. Murine myeloid progenitors 32Dcl3 cells (Valtieri M, 1987) were maintained in Iscove's modified Dulbecco's medium (IMDM; Life Technologies) supplemented with 10% of heat-inactivated FBS (Hyclone), 1X Penicillin-Streptomycin (Life Technologies) and 1 ng/ml of murine interleukin 3 (IL3; Peprotech). To initiate differentiation, 32Dcl3 cells were washed twice in 1X phosphate-buffered saline before transferring to IMDM with 20 ng/ml GCSF (Amgen). For stable retroviral transduction, the 32Dcl3 cells were subjected to electroporation using the Amaxa Nucleofector system (Lonza) or to retroviral RetroNectin system (Takada). Subclones were screened through limiting dilution in 0.2 μM of puromycin or 1.2 mg/ml of neomycin (Sigma-Aldrich). Protein induction was made through addition of 20 ng/ul 4'-hydroxytamoxifen into IMDM. For suppression of kinase activity, PP2 kinase inhibitor was provided at 20 μM final concentration. 32Dcl3 stable clone (77-10) carrying the pBabePuro-shSHP2 retroviral expression vector was previously described (Zhang L, 2011).

2.2 Plasmids

The gene encoding the mouse Runx1b isoform and its mutant variants 5F, 5D, and 2F variants were cloned into pcDNA3 (CMV). Generation of the 4F, 4D, 1F, and 1D mutant variants was done through restriction digestion of the *BamH*I site that separates the Y260 from the other 4 residues at the C'-terminal tyrosine cluster. The 2F*, 2E, 2E* mutant variants were synthesized (Blue Heron) and were ligated to the 584bp *BamH*I-*EcoR*I fragments. pEF1α-birA, pEF1α-birA-FLAGBio-Runx1 and its tyrosine mutants were previously described (Huang H, 2012). The pBabePuro-Runx1(4F)-ER(T) and pBabePuro-Runx1(4E)-ER(T) were made from pBabePuro-Runx1-ER(T) by replacing the 589bp *BamH*I-*Mlu*I fragments. CBF_4-TK-Luc, CMV-βGal, pCEFL-Src(E381G), CMV-CBFβ, CMV-HA-Cdc20, CMV-HA-Cdh1 were previously reported (Zhang L, 2008; Bigg JR, 2006; Bjorge JD, 1995; Ogawa E, 1993). CMV-HA-Cdc20, CMV-HA-Cdh1, CMV-HA-Ubiquitin was acquired from commercial source (Addgene). pBabePuro-Src-ER(T) was generated by linking the DNA encoding the ERα ligand-binding domain C-terminal to the DNA of Src(E381G) in pBabePuro retroviral expression plasmid.

2.3 Luciferase Reporter Assay

Ten-thousand HEK293T cells were plated into each well of the 24 well cell culture dish, and was incubated overnight in the 5% CO_2 incubator. Five ng of pcDNA3-mRunx1b plasmid DNA was co-transfected with 2 ng of beta-galactosidase and minimal-TK-Luc, either with or without 40 ng of activated Src. After 48 hours of transfection, the

cells were lysed and the luciferase and beta-galactosidase activity were then measured using the luminometer.

2.4 mRNA Expression and Quantitative PCR

Total mRNA was extracted with the NucleoSpin RNA II kit (Machery-Nagel) following the manufacturer's instructions. First-strand synthesis of cDNA was prepared at 42ºC for 1 hour using the ImProm-II™ Reverse Transcription System (Promega) and the provided oligo dT primers. Quantitative polymerase chain reaction (PCR) was carried out using 5 ng of cDNA product, iQ SYBR Green (Bio-Rad), and primer pairs targeting *Runx1, Cebpα, PU.1,* or Actin. Details of oligonucleotides (Sigma-Aldrich) are listed in Table 1.

Oligonucleotide	Sequences (5'-3')
Runx1-F	CACCGTCATGGCAGGCAAC
Runx1-R	GGTGATGGTCAGAGTGAAGC
Cebpα-F	CAAGAACAGCAACGAGTACCG
Cebpα-R	GTCACTGGTCAACTCCAGCAC
Pu.1-F	CCTTCGTGGGCAGCGATGGA
Pu.1-R	TGTAGCTGCGGGGGCTGCAC
Actin-F	GACCTCTATGCCAACACAGT
Actin-R	AGTACTTGCGCTCAGGAGGA
Cebpα-enhR3-F	AACAGGAAAGATGGCACCAG

Cebpα-enhR4-R	CCACACCCCTCTATGTGATG
Cebpα-2500F	TTCCTTATCCTTCCAGAGACTTCC
Cebpα-2500R	GATTTCCAGCCTCCCGTGTGATG
Pu.1 enhF	CTGGTGGCAAGAGCGTTTC
Pu.1 enhR	CCACATCGGCAGCAGCAAG
Actin-1500F	GGGAAAGTTCTCTCAGGGTTGG
Actin-1500R	TGCTGTGAACTGGAAACACACC

Table 1: Sequences of oligonucleotides used for quantitative PCR.

2.5 Protein Expression and Western Blots

Whole cell lysates were prepared using the Laemmli sample buffer while the nuclear extracts were prepared using 400 mM of NaCl. Whole cell lysates or nuclear extracts were resolved in SDS-PAGE and then transferred to a PVDF membrane that was pre-moistened with methanol. The membrane was probed with primary antibody overnight at 4^{o}C, followed by incubation for 1 hour with secondary antibodies that were conjugated with HRP. Primary antibodies used were RUNX1 (Active Motif), C/EBPα (14AA), PU.1 (D-19), HA (Y-11), ERα (MC-20), or Lamin B (M-20; Santa Cruz), HA (16B12; BioLegend), P-Tyr (4G10, Millipore), HDAC1 (ab7028) or HDAC3 (3G6; Abcam), FLAG (M2) or β-actin (AC-15; Sigma), GAPDH (14C10; Cell Signaling), anti-V5 (Invitrogen), CBFβ (Cao W, 1997). Protein bands were then visualized using chemiluminescence detection system.

2.6 Electrophoretic Mobility Shift Assay

Nuclear extracts from transfected 293T cells were prepared in 400 mM NaCl. Protein products were also obtained using the rabbit reticulocyte lysate in the *In vitro* TnT® Coupled Transcription Translational System (IVT; Promega), with and without 25 μM of MG132 (Sigma). Equal amount from each nuclear extract or IVT sample was subjected to electrophoretic mobility shift assay as described (Guo H, 2012). The oligonucleotide used was derived from the Runx1 binding site on the myeloperoxidase (MPO) promoter and was radio-labeled with 0.0032 mM of αP^{32}-dCTP. The oligonucleotide pairs are: Runx1MPO-T (5'-CTAGACTGACCATTAACCACAACCAG TTG-3') and Runx1MPO-B (5'-CTAGCAACTGGTTGTGGTTAATGGTCAGT-3'). The protein-oligo complexes were transferred from the gel onto a PVDF membrane. Then, the radioactive signals were exposed to and detected using autoradiography.

2.7 Chromatin Immunoprecipitation (ChIP) Assay

Total cell extracts from 1 million 32Dcl3 stable cells expressing Runx1 or its variants were subjected to ChIP assay with 2 mg ER or normal rabbit IgG. Next, quantitative PCR was performed using 0.5 μL precipitated genomic DNAs and 1 μg/μL of the following oligonucleotide pairs: Cebpα-enh and Cebpα-enhR3-F, Cebpα-2500F and Cebpα-2500R, Pu.1 enhF and Pu.1 enhR, Actin-1500F and Actin-1500R (Table 2.1).

2.8 Protein Immunoprecipitation

Whole cell lysates of transfected HEK293T cells were extracted and then were measured and equalized using the Bradford assay. Coimmunoprecipitation (Co-IP) of Runx1 or its variants was carried out using HA-Ubiquitin. The co-IP buffer consisted of 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.2% Nonidet P-40, 0.2% Triton X-100, 0.2% sodium deoxycholate, 10% (w/v) glycerol, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and 1X protease inhibitor cocktail (Sigma) as described previously (Guo H, 2011). Tyrosine phosphorylation of Runx1 was assessed with similar IP procedures using anti-ERα in whole cell lysates of 32Dcl3 stable clones expressing Runx1-ER(T) or its variants, which were pre-treated with 1.25 mM Na_3VO_4 for 15 minutes to inhibit activity of endogenous protein tyrosine phosphatases. Interactions between Runx1 or its variants and CBFβ were demonstrated using HEK293T cells co-expressing pEF1α-FLAGBio-Runx1 and pEF1α-birA. The protein complexes were pulled-down with streptavidin agarose (Novex®). All immunoprecipant was analyzed with Western Blot.

2.9 Statistical Analysis

Means and standard error are indicated in figures 2, 3 5 and 8, and comparisons for significance were accessed using the Student T-test. Band intensities on Western Blot were quantified with the open software ImageJ managed by the National Institutes of Health.

CHAPTER 3: RESULTS

3.1 Tyrosine phosphorylation of Runx1 increases transactivation

There are a total of 15 tyrosine sites on Runx1, 10 of which are conserved (Huang H, 2012). The tyrosine residues that are modified by Src are Y260 in the transactivation domain (TAD) and Y375, Y378, Y379 and Y386 in the C-terminal repression domain (REP) (Huang H, 2012; Figure 2A). Tyrosine residues (Y) are switched to phenylalanine (F) to confer a phospho-null phenotype. On the other hand, Y residues are replaced by glutamate (E) or aspartate (D) to mimic the constitutive phosphorylation state of Runx1 by Src. Several tyrosine mutant variants were generated to study the effect of tyrosine phosphorylation on Runx1 transactivation and DNA binding. The combinations of the mutated tyrosine residues are listed in Figure 2A, bottom panel.

pCMV-Runx1 and its variant mutants of equal amounts were co-transfected with CBF_4-TK-Luc and CMV-βGal in the HEK293T cells. The transfected cells were used to investigate the Runx1 protein expression as well as for the luciferase reporter assay. CBF_4-TK-Luc contains four repeats of Runx1 consensus sites, located at the upstream of a minimal thymidine kinase promoter and luciferase cDNA (Figure 2B). When Runx1 protein binds to CBF_4, the transcription of the luciferase gene will be activated, which is driven by the TK promoter. Fold–activation changes were reported as the ratio of luciferin signals to the β-galactosidase signals after normalizing to the empty CMV vector control. The transactivity of Runx1-WT was 5-fold higher than the CMV vector, while the transactivity of 5F and 4F was only about 2-fold. Transactivity of 1F was similar to that of WT. In contrast, 5D showed the highest transactivity at about 10-fold,

followed by 4D and 1D (Figure 3A, top). The distinctive levels of transactivation in these mutant variants reflected that the phosphorylation of each tyrosine residue has a different impact on Runx1 transactivity.

To map out which tyrosine residues are critical in Runx1 transactivity, a similar luciferase reporter assay was repeated using other Runx1 mutant variants, 2F*, 2E*, 2F, 2E, and different mutants with single a tyrosine mutation (Figure 3B, left). Again, 2F* and 2F had low transactivity, while 2E* and 2E displayed up to an 8-fold increase in transactivity. The other single F and 2F mutant variants were expressed in the pEF1a vector. Although their protein expression was similar to the Runx1-WT in Western Blot, the transactivation of 2F again showed reduced transactivation while the single F mutant variants manifested similar transactivity as that of WT (Figure 3B, right). This observation further suggests that at least 2 tyrosine sites at the C-terminal REP are required to affect transactivity of Runx1.

It was noted that the total protein expression of 5D and 4D was higher than Runx1 and the other variants, partly contributing to the higher transactivity in the luciferase reporter assay (Figure 3A, bottom). The migration of the Runx1 bands of 5D and 4D were also slightly retarded than those of Runx1 and F mutant variants, which reflected their higher negative charges. However, the higher protein expression of 5F, 4F, 2F* and 2F did not alter the reduced transactivity (Figures 3A and 3B, bottom), therefore, one could confidently conclude that the increased transactivity was due to the effect of tyrosine phosphorylation.

When pCEFL-Src(E381G) was co-expressed with Runx1-WT to assess the effect of cooperation, there was a significant synergistic effect observed in the luciferase reporter assay. Runx1 synergizes with Src to markedly increase the transactivity to about 31X, or at 7.4X higher than Runx1 alone. 5F displayed a minimal effect when combined with Src and only showed a mere 2.6-fold activation, which was similar to the activity of Src alone (Figure 4A). The Runx1-WT expression assessed by Western Blot also exhibited a significant increase in the presence of Src and also migrated more slowly due to the effect of phosphorylation. The 5F protein band, however, only showed minimal increase in expression when co-expressed with Src. Fes and Jak2 are another two major members of non-receptor tyrosine kinases preferentially expressed in myeloid cells that regulate myeloid gene expression during granulopoiesis. Interestingly, in the luciferase reporter assay, Fes minimally increased Runx1 transactivity to only 6.2X but Jak2(V617F) did not increase Runx1 transactivity (Figure 4B). These results not only highlight that the five tyrosine residues are the specific substrates for Src kinase and are important for Runx1 transactivation activity but also give a representation of the maximum transactivity effect of Runx1 when phosphorylated *in vitro*, in which this effect was observed in the luciferase reporter assay using 5D tyrosine mutant.

Runx1 interacts with a cofactor called CBFβ. The formation of the Runx1- CBFβ complex increases the binding affinity of Runx1 to DNA by two-fold (Bae SC, 1994). To evaluate whether transactivation of Runx1 can be improved by the presence of CBFβ, we performed the luciferase reporter assay with and without CBFβ along with Runx1-WT, 5F and 5D. The exogenous CBFβ was expressed much higher than the endogenous CBFβ in HEK293T cells, but this abundance did not increase the Runx1 transactivity (Figure

4C). Surprisingly, transactivation of 5D decreased in the presence of CBFβ. On the other hand, protein levels of WT and 5F increased when co-expressed with CBFβ, supporting the conclusion that CBFβ stabilizes Runx1 and also indicating that basal CBFβ level is limiting compared to WT and 5F expression.

3.2 Tyrosine phosphorylation of the C-terminal cluster activates myeloid-specific genes

In order to study the effect of tyrosine phosphorylation of Runx1 in the myeloid setting, we generated 32Dcl3 progenitor cells that stably expressing the Runx1-WT-ER(T) and its variants 4F-ER(T) and 4E-ER(T) in pBabePuro retroviral vectors. These cells contain both endogenous Runx1 and the ectopically expressed Runx1-ER.

First, we demonstrated that tyrosine phosphorylation of Runx1 occurred in myeloid cells. The 32Dcl3 cells stably expressing the WT-ER(T) were cultured in growth media with the addition of either mIL3 or G-CSF cytokines, then pre-treated with PP2 pan-tyrosine kinase inhibitor or in DMSO vehicle control for 24 hours before total protein lysates were harvested, which were then used for immunoprecipitation with ERα antibodies, followed by SDS-PAGE and Western Blot. The level of tyrosine phosphorylation in Runx1 was detected using anti-phospho-tyrosine antibodies. Tyrosine phosphorylation was observed in the 32Dcl3-pBabePuro-Runx1-ER(T) cells grown in both mIL3 and GCSF. After PP2 treatment, the phosphorylation level was reduced to approximately 50% relative to that without PP2 treatment, as confirmed by densitometry (Figure 5A). Therefore, this observation supports that tyrosine phosphorylation occurred

in 32Dcl3 myeloid progenitors and Src inhibition reduces WT-ER(T) phosphorylation grown in both mIL3 and G-CSF.

We also compared the tyrosine phosphorylation level of Runx1-ER(T) to its phospho-null mutant 4F-ER(T) (Figure 5B). Two 32Dcl3 subclones expressing similar levels of WT-ER and 4F-ER(T) respectively were cultured in mIL3 cytokines and were treated with 4HT for 24 hours. Total protein lysates from these subclones were then immunoprecipitated with ERα antibodies followed by Western Blot to assess the expression for Runx1 and phopho-tyrosine. The phosphorylation levels of both subclones of 4F-ER(T) was 2-fold lower than that of WT-ER(T), indicating that the 4F-ER(T) was deficient in tyrosine phosphorylation. The reduced level of phosphorylation was further verified by densitometry.

In the luciferase reporter assay in 293T cells, Runx1 displayed high transactivation activity when at least 2 tyrosine residues, Y375 and Y378 or Y379 and Y385 were phosphorylated. The optimal effect of Runx1 transactivation on myeloid cell – specific target genes was further evaluated in the WT-ER(T), 4F-ER(T) and 4E-ER(T) 32Dcl3 pooled transductants. C/EBPα is essential to the differentiation of granulocytes and monocytes, whereas PU.1 is crucial to the formation of monocytes. We predicted that the tyrosine phosphorylation of Runx1 contributes to the downstream regulation and activation of *Cebpα* and *PU.1*. Total protein lysates were extracted form the pooled transductants and were analyzed for the expressions of Runx1-ER, C/EBPα or PU.1, with and without 4HT induction for 24 hours. On Western Blot, the expressions of Runx1-WT-ER(T), 4F-ER(T) and 4E-ER(T) were relatively even and similar after 4HT induction. However, only WT-ER(T) and 4E-ER(T) caused an elevated expressions of

endogenous C/EBPα and PU.1(Figure 6A), as confirmed by densitometry. Our results clearly exhibited that expressions of Runx1-WT-ER(T) and to a lesser extent 4E-ER(T) increased protein production of endogenous C/EBPα and PU.1, while the 4F-ER(T) was ineffective

The protein expression profiles of C/EBPα and PU.1 were verified with quantitative expression analysis of *Cebpα* and *PU.1* mRNA, which is a more direct measurement of *Cebpα* and *PU.1* gene activation or transcription. Total cellular RNAs were extracted from the same pooled transductants and were evaluated by quantitative RT-PCR. Similar to their protein expression, WT-ER(T) and 4E-ER(T) exhibited 2-fold higher mRNA expression of *Cebpα* and *PU.1* relative to the Puro vector control but not with 4F-ER(T) (Figure 6B). This further confirmed that phosphorylation of the C-terminal tyrosine clusters on Runx1 were important to trigger the activation of *Cebpα* and *PU.1* transcription, resulting in higher mRNA levels and higher protein expressions.

3.3 Loss of tyrosine phosphorylation of Runx1 reduces its DNA binding affinity

The activation of *Cebpa* and *PU.1* transcription could be a consequence tyrosine-phosphorylated Runx1 binding to its consensus sequences at the regulatory promoter and enhancer regions of *Cebpa* and *PU.1* respectively. The binding of Runx1 on *Cebpa* and *PU.1* promoter had previously been reported (Guo 2012; Huang G, 2008). Therefore, we proceeded to examine the binding affinity between Runx1 and its mutant variants using

Electrophoretic Mobility Gel Shift (EMSA) and Chromatin-Immunoprecitation (ChIP) assays.

HEK293T cells were transfected with CMV-Runx1, 5F, 5D, 4F or 4D. Nuclear extract and cytoplasm prepared from these transfected cells were resolved on 8% SDS-PAGE to determine the exact compartmentalization of the Runx1 protein. Consistent with Runx1 characterization in earlier reports (Lu J, 1995; Kanno 1998), Runx1 protein was identified in the nuclear extract and not in the cytoplasm (Figure 7A). Lamin B is a protein specifically located in the nuclear compartment (Moir RD, 2000) while GAPDH was more abundant in cytoplasm (Mazzola JL, 2003), as shown in Figure 7A.

Protein expression of 5D and 4D of the transfected HEK293T cells was consistently higher than Runx1 and the phospho-null F mutant variants. This phenomenon was also present when resolving the nuclear extract of transfected HEK293T cells, and therefore must be taken into account when running the mobility shift assay. In order to evaluate whether the higher protein expression of 4D was not due to the stabilizing effect of endogenous CBFβ in HEK293T cells, we co-transfected Runx1, 4F and 4D together with CBFβ, and then inspected the Runx1 protein expressions in the nuclear extracts. Presence of CBFβ did not increase the protein expression of 4D, but rather caused a slight reduction. Surprisingly, 4F showed a slightly higher protein expression in the nuclear extract (Figure 7B, right). The reason for this phenomena was unclear but it was further confirmed by the higher 4F and lower 4D total protein lysates in a separate Western Blot (Figure 7C).

The input amount of Runx1 nuclear extracts was adjusted accordingly for EMSA so that equal amounts of nuclear proteins from transfected HEK293T were utilized for binding to the radio-labeled Runx1 consensus oligonucleotides. The DNA-protein complex was then resolved, followed by exposure to autoradiography to detect the presence of DNA-protein complex of Runx1 or of its mutant variants. Of note, because Runx1-WT consistently expressed lower protein expressions compared to 5F and 4D, two concentrations of Runx1 protein were used; one that was identical to all the mutant variants used and one that was twice that concentration, as indicated by the italicized label in Figure 8A. Runx1 and its phospho-mimetic mutant variants, 5D and 4D bond more strongly to the consensus sequences. Conversely, 5F, 4F, 2F* and 2F displayed minimal binding (Figure 8A, left). As 5D and 4D always showed higher protein expressions, I also reduced the input amount for EMSA but this effort failed to generate DNA-binding. This might reflect the possibility of the simultaneous dilution of endogenous CBFβ or other cooperating factors in HEK293T cells when reducing the input nuclear protein of 5D or 4D, therefore ameliorating the occurrence of DNA binding (data not shown).

As discussed earlier, CBFβ increased DNA binding affinity of Runx1. Therefore, when provided with exogenous CBFβ, the overall binding affinity of Runx1-WT, 4F and 4D to the consensus sequences was increased. However, 4F still showed weaker binding compared to Runx1 and 4D (Figure 8A, right).

We were also concerned that the endogenous CBFβ would affect the binding of Runx1 in the EMSA assay. Therefore, EMSA was repeated using proteins generated *in vitro* with cellular components isolated from reticulocytes, provided in the IVT system

(*In vitro* transcription-translation, Promega). Again, in Western Blot, 5D demonstrated the highest protein expression, followed by Runx1-WT, then 5F, although equal volumes of translated proteins were analyzed (Figure 8B, top). The doublets observed in 5F were not degradation products as verified by the addition of MG132 proteosomal inhibitor (Figure 8B, bottom). The volume of IVT products of WT, 5F and 5D was adjusted accordingly based on densitometry to equalize the total input amount for EMSA, either alone or with CBFβ. The absence of CBFβ impaired the ability of Runx1 to bind to its consensus sequence but the binding was rescued significantly when EMSA was carried out with CBFβ (Figure 8C). However, the binding of 5F was again weaker compared to WT and 5D. Therefore, this result suggests that tyrosine phosphorylation of Runx1 neither mimics the function of CBFβ nor relieves its dependence on CBFβ for optimal DNA binding.

The differential DNA binding of Runx1 and its tyrosine mutant variants can be further validated using 32Dcl3 myeloid progenitor cells that stably expressing Runx1-WT-ER(T), 4F-ER(T) and 4E-ER(T). The ability of the Runx1-WT, 4F or 4E to bind the enhancer regions of endogenous *Cebpα* and *PU.1* was assessed with ChIP assay. After the DNA-protein complex was immunoprecipitated in the 32Dcl3 stable cell lines expressing these transgenes, quantitative PCR was performed to measure the abundance of the regulatory DNA. Runx1-WT bound strongly to the +37 kb *Cebpα* enhancer and the -14 kb *PU.1* enhancer at 15-20 fold higher, which contained functional Runx1 binding sites but not at the actin promoter or the -2.5 kb region of *Cebpα* locus, which did not harbor any Runx1 binding consensus sequences (Figure 9A). 4E-ER(T) showed 15-20 fold higher binding to *Cebpα* and *PU.1* enhancers, similar to that of WT-ER(T). 4F-

ER(T) displayed the opposite result, in which the binding was minimal (Figures 9B and 9C). Consistent with the EMSA data, Runx1-WT and 4E manifested significant increased binding affinity for the 2 enhancers that contain functional Runx1 binding sites. This result also reinforces our observations of *Cebpα* and *PU.1* mRNA and protein expressions in Figures 6A and 6B, in which tyrosine phosphorylation of Runx1 played a crucial role in triggering the expression of C/EBPα and PU.1, partly through its DNA binding to *Cebpα* and *PU.1* enhancers.

3.4 Tyrosine phosphorylation stabilizes Runx1 and protects it from ubiquitin-mediated degradation

Phospho-mimetic Runx1 – 5D and 4D showed a higher expression in Western Blot compared to 5F and 4D (Figure 3A). In addition, Runx1 protein expression was also elevated in the presence of Src (Figure 4A). These two observations suggested that tyrosine phosphorylation might contribute to the stability of the Runx1 protein and thus prompted us to further inspect the half-life of these protein products with the help of cycloheximide. Cycloheximide (CHX) blocks protein synthesis by interfering with its elongation step (Schneider-Poetsch, 2010). Therefore, the amount of protein that would be detected after treating the cells with CHX would reflect the rate of degradation or accumulation of that particular protein.

We expressed Runx1-WT alone or with Src(E381G) in HEK293T cells, treated the transfected cells with cycloheximide at specific time-points, and then monitored the Runx1 protein expressions over 8 hours (Figure 10). Runx1-WT protein expression by

itself was moderate at time 0 and began to showed reduction between hour 4 and hour 6 after CHX addition. On the contrary, Runx1-WT expression was markedly increased at time 0 in the presence of Src(E381G), and this elevated expression maintained through the 8 hours of analysis. This result supported our observations in Figure 4A that Src could dramatically increase and stabilize the Runx1 expression. Furthermore, it also suggests that a large amount of exogenous Runx1 protein was not tyrosine phosphorylated due to the limiting level of endogenous Runx1 in HEK293T cells.

Protein expressions of 5D, 5F, 4D and 4F were also examined with CHX. 5D and 4D showed identical protein expression profiles as that of WT + Src(E381G). Beginning from time 0, 5D and 4D were expressed relatively higher than WT, and this level was constant for 8 hours after CHX treatment. In contrast, while 5F and 4F protein expressions were higher than WT, the expression levels started to diminish earlier than WT, which was between hour 2 and hour 4 post-CHX treatment (Figure 10). These results indicate that tyrosine phosphorylation contributes to the stability of Runx1 proteins and therefore may affect the higher transactivation potential of Runx1.

CBFβ protein was known to protect Runx1 from ubiquitination-mediated degradation and therefore stabilizes Runx1 (Huang G, 2001). We also co-transfected CBFβ together with WT, 5F and 5D in HEK293T cells and compared their Runx1 protein expressions (Figure 10). Similar to the effect of Src(E381G), Runx1 protein expressions of WT, 5F and 5D were higher than that of WT alone, and these elevated expressions were maintained throughout the 8 hours in the presence of CBFβ. This observation was not surprising given that CBFβ was known to increase the stability of Runx1 transcription factor (Huang G, 2001).

However, it might be possible that the binding kinetics between Runx1 and CBFβ would be altered in response to tyrosine phosphorylation. To further investigate the association of CBFβ to Runx1-WT, 5F or 5D, we took advantage of the availability of pEF1α vectors expressing FLAGBio-Runx1-WT, 5F or 5D. The incorporation of biotin molecules onto the Runx1 proteins enables protein pulled-down using streptavidin agarose. This method has been established and widely used for affinity purifications (Kim J, 2009). Another reason to choose streptavidin pull-down instead of Runx1 immunoprecipitation was due to the concern that Runx1 antibodies might interfere with Runx1- CBFβ interactions. When equal input amounts of FLAGBio-Runx1-WT, 5F or 5D total protein lysates were pull-down with strepavadin agarose, we noticed that CBFβ bonded at similar strength to each of these protein products (Figure 11B). Hence, the increased stability of non-phophorylated Runx1 in the presence of CBFβ was independent of the physical binding interaction between CBFβ and Runx1.

Protein stability is related to its rate of degradation at physiological condition. There are several molecular mechanisms that facilitate protein degradation, and one of them is ubiquitinylation-mediated proteosomal pathway (UPP). To detect the differential interaction of Runx1 and its mutant variants to ubiquitination, I co-transfected the HEK293T cells with WT, 5F and 5D along with HA-tagged ubiquitin, and carried out co-immunoprecipitation using Runx1 antibodies and with an equal input amount of total cell lysate (Figure 11A). 5F displayed the strongest association with HA-tagged ubiquitin, suggesting that 5F was a better target substrate for ubiquitins. In contrast, WT showed only moderate binding to HA-tagged ubiquitin, while 5D displayed minimal binding to HA-tagged ubiquitin (Figure 11A, right). In an attempt to detect binding of ubiquitin to

5D, we increased the loading volume to 6X higher, and obtained the autoradiograph at 3X longer exposure time. In spite of this, the co-immunoprecipitated HA-tagged ubiquitin bands were still barely visible (Figure 11A, right). It should be noted that the molecular weight of Runx1 protein is 55 kDa, which is similar to that of immunoglobulin heavy chain (IgH). Therefore the Runx1 bands were masked by the intense IgH bands on Western Blot.

Ubiquitins are transferred to the target proteins through the catalytic action of Ubiquitin Ligases. Anaphase Protein Complex (APC) is a major Ubiquitin Ligase III in the UPP degradation system, in which its catalytic functions are assisted by two adaptor proteins, Cdc20 and Cdh1. One early report demonstrated that Cdc20 promotes the degradation of phosphorylated Runx1 at Serine-303, whereas Cdh1 targets for total Runx1. (Biggs JR, 2006).

Finally, to determine the ability of Cdc20 or Cdh1 to mediate Runx1 degradation, we compared the protein expressions of Runx1-WT, 5F and 5D after co-transfecting them with either Cdc20 or Cdh1. Runx1-WT was resistant to Cdc20 but was gradually degraded in the presence of Cdh1 in a dose-dependent manner (Figure 12A). Reduction of Runx1-WT protein expression was already visible with 1 μg of Cdh1 compared to without Cdh1, and its expression dropped to 5-fold lower in 5 μg of Cdh1, as assessed with densitometry. Similar to WT, 5F and 5D did not respond to 1 μg or 5 μg of Cdc20. Furthermore, 5D appeared to become about 3 times more stable in both amounts of Cdc20 and the reason for this phenomenon is unclear. Protein expression of 5F was 2/3 lower at 1 μg or was 1/3 lower at 5 μg of Cdh1. Reduction of 5D protein expression was only observed in 5 μg of Cdh1, which was 2/3 lower compared to without Cdh1 (Figure

12B). The observations of WT, 5F and 5D in Cdh1 suggest that the non-phosphorylated form of Runx1 is the preferred substrate of Cdh1 in the UPP system. It also raises the possibility that the markedly increased stability of 5D is partially due to protection from Cdc20 or Cdh1-mediated degradation. Further evaluations are needed to elucidate the underlying molecular mechanisms, the structural interactions between Runx1 and Cdh1, and the consequences of these interactions.

3.5 Exogenous Src activates Runx1 and C/EBPα expression in SHP2-knock down 32Dcl3 myeloid progenitors

As we gathered more evidences supporting the role of Runx1 tyrosine phosphorylation in C/EBPα induction, we wanted to further examine whether SHP2 tyrosine phosphatase has an effect on Runx1 and C/EBPα productions. A recent publication showed that blocking of SHP2 activity in 32Dcl3 myeloid progenitor cells suppressed its endogenous expression of C/EBPα in response to G-CSF (Zhang L, 2011). The 32Dcl3 myeloid progenitor cells established in this report, that stably expressing RNA interference of SHP2 (77-10) were utilized in this investigation. A second retroviral vector, pBabeNeo carrying the cDNA of Src(E381G) upstream of ER(T) element was transduced into 32Dcl3-77-10. The pooled double transductants were cultured for 24 hours, and for another 24 hours with 4HT addition. Equal amounts of the total protein lysates were used to detect the protein expression of Runx1 and C/EBPα. In both 32Dcl3-77-10, with and without the pBabePuro retroviral vector, Runx1 expression level was moderate. Moderate elevation of Runx1 expression could be observed after Src was

introduced compared to the pBabeNeo control (approximately 1.3-fold), implying that Src was acting downstream in epistasis of SHP2. Consistent with the effect of cooperative interaction between Src and Runx1, the diminished expression of C/EBPα in 32Dcl3-77-10 was at least partly rescued in the presence of exogenous Src, in which its protein expression was about 2.2-fold higher (Figure 13). This result lends additional support to the idea that SHP2 mediates Src to activate Runx1 and that Runx1 activates C/EBPα, as illustrated in Figure 1.

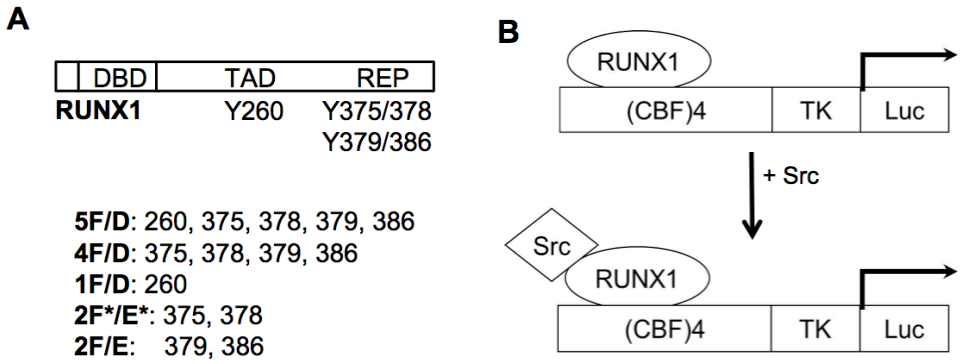

Figure 2: Schematic diagrams of murine Runx1b and Runx1 luciferase reporter construct. A. Diagram describing the locations of DNA-binding runt domain (DBD), transactivation domain (TAD) and repression domain (REP), with sites of tyrosine modifications mapped. The 5 tyrosine residues modified in this study are indicated (top), and the combinations of altered tyrosine residues in Runx1 mutant variants are listed (below). B. Runx1 luciferase reporter gene construct consists of 4 binding sites of Runx1 at the 5'-terminal [$(CBF)_4$] and the luciferase reporter gene at the 3'-terminal (Luc) preceded by the minimal thymidine kinase promoter (TK). Runx1 protein binds to the $(CBF)_4$ region thus activating the transcription of luciferase gene via TK promoter. SRC interacts with Runx1 to affect differential transcription levels of luciferase enzymes. Interaction of Src and Runx1 occurred via protein-protein interaction.

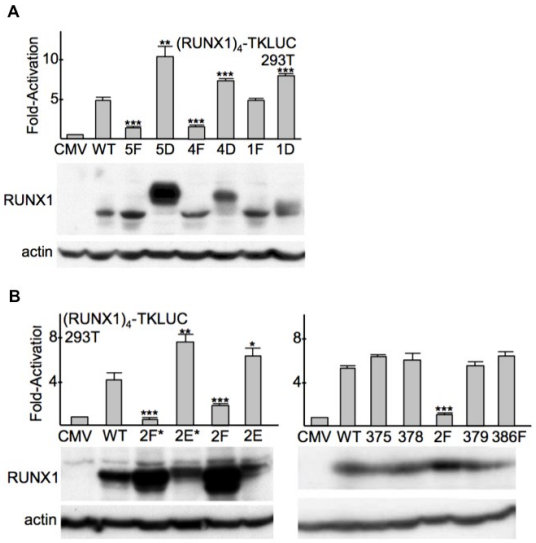

Figure 3: Transactivation and expression of Runx1 and its tyrosine mutant variants. A. 1×10^5 HEK293T cells plated on a 24 well plate were transiently co-transfected with 150 ng of (CBF)4-TK-Luc, 0.8 ng of CMV-β-Gal and 5 ng of CMV vectors expressing wild-type Runx1 (WT) or its respective tyrosine mutant variants or vector only. The luciferase and beta-galactosidase activity was measured after 48 hours. Fold activation was expressed as the ratio of luciferase and beta-galactosidase activity after normalizing to that of CMV vector alone controls, which was set to value 1. Mean and SE were obtained from three determinations. *-$p<0.05$, **-$p<0.01$, ***-$p<0.001$(top). 4×10^5 HEK293T cells plated on a 6 well plate were transfected by 1 μg of CMV vectors expressing wild-type Runx1 (WT) or its respective tyrosine mutant variants or vector only. Equal quantities of total protein lysates were separated on 8% SDS-PAGE after 48 hours, followed by the assessment of Runx1 and β-actin expressions by Western Blot (bottom). B. Luciferase reporter assay (top) and protein expression analysis (bottom) was repeated as described in A, using CMV vectors expressing WT or its respective tyrosine mutants (left) or pEF1α vectors expressing FLAGBio-tagged WT or its single tyrosine mutants (right).

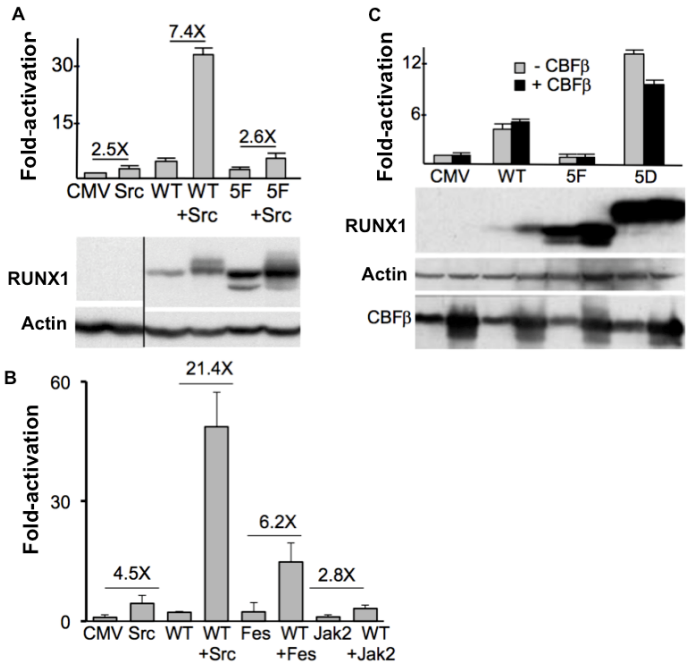

Figure 4: Transactivation and expression of Runx1 and its tyrosine mutant variants in the presence of protein tyrosine kinases and CBFβ. For all fold-activation shown for luciferase reporter assay, mean and SE were obtained from 3 determinations. A. Luciferase reporter assay (top) and protein expression analysis (bottom) were performed as described in Figure 2, with or without 40 ng of pCEFL-Src(E381G). The fold effects of Src expression on the average activity of CMV vector, -WT or -5F alone are also indicated. B. Luciferase reporter assay was carried out as described in A, using 40 ng of pCEFL-Src(E381G), Fes or MIG-Jak(V614F). Fold changes between CMV-WT without versus with protein tyrosine kinase are indicated. C. Luciferase reporter assay (top) was repeated as described in A, with or without 1 μg of CMV-CBFβ. Relative fold activations are shown. Equal quantities of total protein lysates were separated on 8% SDS-PAGE after 48 hours, followed by the assessment of Runx1, β-actin and CBFβ expressions by Western Blot (bottom).

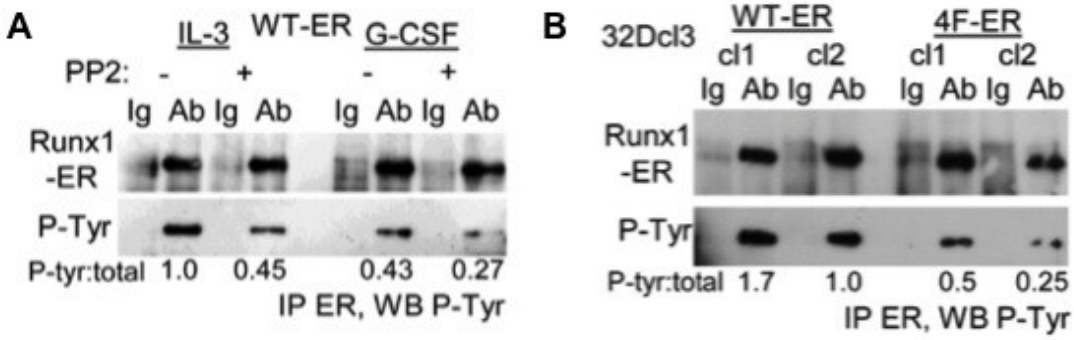

Figure 5: Tyrosine phosphorylation of exogenous Runx1. A. Equal numbers of 32Dcl3 cells stably expressing WT-ER(T) were grown in either mIL3 or 24 hours after transfer to G-CSF cytokine, with and without addition of 20 μM PP2 or DMSO control for 8 hours. Total protein lysate was subjected to ERα immunoprecipitation, followed by 10% SDS-PAGE and Western Blot to assess tyrosine phosphorylation levels. The ratios of phosphorylated to total WT-ER(T) are shown. B. Equal quantities of total protein lysates extracted from 2 subclones each of 32Dcl3 stable cells expressing WT-ER(T) and 4F-ER(T) were subjected to mouse ERα immunoprecipitation. The immunoprecipitant was resolved in 10% SDS-PAGE and was transferred to PVDF membrane followed by immunoblotting using phospho-tyrosine and ERα antibodies. The ratios of phosphorylated to total WT-ER(T) and 4F-ER(T) are indicated.

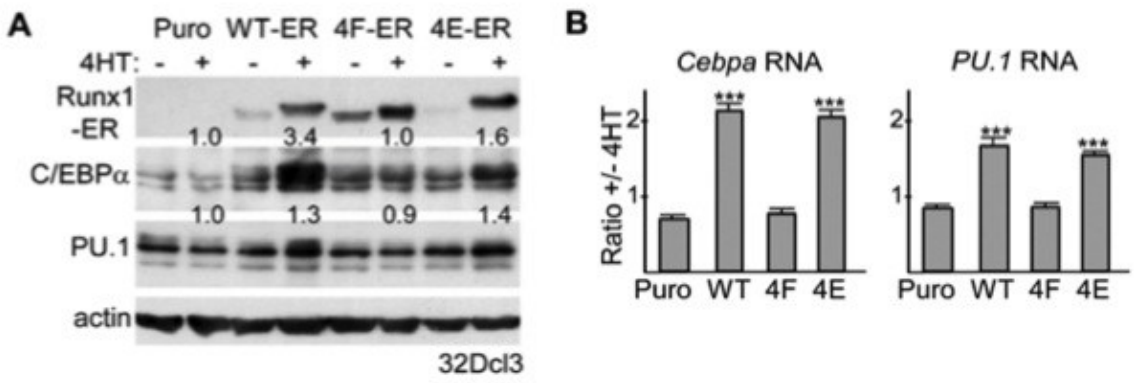

Figure 6: Protein and mRNA expression of Runx1 molecular targets. A. Equal quantities of total protein lysates equivalent to 2×10^5 of 32Dcl3 cells that stably expressed pBabePuro vector, Runx1-WT-ER(T), 4F-ER(T) or 4E-ER(T) cultured in the absence or presence of 4-hydroxytamoxifen (4HT) for 24 hours were separated in 8% SDS-PAGE and were subjected to Western Blot using ERα, C/EBPα, PU.1 or β-actin antibodies. Fold-increase induced by 4HT, normalized to β-actin is measured by densitometry. B. Total RNA from the same cell cultures as described in A were subjected to mRNA expression analysis. After reverse-transcription, 5 ng of cDNA was used for quantitative PCR to assess the expression level of *Cebpa* and *PU.1*, with Actin as internal control. The relative expressions of *Cebpa* or PU.1 before and after 4HT induction were normalized to pBabePuro vector, in which the value is set to 1. Mean and SE were obtained from three determinations. ***-$p < 0.001$.

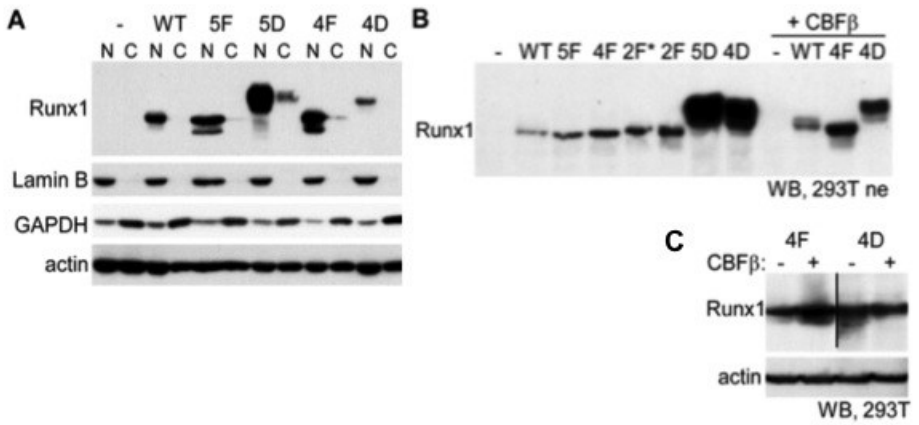

Figure 7: Localization and expression of Runx1 in nuclear compartment. A. 3×10^6 HEK293T cells plated on a 10 cm culture dish were transiently co-transfected with 6 μg of CMV vectors expressing wild-type Runx1 (WT) or its respective tyrosine mutant variants or vector only. Nuclear and cytoplasmic extracts from equal cell numbers were harvested after 48 hours and were resolved in 8% SDS-PAGE, followed by immunoblotting using Runx1, Lamin B, GAPDH or b-actin antibodies. B. Similar transient transfection was repeated as described in A, using 6 μg each of CMV vectors expressing Runx1-WT, 5F, 4F, 2F*, 2F, 5D, 4D or 3 μg of each of WT, 4F, 4D with or without 3 μg of CMV-CBFβ. Runx1 protein level is assessed from the nuclear extract prepared 48 hours later using Western Blot. C. 4×10^5 HEK293T cells plated on a 6 well culture dish were transiently co-transfected with 1 μg of CMV vectors expressing 4F or 4D with or without 3 μg of CMV-CBFβ. Total protein lysates were harvested after 48 hours, and subjected to Western Blot using Runx1 and β-actin antibodies.

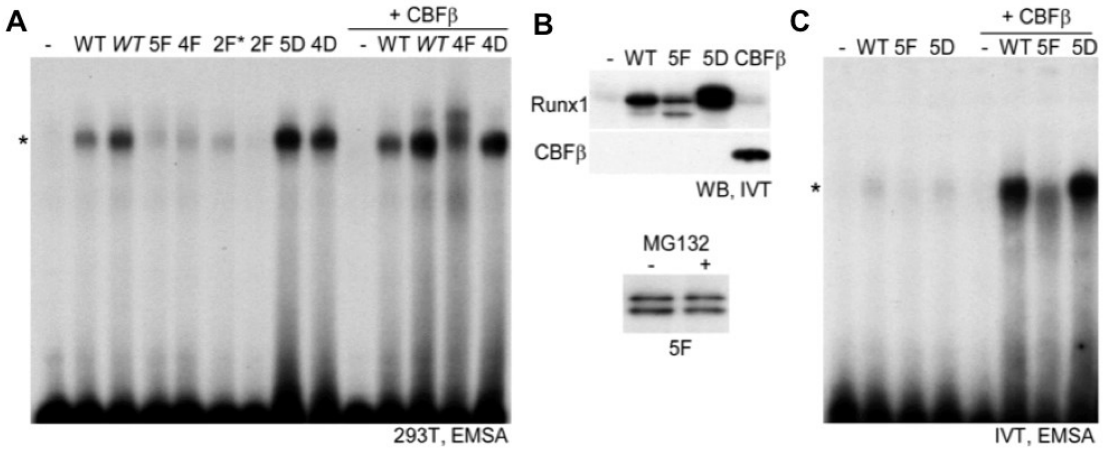

Figure 8: Tyrosine phosphorylation of Runx1 mediates DNA binding. A. 6 μg of nuclear extracts, individually prepared from transiently transfected HEK293T cells as described in Figure 6B, were subjected to electrophoretic mobility gel shift analysis (EMSA). Prior to EMSA, the nuclear extracts were mixed with a radio-labeled double-stranded DNA containing a Runx1 binding consensus sequence found at the myeloperoxidase promoter. Lanes with 12 μg of WT or WT with CBFβ were included, labeled as *WT*. "*" marks the location of the specific gel shift band. E. *In vitro* transcription-translational (IVT) products were generated from 1 μg of linearized CMV vectors expressing Runx1-WT, 5F, 5D or CMV vector expressing CBFβ. Equal volumes of IVT products including the negative control with reticulocyte lysate alone were then separated in 8% SDS-PAGE and were subjected to Western Blot to access the expression of Runx1 and CBFβ (top). Similarly, IVT products of 5F with and without treatment of 25 μM MG132 protease inhibitor were analyzed (bottom). C. After equalizing IVT products of WT, 5F or 5D using densitometry, equal amounts of each sample, with and without 7 μl CBFβ, were subjected to EMSA.

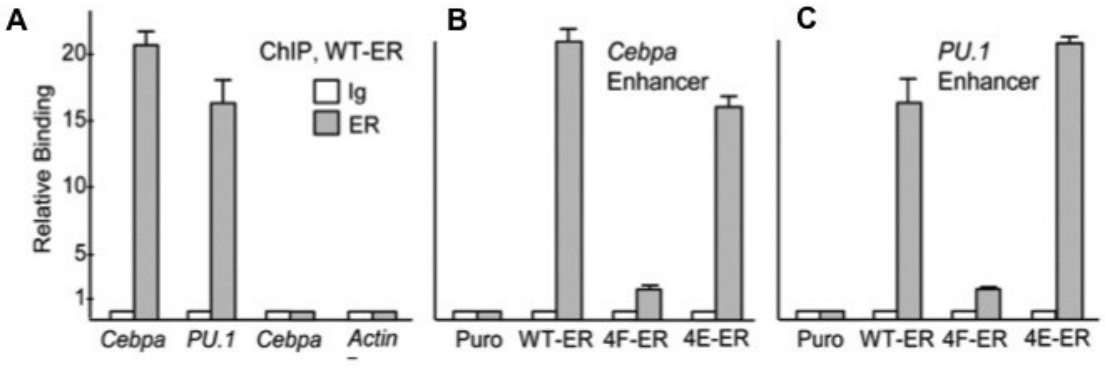

Figure 9: Tyrosine phosphorylation of Runx1 promotes binding to endogenous promoters and enhancers assessed with Chromatin Immunoprecipitation (ChIP). A. 1×10^6 32Dcl3 cells stably expressing Runx1-WT-ER(T), 4F-ER(T), 4E-ER(T) or vector only (Puro) were cultured for 24 hours after addition of 4HT, and were subjected to ChIP assay using 2 mg of IgG (Ig) or rabbit anti-ERa (ER) antibodies, followed by quantitative PCR using primer pairs targeted to +37 kb *Cebpa* enhancer, -14 kb *PU.1* enhancer, -2.5 kb *Cebpa* promoter, or β-actin promoter. The relative binding of ER to each of these DNA elements in WT-ER(T) compared to that of Ig is shown. Mean and SE were obtained from three determinations. ChIP was performed as described in A to evaluate the relative binding of +37 kb *Cebpa* enhancer (B) or -14 kb *PU.1* enhancer (C) between WT-ER(T), 4F-ER(T), 4E-ER(T) or vector only (Puro).

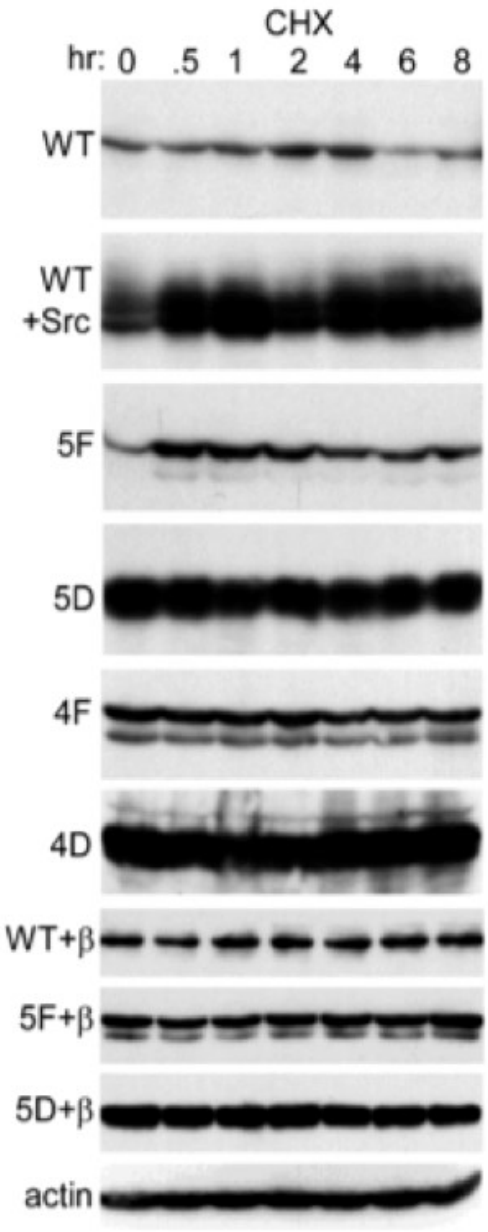

Figure 10: Src or tyrosine phosphorylation of Runx1 enhances the stability of Runx1 protein. 4×10^5 HEK293T cells plated on a 6 well culture dish were either transiently transfected with 1 μg of CMV vectors individually expressing WT, 5F, 5D, 4F or 4D, or co-transfected with 0.25 μg of CMV-WT + 1 μg pCEFL-Src or 1 μg of CMV-WT, 5F, 5D + 1 μg CMV-CBFβ for 48 hours. Cycloheximide was added into the transfected cells 0.5 – 8 hours before total protein lysates were prepared from equal number of cells. The expressions of Runx1 isoforms were assessed by 8% SDS-PAGE and immunoblotting with Runx1 antibodies or β-actin as a loading control. Data presented here were the representative of separate experiments of 2-3 repetitions.

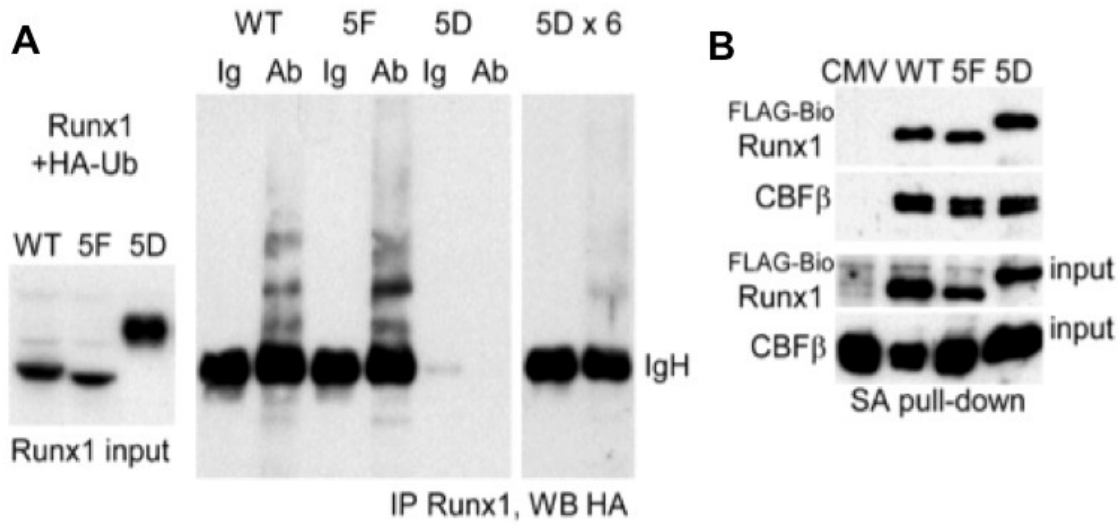

Figure 11: Tyrosine phosphorylation protects Runx1 from ubiquitinylation but does not affect the binding affinity to CBFβ. A. 4×10^5 HEK293T cells plated on a 10cm culture dish were transiently co-transfected with either 2 μg of CMV-WT, 1 μg of -5F, or 0.4 μg of -5D + 3 μg of pCMV-HA-ubiquitin (HA-Ub). Total protein lysates prepared from these transfected cells 48 hours later were subjected to Western Blot (left) and were also immunoprecipitated with IgG (Ig) or mouse-anti-HA antibodies (HA) (middle and right). Amount of protein lysate loaded for 5D was adjusted to 1/12 (left and middle) or 1/2 (right) that of WT and 5F. B. 4×10^5 HEK293T cells plated on a 10cm culture dish were transiently co-transfected with 1.5 μg of CMV-CBFβ + 1.5 μg of pEF1α-birA + 3 μg of pEF1α-FLAGBio-WT, 5F or 5D. Total protein lysates were harvested after 48 hours, and equal amount of protein lysates were subjected to immunoprecipitation using streptavidin agarose, followed by 12% SDS-PAGE and Western Blotting with Runx1 and CBFβ antibodies. Input amount was 2.5% of the total protein lysate used for immunoprecipitation.

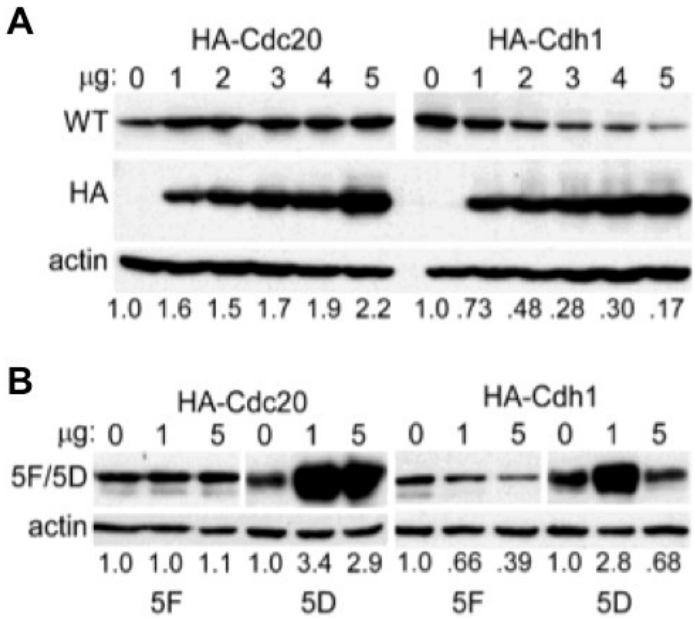

Figure 12: Non-tyrosine phosphorylated Runx1 is a specific substrate of Cdh1, the adaptor protein of APC ubiquitin ligase III, in a dose-dependent manner. A. 4×10^5 HEK293T cells plated on a 6 well culture dish were transiently co-transfected with 2 μg of CMV-WT and 1 to 5 μg of pCMV-HA-Cdc20 or Cdh1 expression vectors. Equal amounts of total protein lysates were resolved in 8% SDS-PAGE followed by the assessment of Runx1, HA and β-actin expressions by Western Blot. The relative intensities of the Runx1 bands after normalizing to β-actin bands are shown below each lane. Data shown is the representative experiment from 4 replicates. B. Similar transient co-transfections were carried out as described in A using 1 μg of CMV-5F or 0.5 μg of CMV-5D and 1 or 5 μg of pCMV-HA-Cdc20 or Cdh1 expression vectors, followed by analysis of Runx1, HA and β-actin expressions using Western Blot. The relative intensities of the Runx1 bands after normalizing to β-actin bands are shown below each lane.

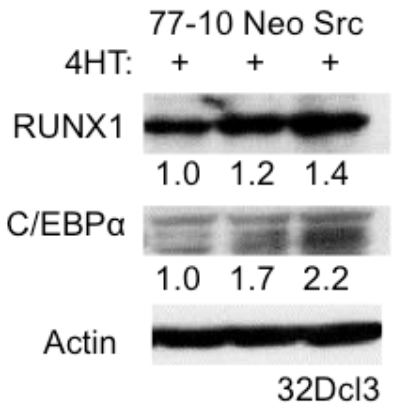

Figure 13: Exogenous Src induced Runx1 and C/EBPα expressions in SHP2 deficient 32Dcl3 myeloid progenitor. 32Dcl3 cells harboring a pBabePuro retroviral vector that constitutively repressing the SHP2 mRNA expression were transduced again with pBabeNeo retroviral vector, or pBabeNeo carrying the cDNA of Src-ER(T). Equal quantities of total protein lysates equivalent to 2×10^5 of 32Dcl3 stable double transductants cultured in the presence of 4-hydroxytamoxifen (4HT) for 24 hours were separated in 8% SDS-PAGE and were subjected to Western Blot using Runx1, C/EBPα, or β-actin antibodies.

CHAPTER 4: DISCUSSION AND CONCLUSION

In this study, we have demonstrated that tyrosine phosphorylation of Runx1 at residues Y260, Y375, Y378, Y379 and Y386 by Src or potentially other tyrosine kinases increases its transactivation potency and DNA binding ability to its consensus sequences, resulting in the induction of the *C/ebpα* and *PU.1* mRNA and protein expression. The study also showed that tyrosine phosphorylated Runx1 is more stable due to its longer half-life, binds less to ubiquitin and is protected from the Cdh1-mediated ubiquitin proteosomal pathway.

Tyrosine phosphorylation of Runx1 has only been recently reported in megakaryocytes and thymocytes (Huang H, 2012). However, Runx1 transactivation upon tyrosine phosphorylation in these and all other blood lineages remained to be investigated. Src phosphorylates Runx1 at Y260, and four tyrosine residues at the C-terminal, Y375, Y378, Y379, and Y386. In the luciferase reporter assay on HEK293T cells, we demonstrated that Src strongly synergized with Runx1 to increase its transactivation. Mutation of the five tyrosine residues to phenylalanine (Runx1-5F) to block phosphorylation obviated Runx1 transactivity. Interestingly, mutations of the C-terminal cluster (Runx1-4F, 2F or 2F*) to phenylalanine were sufficient to abolish Runx1 transactivity but not the mutation of individual tyrosine to phenylalanine. This observation shows that either Y375/Y378 or Y379/Y386 could significantly affect the transactivation potency of Runx1. It has been shown that a combinatorial phosphorylation effect from multiple tyrosines on G-CSF receptor contributes to proliferation, differentiation and survival signals in 32Dcl3 myeloid progenitors (Ward AC, 1999). Combination of different multiple phosphorylation sites on Runx1 led to different levels

of hematopoietic development (Yoshimi M, 2012). Therefore, mapping the exact combination of tyrosine residues modified by Src to influence Runx1 transactivity could be important step to elucidate the signaling pathways in myeloid progenitors.

In addition to Src, several other tyrosine kinases are activated in G-CSF signaling, contributing to the maturation of myeloid progenitors (Miranda MB, 2007). Although we found that the level of tyrosine phosphorylation of the exogenous Runx1-ER expressed in 32Dcl3 cells was significantly reduced upon treatment with PP2, a non-selective Src kinase inhibitor, we could not be certain that Src was the most relevant tyrosine kinase to regulate the optimal Runx1 transactivation. Therefore, we further evaluated the ability of two other tyrosine kinases, Fes and Jak2 that are abundantly expressed in myeloid lineages, to affect Runx1 transactivation. We showed that both activated Fes and activated Jak2 failed to increase the transactivity of Runx1 in the luciferase reporter assay, implying that Runx1 is not their substrate of choice. This observation was unexpected, as Fes and Jak2 are expressed during myeloid differentiation and their activation upregulate Runx1 targets, C/EBPα and PU.1 (Kim J, 2004; Irino T, 2011). It is possible that Fes and Jak2 may mediate other functions of Runx1 aside from transactivity in granulopoiesis, as precedent studies have reported that members of the Src family kinases could affect both proliferation and differentiation of myeloid progenitors (Mermel CH, 2006). In the future, we could expand our investigation to other hematopoietic cell-specific tyrosine kinases that may display overlapping functions with Src. For example, Jak3 expression increased during granulopoiesis in 32Dcl3 cells, and Hck is highly expressed in granulocytes (Rane SG, 2002; English BK, 1996), and therefore may regulate Runx1 transactivity.

In order to discover the biological effects of phosphorylated Runx1 in a myeloid setting, 32Dcl3 mouse progenitor cell line were used as the study model. 32Dcl3 are bipotential, non-tumorigenic myeloid progenitor cells derived from the murine bone marrow, and are strictly interleukin-3 (IL3) dependent for cell proliferation and survival (Valtieri M, 1987). Replacement of G-CSF into the growth media triggers its terminal differentiation into functionally mature granulocytes (Guchhait P, 2003).

The exogenous Runx1 (WT-ER) and its phospho-mimetic C-terminal tyrosine mutant (4E-ER) induced the transcription of endogenous *Cebpα* and *PU.1* and their protein production in 32Dcl3 myeloid cells. Mutation to phenylalanine (4F-ER) obviated this effect. Diminished C/EBPα transcription, resulting in inhibition of myeloid differentiation, may contribute to leukemic transformation in AML cases associated with decreased RUNX1 activity (Guo H, 2012). To provide further support on the effect of phosphorylation that is controlled by Src and SHP2, protein expressions of endogenous Runx1 and C/EBPα were down-regulated in SHP2 knock-down 32Dcl3 cells but was partially rescued after induction of exogenous Src. This observation is consistent with the previous report by Zhang *et al.*, in which the authors demonstrated that exogenous Runx1 (WT-ER) could moderately restored the diminished C/EBPα expression in the SHP2 knock-down 32Dcl3 cells (Zhang L, 2011). Therefore, reduced tyrosine phosphorylation in Runx1, either due to diminished Src function, enhanced SHP2 activity or loss of tyrosine residues on Runx1, could potentially reduce Runx1 activity, thereby leading to down-regulation of C/EBPα, blocking granulopoiesis and contributing to myeloid transformation.

Of note, three phopho-mimetic mutants, 4E, 2E, and 2E* were generated by replacing tyrosine (Y) residues with glutamate (E) residues rather than aspartate (D) residues. Glutamate has an extra methyl group and thus could be a better representative of a bulky tyrosine residue.

Hong *et al.* demonstrated that endogenous Runx1 binds to consensus sites at the *Cebpa* promoter and the +37kb enhancer region (Guo H, 2012). We further determined that the exogenous Runx1-4E-ER as well as WT-ER also bound to endogenous *Cebpa* enhancer in 32Dcl3 progenitor cells, but not 4F-ER. This was the first demonstration that tyrosine phosphorylation of Runx1 is required to mediate the physical interaction of Runx1 protein to the *Cebpa* and *Pu.1* enhancers.

We have also demonstrated that, in general, Runx1-4D and -5D bound strongly to the consensus sequences as assessed with EMSA. Conversely, Runx1-4F and 5F exhibited reduced DNA binding, which also reflected the loss of binding of Runx1 to enhancers of endogenous *Cebpa* and *Pu.1* in the absence of tyrosine phosphorylation. Whether Runx1 tyrosine phosphorylation facilitates its binding to enhancers of other target genes remains to be determined.

Runx1-5D and 4D phospho-null tyrosine mutants repeatedly displayed higher protein expressions compared to WT, 5F and 4F. Upon treatment with cycloheximide, elevated protein expression of Runx1-5D and 4D could sustain for 8 hours. This observation was consistent with the earlier report by Huang H *et al.*, and provided additional support that tyrosine phosphorylation enhances Runx1 stability (Neel BG, 2012; Huang H, 2012).

Apart from displaying a longer half-life, Runx1-5D also showed weak binding to ubiquitin and was resistant to Cdh1-mediated degradation compared with Runx1-WT. Conversely, Runx1-5F showed a shorter half-life in cycloheximide, bound strongly to ubiquitin, and was sensitive to Cdh1-mediated degradation. Interestingly, Cdc20 did not mediate the degradation of Runx1-WT and -5F, and could even stabilized Runx1-5D. Both Cdh1 and Cdc20 adaptor proteins are responsible for the substrate recognition and activating mechanism for anaphase-promoting complex (APC), and they can interact with specific substrates either independent of or dependent on APC (Pfleger CM, 2001). Zhang's group demonstrated that Cdc20 preferentially targeted phosphorylated Runx1 whereas Cdh1 could promote the degradation of total Runx1, which consists of both phosphorylated and non-phosphorylated Runx1 (Biggs JR, 2006). Our results showed the opposite; only Runx1-5F was targeted by Cdh1 for degradation, and all forms of Runx1 was protected from Cdc20-mediated degradation. Hence, the differential interactions of tyrosine phosphorylated Runx1 with Cdh1 and Cdc20 could potentially reflect their substrate preferences.

Transactivation of transcription factor is closely correlated with proteosomal degradation. Transcription factor with higher transactivity is degraded by proteasome at a faster rate (Molinari E, 1999). Our observation, again, contradicts this previous report. Runx1-5D showed the highest transactivity, but it was resistant to Cdh1 and Cdc20-mediated proteosomal degradation. In order to further characterize the effect of Cdh1 on Runx1-WT, -5F and -5D, the physical association and the binding motifs of Cdh1 on Runx1 need to be identified. In addition, determining whether Cdh1 also mediates

degradation of Runx1-4F ad -4D will elucidate the relationship between tyrosine phosphorylation, transcriptional activity, and proteosomal degradation.

Luciferase reporter assay, EMSA, and cycloheximide assay were repeated with the addition of exogenous CBFβ, which was produced either through co-transfection in 293T cells or generated separately using IVT. As discussed in Chapter 1, CBFβ increases the DNA binding affinity and stability of Runx1 (Gu T-L, 2000). We observed a marked increased DNA binding of Runx1, and its tyrosine mutants to consensus sequence in the presence of CBFβ, and both Runx1-WT and -5F protein expression were stabilized across 8 hours after cycloheximide treatment. However, the reduced transactivation of Runx1-5F was not rescued by the addition of CBFβ. Furthermore, the binding affinity of Runx1-WT, -5F and -5D to CBFβ was at similar levels as assessed by co-immunoprecipitation assay. These results indicated that phosphorylation at Runx1 C-terminal tyrosine cluster did not modify its binding interaction with CBFβ nor limit the access of CBFβ to its Runt DBD, although their association markedly increased the DNA binding affinity. In addition, Runx1-5D, alone, displayed minimal DNA binding in EMSA, implying that Runx1 tyrosine phosphorylation was insufficient to mimic or replace the function of CBFβ. It is possible that the allosteric changes at the Runx1 C-terminal after tyrosine phosphorylation do not affect Runx1-CBFβ heterodimerization.

Runx1 molecule can serve as a scaffolding bridge to many co-regulators. Multiple cellular molecules form multimeric complexes with the different domains on Runx1 to modulate cell-specific gene expressions after exposure to extracellular stimuli. C-terminal domain of Runx1 is important in the interaction with the N-terminal of p300 to increase DNA binding (Yamaguchi Y, 2004). Their association along with CREB binding protein

(CBP) contributed to the induction of granulocyte differentiation in response to G-CSF signaling (Kitabayashi I, 1998). Endogenous HDAC1 and HDAC3 were also shown to bind to C-terminal of endogenous Runx1 to markedly reduce its transactivity, and this interaction was impaired after Cdk-mediated S424 phosphorylation (Hong G, 2011). RUNX1-RUNX1T1 recruits HDACs to repress gene expressions for granulocyte differentiation (Hayashi Y, 2000, Gelmetti V, 1998). Therefore, it had been proposed that HDAC inhibitors or other epigenetic therapeutic approaches could be beneficial to reverse the granulocytic differentiation block caused by Runx1 mutation (Gozzini A, 2003; Klisovic MI, 2003).

Tyrosine phosphorylation of Runx1 is also one of the tightly orchestrated PTM that affect interactions between Runx1 and various molecular partners, and can further cross-talk with other PTM in the same protein to fine-tune signaling pathways (Hunter T, 2014). Tyrosine modifications on Runx1 can reduce its interactions with transcriptional co-repressors to complement its ability to activate its transactivation potential (Lam K, 2012). Based on the above observations, we could predict that the mutation of the C-terminal tyrosine cluster (Y375, Y378, Y379 and Y386) to phenylalanine or to aspartate may influence the recruitment of HATs and HDACs. The identification of physical associations between HDACS and Runx1 tyrosine mutants was not included here, but Guo H continued the effort beyond this study, and the observation was reported recently (Leong WY, 2016). We demonstrated that mutation of C-terminal tyrosine cluster to aspartate greatly reduced its association to HDAC1 and HDAC3, and the mutation to phenylalanine reversed this effect. Consistent with the finding of Hong *et al*., this result

highlighted the functional importance of C-terminal domain of Runx1 in terms of interaction with epigenetic factors.

Full length Runx1 exhibits auto-inhibition, partly due to its 3-dimensional conformation determined by circular dichroism spectroscopy. (Tang Y-Y, 2000). C-terminal Runt DNA binding domain has also been shown to inhibit DNA binding or heterodimerization of CBFβ (Gu T-L, 2000). Therefore, it is possible that tyrosine phosphorylation or other PTM of the Runx1 C-terminal domain is necessary to relieve inhibitory effects of its transcriptional activity. Besides HATs and HDAC binding, Runx1 interacted with PMRT1/4/6, mSin3A, SUV39H1, CHIP and many other co-regulators (Chuang LSH, 2013; Shang Y, 2009). Therefore, it will be important in future studies to investigate the effect of Runx1 tyrosine modifications on the interaction with HATs and these additional co-activators and co-repressors.

Tyrosine phosphorylation represents one of many PTM occurring in Runx1, but its biological effects on myeloid cell differentiation are significant (Neel BG; 2012). Given that tyrosine phosphorylated Runx1 showed higher transactivation, increased DNA binding affinity, and could induce endogenous C/EBPα and PU.1 expressions in 32Dcl3 myeloid progenitors, we might predict that Runx1-5D would promote granulocyte differentiation. However, Cantor's group described that exogenous Runx1-5D inhibits the development of CD8+ T cells in Runx1-deleted marrow cells and blocked the maturation of megakaryocytes in the erythroid cell line. During megakaryopoiesis, Runx1 suppressed KLF1 promoter to inhibit erythropoiesis (Kuvardina ON, 2015). Hence, it may be possible that Runx1-5D transactivation can simultaneously silence the genes required for thymocyte development and megakaryopoiesis while activating gene

expressions specific for maturation of granulocytes or other blood lineages. The effect of tyrosine phosphorylated Runx1 could be lineage dependent. Therefore, it is important to determine the effect of Runx1 tyrosine phosphorylation in granulopoiesis. Of note, this study was conducted by Guo H, in which exogenous Runx1-4E rescued the granulocyte formation in the lineage-negative bone marrow cells obtained from Runx1-deficient mice (Leong WY, 2016).

Combining all these observations, we conclude that Src phosphorylation of Runx1 at tyrosine residues increases its transactivity potency and DNA binding ability independent of its cofactor CBFβ, improves its stability by abolishing ubiquitination-mediated proteosomal degradation, thereby inducing *Cebpα* and *PU.1* mRNA and protein expression, which are the biological events that promote granulocyte differentiation. The findings that tyrosine phosphorylation of Runx1 by Src precedes the induction of C/EBPα also provide strong evidences to our proposed model of G-CSF signaling pathway (Figure 1), in which Src mediates the activation of Runx1 and that Runx1 activates C/EBPα in granulopoiesis.

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Abbreviations:

4HT	4-hydroxytamoxifen
AML	Acute myeloid leukemia
APC	Anaphase promoting complex
CBF	CREB binding protein
CBFβ	Core binding factor β
CBFβ-SMMHC	Core binding factor β-smooth muscle myosin heavy chain
Cdc20	Cell division cycle 20
Cdk	Cyclin-dependent kinase
C/EBPα	CCAAT/enhancer binding protein alpha
ChIP	Chromatin immunoprecipitation
CHX	Cycloheximide
Co-IP	Co-Immunoprecipitation
CREB	cAMP response element-binding protein
DBD	DNA binding domain
DMEM	Dulbecco's modified Eagle medium

DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
ER	Estradiol receptor
ERK	Extracellular signal-regulated kinases
FAB	French-American-British
FBS	Fetal Bovine Serum
FLT3	Fms-related tyrosine kinase 3
FLT3-ITD	FLT3-internal tandem duplication
FPD	Familial platelet disorder
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-monocyte colony stimulating factor
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HIPK1/2	Homeodomain-interacting protein kinase 1/2
HSC	Hematopoietic stem cell
IMDM	Iscove's modified Dulbecco's medium

IL3	Interleukin-3
IL6	Interleukin-6
IVT	In vitro transcription-translation
JAK2	Just another kinase 2
KLF1	Krueppel-like factor 1
LOH	Loss of heterozygosity
MDS	Myelodysplastic syndromes
MPO	Myeloperoxidase
MPS	Myeloproliferative syndromes
NaCl	Sodium chloride
NaF	Sodium fluoride
Na_3VO_4	Sodium orthovanadate
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PP2	Pyrolopyrimidine 2
PRMT1	Protein arginine methyltransferase 1

PTM	Post-translational modifications
PVDF	Polyvinylidene fluoride
SCF	Stem cell factor
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide agarose gel electrophoresis
SFK	Src family kinases
SHP2	Src homology tyrosine phosphatase
STAT3	Signal transduction and activator of transcription 3
Runx1	Runt-related transcription factor 1
REP	Repression domain
TAD	Transactivation domain
WT	Wild-type

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Molecular and cellular biologist with 15 years of research experience in cancer biology, microbiology, immunology and hematopoiesis. Adept at high-content screening, bioassay design and execution integrating nanotechnology and robotics; proficient in handling infectious clinical samples. Led, supervised and managed cross-disciplinary team-based projects at publicly funded institutes and a private start-up biotechnology company. Inventor of high throughput miniaturized cellular-based assays in a US patent application. Highly interested in fundamental or translational scientific research on the diagnosis and treatments of cancers or infectious diseases.

EDUCATION

2011-2016 *(Graduation: May 2016)*
Ph.D. candidate, Johns Hopkins University School of Medicine
Thesis: Transcription factor RUNX1 phosphorylation by SRC favors granulopoiesis
Thesis mentor: Alan Friedman, M.D.

2002-2004
Master of Science (M.Sc.), National University for Singapore
Thesis: Molecular characterization of toxins in *Clostridium difficile*
Thesis mentor: Keang-Peng Song, Ph.D.

1998-2001
Bachelor of Science (B.Sc.), University of Malaya
Thesis: Single-stranded Conformation Polymorphism Analysis of Variability of the *rpoS* sequence in the environmental and clinical isolates of *Salmonella typhi*
Thesis mentors: Kwai-Lin Thong, Ph.D. & Zulqarnain Mohamed, Ph.D.

PROFESSIONAL EXPERIENCE

Research and Leadership Experience

Aug 2012-Present
Graduate Researcher (Mentor: Alan Friedman, M.D.)
Johns Hopkins Uni. School of Medicine, Pathobiology Graduate Program
- Investigate the role of tyrosine phosphorylation of the RUNX1 transcription factor by Src kinases in the control of granulocyte differentiation using biochemical and cell-based functional assays.

Sep 2010-July 2011
Senior Research Officer (Advisor: Christopher Wong, Ph.D.)
Genome Institute of Singapore (Singapore)
- Led and supervised a team of 4 in a large scale siRNA library screening set-up to carry out the whole genome human gene knockdown functional assay on different cancer cell lines using the High Content Screening platform facilitated by an automation handling system; the entire system then became available to all users at the institute.
- Validated the Proseek IP-10 Validation Kit from Olink Biosciences, who then published the result on their website.

Aug 2009-Aug 2010
Application Manager in Biology (secondment)
Curiox Biosystems Pte. Ltd. (Singapore)
- Led a team of 3 in the evaluation of the performance of the DropArray™ platform using miniaturized high content cell-based phenotypic assays on various cell lines.

- Led the beta-testing of DropArray™ platform at site of client (Sanofi-Aventis, Bridgewater campus NJ, USA).
- Established the protocols for miniaturized high content cell-based assays using DropArray™ platform, which were included in a US patent application submitted in 2013 (Application No. 61/711725).

Sep 2004-July 2009 **Senior Research Officer** (May 2008-July 2009), **Research Assistant** (Sep 2004-Apr 2008)
(Advisors: Christopher Wong, Ph.D., Lance D. Miller Ph.D. & Kartiki Desai, Ph.D.)
Genome Institute of Singapore (Singapore)

- Validated the breast cancer biomarkers identified from a comprehensive microarray assay using siRNA knock-down functional test and immunohistochemistry staining.
- Optimized the protocol for NimbleGen microarray-based pathogen chip, which was developed as a quick and simple diagnostic tool for virus and bacteria pathogen detection from clinical samples of Dengue patients, SARS patients and H1N1 Influenza patients during multiple national and international epidemics; the Pathogen Chip technology is now commercialized under the company PathGEN Dx Pte. Ltd.

Other Work Experience

2001 **Assistant Nurse**
Ranjoth Singh Obstetric and Gynaecology Specialist Clinic, Ipoh (Malaysia)

- Assisted doctors and patients during clinic visits; e.g. measured blood pressures, prepared diagnostic tests.
- Facilitated communications between doctors and patients; e.g. translated English medical terms to Mandarin or Cantonese, explained doctors' instructions for taking medicines.

1995 **Piano accompanist,** City School of Ballet, Ipoh (Malaysia)

SCIENTIFIC PUBLICATIONS AND PRESENTATIONS

Peer-Reviewed Journals

1. ***WY Leong***, H Guo, O Ma, H Huang, AB Cantor, AD Friedman; Runx1 phosphorylation by Src increases trans-activation via augmented stability, reduced HDAC binding, and increased DNA affinity, and activated Runx1 favors granulopoiesis (2016); Journal of Biological Chemistry, 291(2): 826-836.

2. LA Zaritsky, A Dery, ***WY Leong***, L Gama, JE Clements; Tissue specific interferon alpha subtype response to SIV infection in brain, spleen and lung (2013); Journal of Interferon & Cytokine Research, 33(1): 24-33.

3. J Zhang, X Liu, A Datta, KR Govindarajan, WL Tam, J Han, J George, CW Wong, K Ramnarayanan, TY Phua, ***WY Leong***, YS Chan, N Palanisamy, ET Liu, R Krishna Murthy Karuturi, B Lim, LD Miller; RAB11FIP1/RCP is a Novel Breast Cancer Promoting Gene with Ras Activating Function (2009); Journal of Clinical Investigation, 119(8): 2171-2183.

4. WH Lee, CW Wong, ***WY Leong***, LD Miller, WK Sung; LOMA: A Fast Method to Generate Efficient Tagged-random Primers despite Amplification Bias of Random PCR on Pathogens (2008); BMC Bioinformatics, 9:368.

5. CW Wong, CWH Lee, ***WY Leong***, SWL Soh, CB Kartasasmita, EAF Simoes, ML Hibberd, W-K Sung & LD Miller; Optimization and Clinical Validation of a Pathogen Detection Microarray (2007); Genome Biology, 8:R93.

Posters

1. ***WY Leong***, H Guo, O Ma, S Cooper, H Huang, AB Cantor & AD Friedman; Src Activates RUNX1 in Myeloid Cells to Mediate Granulopoiesis; 10th International Workshop on Molecular Aspects of Myeloid Stem Cell Development and Leukemia, Cincinnati, Ohio, USA, 4-7 May 2014.

2. AD Friedman, ***WY Leong***, H Guo, O Ma, AB Cantor & H Huang; Src Kinase can Activate RUNX1 Activity via Phosphorylation of C-terminal Tyrosines and Activated RUNX1 Stimulates Granulopoiesis; 2013 Meeting of the American Society of Hematology, New Orleans, Louisiana, USA, 7-10 Dec 2013.

3. ***WY Leong***, FL Soh, BL Han, FL Yan, LD Miller & CW Wong; Cell based Assays Suggest Role for RAB11F1P1 in Cell Cycle and Apoptosis; American Association for Cancer Research (AACR) Annual Meeting 2008, San Diego Conventional Center, San Diego, California, USA, 12-15 Apr 2008.

4. CW Wong, CWH Lee, ***WY Leong***, S Soh, ML Hibberd, KW-K Sung, LD Miller; Pathogen Detection Microarray – from Lab to Bedside; Keystone Symposia – Respiratory viruses of Animals: Causing Disease in Humans, Swissotel the Stamford/Biopolis, Singapore, 10-15 Dec 2006.

5. ***WY Leong***, R. Das, KP Song; Molecular characterization and prevalence of *Clostridium difficile* in Singapore; 14th European Congress of Clinical Microbiology & Infectious Disease, Prague, Czech Republic,1-4 May 2004.

6. ***WY Leong***, R. Das, KP Song; Molecular characterization and prevalence of *Clostridium difficile* in Singapore; 7th NUS-NUH Annual Scientific Meeting, National University of Singapore, 30 Sept 2003.

Patent

1. Patent application publication: High throughput miniaturized assay system and methods
Application number: 14/050, 321; Publication number: US 2014/0235468 A1

Featured Work

1. ***WY Leong***, C Wong, M Hibberd, EE Ong; Validation Data for Proseek Assay Development Kit, IP-10, Olink Biosciences.
http://demo.olink.com/products-services/proseek/applications/proseek-ad-ip-10

TEACHING EXPERIENCE

July 2015 — **Summer Program Teaching Assistant**
Johns Hopkins University, Krieger School of Arts & Sciences, Department of Biology

- Taught DNA replication and transcription to 48 freshman and advanced pre-college students.
- Designed learning objectives, assessment activities, examination questions.
- Taught laboratory sessions and demonstrated laboratory techniques.

Sep 2012 – Oct 2012 — **Graduate Student Teaching Assistant**
Johns Hopkins University, Pathobiology Graduate Program

- Co-designed selected teaching and assessment materials for "Mechanisms of Diseases" course.
- Tutored selected graduate students on the lecture materials.

2002-2004 **Graduate Student Tutor**
National University of Singapore, Faculty of Medicine, Department of Microbiology
- Taught and assessed 40-50 students in year-round laboratory sessions.
- Tutored approximately 100 students on lecture materials.

1998 **Middle School Science Teacher**
SMJK Ave Maria Convent, Ipoh (Malaysia)
- Taught multiple sessions of basic science to approximately 200 students.

AWARDS AND ACCOMPLISHMENTS

Academic Scholarships and Fellowships

Aug 2015-May 2016 **Teaching Fellowship**, Johns Hopkins University
Preparing Future Faculty Teaching Academy

Aug 2011 **Margaret Lee Fellowship**, Johns Hopkins University School of Medicine (1 year full tuition plus stipend)

July-Aug 2004 **Osaka University Frontier Biosciences Summer School Scholarship**, Japan (7 weeks full tuition plus stipend)
Internship under supervision of Fumio Hanaoka, Ph.D.
(Integrated Biology Laboratories, Cellular Biology Group)

2002-2004 **Postgraduate Research Scholarship**, National University of Singapore, Singapore (2.5 years full tuition and stipend)
M.Sc. degree under supervision of Keang-Peng Song, Ph.D.
(Molecular Pathogenesis Laboratory)

Academic Awards

Sep 2015 **Winner,** Best Oral Presentation, Annual Pathobiology Retreat, Johns Hopkins University School of Medicine

May 2001 **Winner,** Best Oral Presentation, MSMBB-Promega Young Researcher Award for Molecular Biology Competition, Kuala Lumpur, Malaysia

2000-2001 **Dean's Award,** Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia

Employment Awards and Nomination

Nov 2010 **Nomination, A*STAR T-UP Excellence Award**, Biopolis and Fusionopolis, Singapore(for work on DropArray™ platform with Curiox Biosystems; T-UP denotes "Growing Enterprises with Technology Upgrade")

2010 **5-year Long-term Service Award,** Genome Institute of Singapore (GIS)

2007, 2006 **Sport Award,** for serving as committee member of the Recreational Club, GIS

Music Accomplishments

2001 Licentiate Diploma in Music, Australia (LMusA) of the Australian Music Examinations Board in Piano Performing

1996	Licentiate of the Royal Schools of Music in Piano Performing issued by the Associate Board of the Royal Schools of Music, United Kingdom
1995	Fellowship Diploma in Pianoforte issued by Trinity College, London

CERTIFICATION AND ADDITIONAL TRAINING

Certification

2009-2011	Certification on Competency: Dangerous Goods Regulations for Transporting Infectious Substances and Diagnostic Specimens by Air; World Courier Singapore

Additional training at Johns Hopkins University

2015	Leadership Module - Project Management, Summer Teaching Institute
2014	Bootcamp for Technology Entrepreneurs, Science Writing and Communications
2013	Mouse Pathobiology and Phenotyping
2012-2013	Research Ethics

SKILLS

Areas of Expertise

Molecular and Cellular Biology
Hematopoiesis
Microbiology and Immunology
Infectious Diseases
Assay Design and Construction
Instrument Maintenance
Genetically Engineered Mouse Model
Flow Cytometry (Basic)

Languages

English (fluent)
Mandarin (fluent)
Cantonese (fluent)
Malay (proficient)

COMMUNITY SERVICES AND ACTIVITIES (selected)

May 2015	Volunteer, Civic Works Real Food Farm, Baltimore • Transformed vacant lots into community vegetable gardens.
2013-2014	Organizer, "Ignite Hopkins" storytelling events, JHU School of Medicine • Applied for and was awarded a $1,000 grant from JHU Alumni Association.
2013, 2014	Color Me Rad 5K run, Port to Fort 6K run
Mar 2013	Volunteer, Maryland Food Bank
Feb 2013	Interviewer, Recruitment Week, Pathobiology Program, Johns Hopkins Uni.
2007 - Present	Musician, SoundBliss music group, Singapore, www.soundbliss.net
2006-2009	Committee member, Recreation Club, Genome Institute of Singapore
2006-2007	Laboratory representative, Genome Institute of Singapore • Singapore Science Open House, A*SAR YRAP Scholars Visit & Singapore Principals' Forum Visit, International Summit for Young Scientists (ISYS)

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