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REVIEW

Molecular Basis of Hematological Disease Caused by Inherited or Acquired RUNX1 Mutations

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The transcription factor RUNX1 is essential for correct hematopoietic development; in its absence in the germ line, blood stem cells are not formed. RUNX1 orchestrates dramatic changes in the chromatin landscape at the onset of stem cell formation, which set the stage for both stem self-renewal and further differentiation. However, once blood stem cells are formed, the mutation of the *RUNX1* gene is not lethal but can lead to various hematopoietic defects and a predisposition to cancer. Here we summarize the current literature on inherited and acquired RUNX1 mutations, with a particular emphasis on mutations that alter the structure of the RUNX1 protein itself, and place these changes in the context of what is known about RUNX1 function. We also summarize which mutant RUNX1 proteins are actually expressed in cells and discuss the molecular mechanism underlying how such variants reprogram the epigenome setting stem cells on the path to malignancy.

HIGHLIGHTS

- Germline and acquired RUNX1 mutations are associated with FPD and MDS/AML.
- Large deletions and nonsense mutations are more common in germline FPD patients.
- Missense mutations affecting the transactivation domain are more commonly acquired.
- Both loss of function and altered function of RUNX1 contribute to disease.
- Expression and function of many pathogenic RUNX1 proteins remain elusive.

THE RUNX FAMILY OF TRANSCRIPTION FACTORS

RUNX1 is a DNA-binding transcription factor that binds to the DNA consensus sequence TGTGGT as a heterodimer with its cofactor core-binding factor subunit β (CBFβ) (Figure 1). CBFβ does not contact DNA directly, but it is required for efficient binding of RUNX1 [1,2]. The RUNX family of RUNT homology domain (RHD) transcription factors also includes RUNX2 and RUNX3, and all three RUNX proteins play multiple roles contibuting both to normal development and to cancer in various tissues [3,4]. RUNX1 was originally termed AML1 because it was identified as a fusion protein with altered function in acute myeloid leukemia (AML) cells carrying the t(8;21) translocation (RUNX1/RUNX1T1). This fusion replaced the transactivation domain (TAD) of RUNX1 with the repressive domain of the ETO protein [4]. Dysregulated or mutated RUNX1 is able to contribute to leukemia in many ways whereby either too much [5] or too little RUNX1, or altered function of RUNX1, can promote AML [6] or myelodysplastic syndromes (MDS) [7]. Deregulation of RUNX1 or other RUNX proteins is also not just a feature of myeloid malignancy. In one study of T acute lymphoblastic leukemia (T-ALL), mutations of the RUNX1 RHD or TAD were also encountered in 18% of cases [8]. Furthermore, ectopic expression of RUNX2 can promote T-cell lymphoma and other cancers [3,4], while either loss of RUNX3 or reduced RUNX3 expression is a feature of gastric cancer [9]. Mutations or translocations of the RUNX1 and CBFβ genes are now widely seen as major drivers of AML and MDS, and myeloid disorders carrying RUNX1 mutations frequently progress to AML. This review focuses on the molecular impact of different classes of RUNX1 mutations found either in AML as somatic mutations or in familial platelet disorder (FPD) as inherited germline mutations.

RUNX1 COORDINATES BLOOD DEVELOPMENT

Hematopoiesis is the process by which blood cells develop in adults and during embryogenesis. Hematopoietic stem cells (HSCs) are at the apex of this process and are capable of self-renewal as well as differentiation into multipotential progenitors; these progenitors then give rise to myeloid, lymphoid, and erythroid cells. This complex process is carefully orchestrated by a number of transcription factors that control cell type-specific gene expression. When hematopoiesis is

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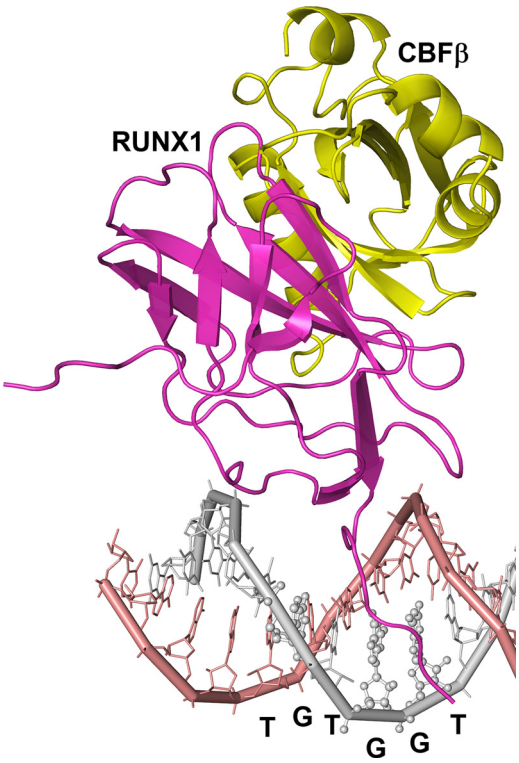

Figure 1 X-Ray crystal structure of the RUNT Homology domain (residues 50–177 of RUNX1b, residues 77–204 of RUNX1c) in association with the heterodimerization domain of CBFβ (residues 1–135) bound to DNA containing the RUNX1 consensus binding sequence TGTGGT. This model is based on structural data from Bravo et al. [1] and has been adapted, with permission, from Bowers et al. [10]. *CBFβ*=core-binding factor subunit β; *FPD*=familial platelet disorder.

perturbed by mutation of these transcription factors, hematological disorders such as leukemia can develop. During embryonic hematopoietic development, the transcription factor RUNX1 is required for the process of endothelial-hematopoietic transition (EHT), which forms blood progenitors [11,12]. Thereafter, RUNX1 is involved in terminal blood development across multiple lineages. Experiments with conditional RUNX1 knockout mice have found that in adults, RUNX1 is required for maturation of megakaryocytes and lymphoid cells and for balanced myeloid differentiation [13,14]. However, RUNX1 does not act alone. The RUNX1 protein contains multiple regions that interact with other proteins to coordinate lineage-specific transcriptional events, and as such, it has been named a master hematopoietic regulator [13,15–19]. The interactions of RUNX1 with other transcription factors are cell type dependent, as is the binding pattern of RUNX1 within the genome; thus, RUNX1 regulates multiple cell fates. For example, RUNX1 activates the expression of CEBPA and PU.1, but then also interacts with these two transcription factors to drive myelopoiesis [18,20–22].

Three isoforms of RUNX1 are expressed: the longest isoform is RUNX1c, RUNX1b has a shorter N-terminus and is expressed from an alternate promoter to RUNX1c, and RUNX1a is missing a large portion of the C-terminus. RUNX1c is expressed primarily in adult blood cells, whereas RUNX1a and RUNX1b expression is primarily during development, with RUNX1b also coordinating adult megakaryocyte/erythroid differentiation [23,24].

RUNX1 MUTATION IN HEMATOLOGICAL DISORDERS

RUNX1 mutations are involved in a variety of hematological disorders, most notably somatic mutations in AML/myelodysplastic syndrome (MDS) and germline in FPD, which may then lead to AML/MDS or other malignancies [25].

The presentation of FPD is very variable. Some patients present in early childhood with thrombocytopenia, but many others present much later in life, even into their fifties or sixties [26]. They may have loss of chromosome 7 or del(7q) even in the absence of transformation to AML/MDS. Most patients have platelet dysfunction, but there is considerable variation between and within families in the propensity to transform to AML/MDS [26–28].

RUNX1-mutated AML is a highly heterogeneous set of myeloid neoplasms [29–31], many of which have their initial phenotype as MDS. The presentation can be as de novo MDS at any stage from single-lineage dysplasia to excess blasts, but the prognosis is frequently poor. This heterogeneity and poor prognosis are also reflected in the presence of RUNX1 mutations in therapy-related myeloid neoplasms. The World Health Organization has provisionally classified AML with mutated RUNX1 as a separate entity, where it occurs in the absence of dysplasia; they note the poor prognosis of this group in a number of studies [32]. Additionally, RUNX1 mutations are found less frequently in MDS/myeloproliferative neoplasms (MDS/MPN) particularly in chronic myelomonocytic leukemia. Occasionally patients may present with acute lymphoblastic leukemia (ALL), of T-cell and less frequently B-cell phenotypes [33]. *RUNX1* mutations typically co-occur with mutations in genes involved in signaling, RNA splicing, or epigenetic modifications.

STRUCTURE, FUNCTION, AND LOCALIZATION OF RUNX1

RUNX1 protein function can be broadly divided into DNA binding and co-factor interactions. RUNX1 contains the RHD, a DNA binding region, which also mediates interactions with its obligate partner CBFβ, illustrated in Figure 1 [34,35]. CBFβ significantly enhances the stability and DNA binding capacity of RUNX1 by altering its conformation when the two proteins are partnered, and is itself commonly mutated in hematological malignancies [2]. The RHD forms an immunoglobulin fold that interacts with DNA directly via loops at either end of the fold [36,37]. Within the RHD, 33 amino acids are predicted to be in contact with CBFβ, with amino acids 136, 140, 176, 186, and 188 found to be specifically required for this contact [35,38]. Fewer amino acids are thought to be directly in contact with DNA, but targeted mutations within amino acids 72, 107, 108, 110, 169, 171, 198, 201, and 204 disrupt DNA binding [38–41].

RUNX1 alone is a weak activator of transcription, and it typically functions within multimeric complexes containing other transcription factors controlling the activities of promoters and enhancers. RUNX1 can be found bound to genes in the presence of either activating or repressive chromatin regulators and other transcription factors. While the RUNX1 TAD recruits co-activators, the C-terminus of the RUNX1b and RUNX1c proteins contains a "VWRPY" domain, a

conserved sequence that allows interactions with Groucho/TLE proteins mediating repression of transcription [42].

Many proteins are known to interact with RUNX1 directly or indirectly, including other transcription factors such as SCL, GATA2, and FLI1 [15,18,43,44] and chromatin modifiers such as p300 and mSin3a [17,45–47]. Initially, the activation domain was presumed to be concentrated within the region of the protein unique to RUNX1b and RUNX1c because of the increased ability of RUNX1b compared with RUNX1a to activate transcription of myeloid genes [48,49]. Functional deletion studies have indicated roles for some of the residues within this region, but not all, with the region between 291 and 371 amino acids having the highest transactivation potential and being involved in regulating the cell cycle [43,50]. Interaction with the transcriptional activator p300 occurs between amino acids 178 and 294 [45], whereas the amino acids following 371 are involved in reduced transcriptional activation of myeloid genes and so are considered inhibitory [50].

As a transcription factor, RUNX1 is located predominately in the nucleus. The protein contains both a nuclear localization signal (NLS) at the end of the RHD at amino acids 194–210 and a nuclear matrix targeting signal (NMTS) within the transactivation domain [51,52]. Within the nucleus, RUNX1 binds to DNA to control gene transcription and may act as a scaffolding protein within the nucleus to organize chromatin [53].

POSTTRANSLATIONAL MODIFICATION OF RUNX1 PROTEIN

The functionality and stability of the RUNX1 protein are modulated through multiple post-translational modifications occurring throughout the regions of the protein. These include moieties that can be phosphorylated, acetylated, methylated, or ubiquitinated to alter RUNX1 activity.

Phosphorylation of serine and threonine residues in RUNX1 is the primary mechanism by which RUNX1 function is altered by protein–protein interactions and signaling. RUNX1 is phosphorylated by multiple signaling molecules. The interaction between RUNX1 and CBFβ promotes phosphorylation of RUNX1 at Ser276 and Thr300/Ser303 in the C-terminus of the protein by HIPK2 [54]. Phosphorylation of these residues promotes phosphorylation of the associated histone acetyltransferase p300, also by HIPK2, which plays a major role in upregulating RUNX1 target gene expression. Interestingly, the oncogenic CBFβ/SMMHC mutation prevents this phosphorylation of RUNX1 through sequestration of HIPK2 by the fusion protein [54].

The product of the proto-oncogene *PIM1* has also been reported to phosphorylate RUNX1 at multiple target sites, leading to increased transactivation of RUNX1 target genes [55]. RUNX1 activity is also modulated by cyclin-dependent kinases (CDKs). CDK 1/2/6 phosphorylate RUNX1 at Ser276 and Ser303 to mediate RUNX1 ubiquitination and degradation [56]. Conversely, CDK1/6 can reduce the interaction of RUNX1 with HDAC1/3 through phosphorylation of Ser48, Ser303, and Ser424, resulting in enhanced activation of bone marrow progenitor differentiation [57,58].

ERK, a key member of the RAS/MAPK pathway, phosphorylates RUNX1 at Ser276 and Ser293, which has been found to enhance the transcriptional activation capacity of RUNX1 in fibroblasts [59] as it disrupts the interaction of RUNX1 with the corepressor SIN3A, stimulating transcription of RUNX1 targets [60]. ERK-mediated phosphorylation of RUNX1 has also been described at moieties Ser303, Ser462, and Thr300 in the RUNX1 C-terminus. This action of the RAS/MAPK signaling pathway on RUNX1 may explain the importance of RUNX1 in maintaining the transcriptome in FLT3-ITD-mutated AML, which has constitutively activated signaling through RAS/MAPK [61]. However, there have also been reports of direct action of FLT3-ITD on RUNX1, where the mutated protein causes phosphorylation of Tyr402, Tyr405, Tyr406, and Tyr413 in the inhibitory domain of RUNX1, which stabilizes the protein, leading to intracellular accumulation; this, in turn, is critical for the onset of AML in cells with FLT3-ITD [5]. Conversely, phosphorylation of tyrosine residues 281, 285, 287, 403, 406, 407, and 414 by SRC family kinases alter the protein–protein interactions of RUNX1 with GATA1 and the SWI/SNF chromatin remodeler complex to negatively regulate RUNX1 activity in CD8 T-cell differentiation and megakaryocyte maturation [62].

Acetylation and methylation also modulate RUNX1 activity. RUNX1 DNA binding activity is augmented by p300 by acetylation of Lys51 and Lys70, although the effect on transcription activation of RUNX1 targets is relatively small [63]. PRMT1 methylates multiple arginine residues in RUNX1 such as Arg233 and Arg237, which disassociates RUNX1 and the corepressor SIN3A, leading to increased transcription of RUNX1 targets [64]. PRMT4 methylates RUNX1 at Arg250, which promotes the assembly of the repressive DPF2 complex, which inhibits expression of miR-233 and therefore differentiation of $CD34^+$ cord blood cells [65].

The RUNX1 protein contains multiple lysine residues, which are the target of ubiquitin ligases, including Lys51, Lys70, Lys110, Lys117, Lys152, Lys171, Lys194, Lys209, and Lys215 [66]. These moieties cluster around the RHD of the protein and are protected from ubiquitination when RUNX1 is bound by CBFβ, MLL, and H3K methyltransferases [67], increasing the stability of the protein when it forms a complex.

RUNX1 AND CELLULAR SIGNALING

The interactions between RUNX1 and various signaling pathways have been investigated in multiple studies addressing the relationships between chronic signaling and RUNX1 depletion in FPD and AML/MDS. Although it has previously been reported that RUNX1 expression can be regulated by the RAS/MAPK pathway [5,61,68], RUNX1 has also been described as directly regulating signaling cascades and being regulated by signaling in a post-translational manner. The STAT3 signaling cascade, which is activated by colony-stimulating factor granulocyte (G-CSF), is negatively regulated by RUNX1 by two mechanisms [69]. First, the RUNX1 target PIAS1 negatively regulates phosphorylation of STAT3, and mutations in RUNX1 reduce the expression of this protein, leading to an increase in STAT3 phosphorylation. Second, the RUNX1 protein physically interacts with STAT3 through the RHD, which attenuates phosphorylation of STAT3. It is possible that similar mechanisms of interaction between RUNX1 and signaling proteins are responsible for the inhibitory effect of RUNX1 on JNK phosphorylation [70], although this mechanism has not been thoroughly investigated.

Regions of homology between different RUNX family members can also indicate potential interactions of RUNX1 with other signaling pathways, such as the PY motif, which is conserved in all RUNX family members has been found to interact with YAP1, a member of the Hippo signaling pathway. Recruitment of YAP1 to RUNX-bound

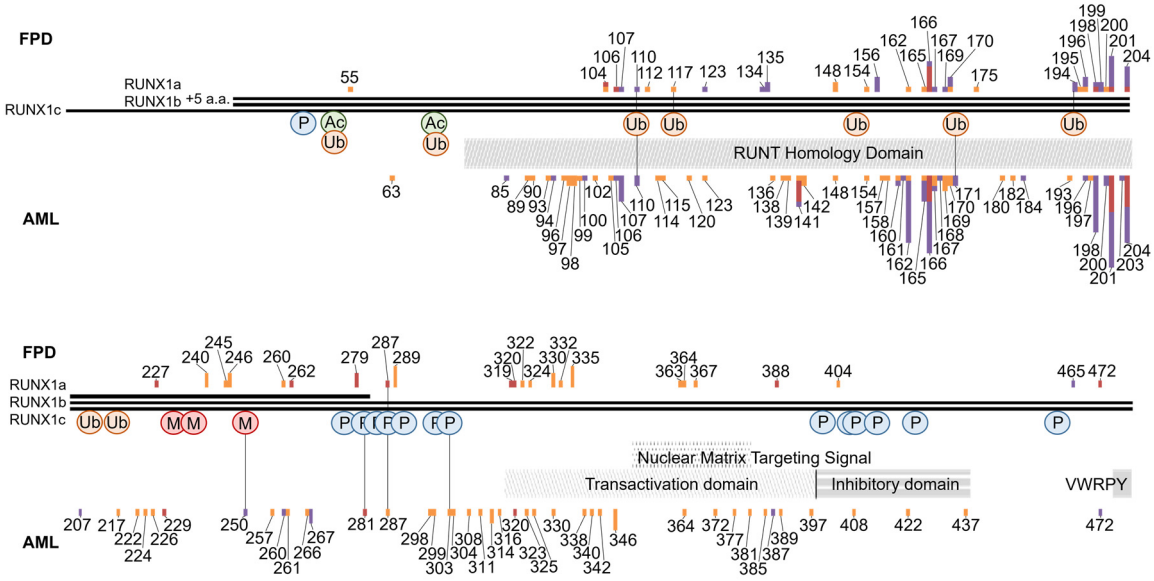

Figure 2 Diagram of the location and type of 88 reported FPD-causing mutations (above the lines) and 189 AML-causing mutations (below the lines), aligned with the three main isoforms of RUNX1, known functional protein domains, and posttranslational modifications. Amino acid numbers are listed according to the RUNX1c isoform. Where mutations coincide with posttranslational modification, this is indicated by a vertical line. Multiple recorded mutations at the same location are shown with additional *vertically stacked squares*. *AML*=acute myeloid leukemia; *FPD*=familial platelet disorder.

promotors enhances transcription, resulting in Hippo signaling inducing RUNX target genes [71,72].

RUNX1 therefore has numerous links to signaling pathways within the cell, and its activity is strongly regulated by the related post-translational modifications.

HOW DO RUNX1 MUTATIONS LEAD TO DISEASE?

The wide variety of RUNX1 mutations seen in myeloid malignancies, as well as the potentially differing impact when they occur somatically or in the germline, provides a unique challenge in resolving how they contribute to disease. RUNX1 is involved in multiple aspects of hematological development, which makes it challenging to elucidate precisely how different RUNX1 mutations contribute to hematological disorders. The effect of RUNX1 mutations can be broadly divided into either haploinsufficiency or dominant negative gain of function phenotypes. The predisposition and transformation to AML seen in FPD families is particulary poorly understood. It is known that FPD is associated with platelet maturation associated with inappropriate CD34 expression, as well as misregulation of other RUNX1 target genes including NFE2, but this also varies between individual mutations [73–77]. To gain insights into the relationship between RUNX1 structure and function, we have collated available mutational data from 101 reported cases of FPD, with or without coinciding AML, where pedigrees were reported or germline transmission is confirmed [26–28,73–76,78–105] (Raghavan, unpublished). We have compared these data with 196 reported cases of AML with mutated RUNX1 without a reported history of FPD, from several sources, while avoiding duplicate reports on the same patient [29,30,81,94,102,105–108] (Raghavan, unpublished). The point mutations (88 FPD, 189 AML) have been plotted onto the RUNX1c protein structure in Figure 2. In line with previous summaries, we see the majority of mutations are confined to the RHD: 54 of 88 in FPD and 144 of 196 in AML. Outside of the RHD, most alterations are frameshift mutations, but missense and nonsense mutations have also been recorded.

Large deletions of the RUNX1 gene have been reported in nine cases of FPD and lead to haploinsufficiency; beyond this, the mutational landscape is more complex. If inherited mutations would all simply lead to haploinsufficiency, consistent phenotypes and a disturbance of blood development would be expected [109,110], which is not in keeping with the heterogeneity observed in RUNX1-mutated hematological disorders. Typically, frameshift mutations, particularly those at the beginning of the gene, are considered to be null alleles, but real experimental evidence for this statement is limited, which we discuss in the following. Similarly, mutations leading to truncated proteins could lead to a loss of functional domains and haploinsufficiency, but could equally be subjected to nonsense-mediated decay of their mRNAs or simply become unstable. Missense mutations equally could either be rendered nonfunctional or gain a dominant negative function. Simon et al. [111] suggest a differing gene signature resulting from both nonsense and frameshift mutations, as opposed to missense mutations, supporting the idea that these proteins are not all simply nonfunctional. Interestingly, the dominant class of mutations varies between the inherited FPD-causing mutations and the sporadic AML-causing mutations (Figure 3), with large deletions, splicing defects, and nonsense mutations occurring roughly twice as often in FPD compared with AML. This fact may indicate that haploinsufficiency of RUNX1 causes FPD more readily than AML.

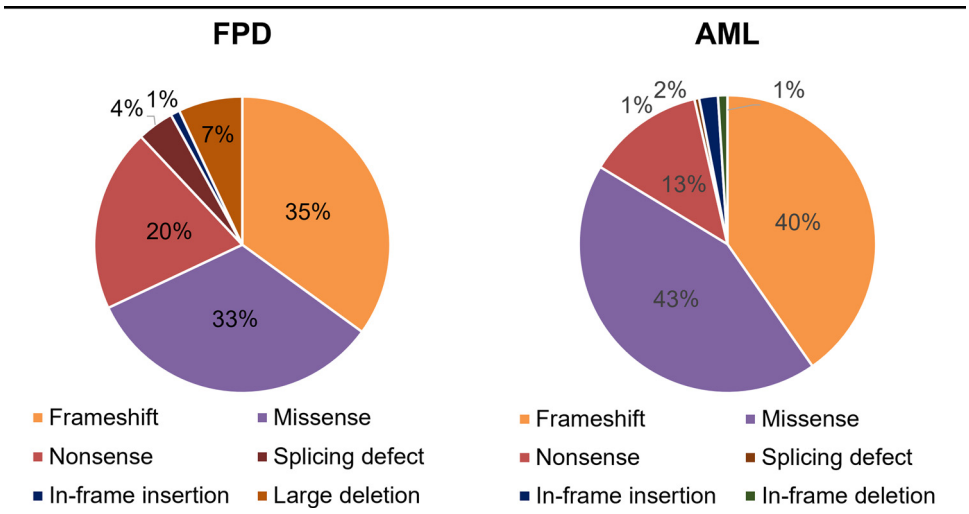

Figure 3 Percentage of each type of mutation reported in Figure 2, with the addition of large deletions, splicing defects, and in-frame insertions/deletions. AML=acute myeloid leukemia; FPD=familial platelet disorder.

HOTSPOT LOCATIONS FOR MUTATIONS

Several amino acids are subject to recurrent mutations in both FPD and AML, such as those at positions 166, 201, and 204. Mutations at these locations were some of the first reported [101], and it is unclear whether these are the most common mutations or are screened for more. Two genomic locations are subject to recurrent mutation in AML only, leading to alterations in amino acids 107 and 141. To our surprise we found several recurrent hotspots for frameshift mutations in FPD—at least three cases were found with frameshift mutations from amino acids 289 and 335, with an additional five frameshift and two nonsense mutations in the region between amino acids 319 and 334. In AML, three frameshift mutations were seen beginning at amino acids 169 in the RHD and 346. Few mutations coincided with amino acids subjected to posttranslational modification with the exception of amino acids 110, 117, and 171, which are all ubiquitinated and have five missense mutations and one frameshift recorded, driving both FPD and AML.

EXPRESSION OF MUTANT PROTEINS IN MODEL SYSTEMS

To correctly understand the behavior of these mutations, we must first know whether the altered proteins can be stably produced. Because of the wide variety of mutations and limited availability of antibodies to probe them, studies on protein expression are generally confined to model systems. Missense mutations would all generally be expected to be produced. However, nonsense and frameshift mutated proteins can be subject to degradation, effectively being null alleles. The variant proteins that have been detected, or have been experimentally confirmed as not detected, and the models used are outlined in Table 1—both nonsense and frameshift proteins have been assayed, with stable proteins produced. Of note, Matheny et al. [41] could not detect the R204X mutant in the thymocytes of 6-week-old mice, either because the protein was degraded (e.g., by nonsense-mediated decay) or because cells containing it were not viable. To our knowledge, the presence of only one mutated protein, L472fsX123, has been confirmed in patient-derived cells [93]; one has been confirmed as not expressed, albeit in myeloproliferative neoplasm patient cells [112]. As the expression of mutated proteins could be an artifact of the expression model systems used, proof of protein expression in patient-derived blood cells would be highly informative.

The model systems used have their own advantages and disadvantages. NIH3T3, HEK293T, REF52, and COS-7 cells are easy to stably express proteins in but do not express endogenous RUNX1 or other myeloid transcription factors; therefore, physiological functions in a context where these proteins are present cannot be studied. Mouse models expressing mutant proteins in the germline allow for a native environment to study FPD-causing proteins; however, particularly for AML-causing mutations, mutant protein expression could be incompatible with development because of blocking of the EHT. Mouse and human pluripotent stem cell differentiation models circumvent this issue and allow the mutant proteins to be induced either before or after the developmental crux point of EHT [113–116], but do not produce adult-type blood cells. Validation of key findings in patient-derived primary cells would therefore be important.

If we assume that results from model systems are applicable to native patient blood cells, the next questions are what functions the mutated proteins retain and how their normal function is altered. Because of the complexity of RUNX1 functions and interactions, this is again challenging to investigate in the context of such a wide range of mutations [117].

DISRUPTION OF RUNX1 ACTIVITY BY MUTATION

RUNX1 is normally localized to the nucleus, but when the NLS is partially or completely absent, as is the case in a number of FPD and AML mutations, the RUNX1 protein is located at least partially in the cytoplasm. Some point mutations also result in disrupted intracellular localization caused by changes in polarity of the protein [84], while

Table 1 Known RUNX1 mutations where translated proteins have or have not been detected and how they were detected

Mutation	Model	Assay
S141X	REF52 [107]	Immunofluorescence
R166X	HEK293T [75]	Immunofluorescence, Western blot
R201X	NIH3T3 [84]	Immunofluorescence
R204X	REF52 [107] COS-7 [41] mESC-derived hematopoietic progenitors [116] NIH3T3 [84]	Immunofluorescence Western blot Immunofluorescence, Western blot Immunofluorescence
S388X	HEK293T [87]	Western blot
C72insfs111X	REF52 [107]	Immunofluorescence
K117fsX101	NIH3T3 [84]	Immunofluorescence
R162fsX177	NIH3T3 [84]	Immunofluorescence
L472fsX123	Patient-derived PBSCs [93]	Western blot
H85N	REF52 [107]	Immunofluorescence
W106R	HEK293T [117]	Western blot
R107C	REF52 [107]	Immunofluorescence
K110N	REF52 [107] NIH3T3 [84]	Immunofluorescence Immunofluorescence
K110E	NIH3T3 [84] HEK293T [117]	Immunofluorescence Western blot
R166Q	NIH3T3 [84] HEK293T [117]	Immunofluorescence Western blot
K194N	HEK293T [102,117]	Western blot
R201Q	NIH3T3 [84] COS-7 [41] HEK293T [75,117] mESC-derived hematopoietic progenitors [116]	Immunofluorescence Western blot Immunofluorescence, Western blot Immunofluorescence, Western blot
R204Q	REF52 [107] NIH3T3 [84]	Immunofluorescence Immunofluorescence
R204X	Mouse [41]	Western blot—not detected
R230X	Patient-derived blood and bone marrow [112]	RT-PCR, mutation-specific primers—not detected

RT-PCR=reverse transcription polymerase chain reaction.

disruption to the NMTS prevents intranuclear targeting, which impairs RUNX1-driven roles in hematopoietic differentiation [118]. Correct localization to the nucleus is therefore essential for RUNX1 function, including the ability to bind DNA; therefore, aberrant localization is one way in which mutations contribute to RUNX1-driven disease.

Many RUNX1 mutations do not disrupt nuclear localization, but instead disrupt the function of the RUNX1 protein within the nucleus. RUNX1 directly binds DNA, which is significantly enhanced by interaction with CBFβ. No mutations were recorded in either FPD or AML at any amino acids associated with CBFβ interaction, with the exception of a single frameshift starting from amino acid 136 seen in AML [35,38]. However, mutations have been observed in both FPD and AML at many, but not all, amino acids thought to be involved in direct DNA contact [38,40]—amino acids 107, 110, 169, 171, 198, and 201 (see Figure 2)—and these are some of the most recurrent mutations detected.

Mutations in both FPD and AML also coincide with several ubiquitination sites, at amino acids 110, 117, 171, and 194 within the RHD (Figure 2), which may have an impact on the stability of the RUNX1 protein and the other functions of the RHD [66]. Notably, two of the residues subject to ubiquitination, lysines 51 and 70, are also the targets of acetylation, but no mutations were observed at these sites.

Mutations in the RUNX1 protein can also lead to altered signaling. The Leu56Ser missense variant of RUNX1 has a mutant allele frequency of 0.012 in the general population and may be associated with a RUNX1-related disorder [119], which impairs platelet function. Homozygous Leu56Ser mice develop thrombocytopenia as a result of a deficiency in PKC signaling, which appears to be due to post-translational mechanisms as no change in protein kinase α (PKCα) or protein kinase β (PKCβ) mRNA levels was observed [119]. However, whether this is an effect of a direct protein–protein interaction of RUNX1 with PKC signaling proteins or of altered expression of an inhibitory protein within the pathway is currently unknown. When

RUNX1 carries FPD-associated mutations such as R201Q and Y287X, attenuation of STAT3 phosphorylation caused by RUNX1−STAT3 interaction no longer occurs [69], indicating that although the Runt domain of RUNX1 is necessary for the protein to interact with STAT3, the carboxyl terminus is required to inhibit STAT3 phosphorylation. While few mutations are observed at sites of posttranslational modification, the question remains whether chronic signaling and inflammation contribute to the transition from FPD to AML.

The mutational spectrum beyond the RHD is less clear. As mentioned previously, mutations in the TAD are far less recurrent than in the RHD—interactions may be mediated by broader ranges of the protein than are required for DNA binding. It is therefore unclear exactly what contribution mutations within the TAD lead to the development of disease, particularly in the case of the missense mutations reported in AML, which are R207P, R250C, N260K, M267I, G387A, and L472P. Studies to identify the roles of the transactivation domains have deleted large regions rather than specific amino acids and looked at general phenotypes such as transcriptional activity [45,46].

No mutations were recorded in the VWRPY domain as illustrated in Figure 2; therefore, blocking transcriptional repression of the RUNX1 complex seemingly does not lead to myeloid malignancy. Indeed, evidence points to this domain being more important in the lymphoid lineages [16]. A large number of mutations are found in both FPD and AML scattered throughout the TAD, including the section between AML1a and the TAD identified by Kanno et al. [50] which is a highly phosphorylation prone amino acid sequence. Nonsense and frameshift mutations were observed at and around residues known to be phosphorylated, suggesting an importance to this region that has not been fully explored.

The majority of nonsense mutations in both FPD and AML are within or at the end of the RHD, thereby eliminating the entire TAD but preserving DNA binding activity, presuming these proteins are produced and able to bind. Differences in histone acetylation were seen in mouse embryonic stem cell–derived progenitors expressing the R204X protein, which is truncated from the last amino acid of the RHD, but changes were modest and did not have an immediate impact on gene expression [116]. This result is likely due to the limited quantity of these proteins, which reach the nucleus because of a disrupted NLS [84,107,116]. Loss of the TAD may therefore confer a mild dominant negative phenotype because of expression of a protein with limited DNA binding capacity capable of variable interaction with chromatin modifiers or transcription factor complexes. The cofactor interactions retained would be dependent on the exact location of the truncation, which would account for some of the heterogeneity seen within disease presentation.

IMPACT OF RUNX1 MUTATIONS ON THE CHROMATIN LANDSCAPE

As outlined, most mutant RUNX1 proteins occur in a heterozygous genetic background in which the wild-type protein is still present. Moreover, the presence of the latter is required for the survival of cells carrying at least some of these mutants, indicating a fine balance between normal and mutant proteins that will have to be played out at the level of the genome [120]. However, only a handful of experiments have studied in detail how different types of RUNX1 mutations causing both AML and FPD affect the chromatin binding activity of wtRUNX1 and the expression of its target genes.

A number of studies used human and mouse ES systems expressing inducible versions of RUNX1 fusion proteins such as RUNX1-ETO and RUNX1-EVI in progenitors differentiated from such cells. This system was previously used to illustrate that RUNX1 is essential for the EHT [11] and that the induction of RUNX1 leads to a profound reorganization of the chromatin landscape, with RUNX1 orchestrating the binding of multiple other transcription factors to gain access to DNA [19,121]. These data confirmed the mouse studies indicating that the expression of these proteins is incompatible with the EHT [114–116,122] and disrupt the earliest instructions for the differentiation trajectory of hematopoietic progenitors. Once expressed in already formed progenitors, both fusion proteins interfere with the binding of wild-type RUNX1, alter chromatin, and lead to the establishment of a preleukemic state with enhanced self-renewal and a skewed differentiation trajectory. However, gene expression changes and alterations in the open chromatin landscape, as well as RUNX1 binding, differed between the two oncoproteins.

Kellaway et al. [116] also studied two RUNX1 point mutants. One (R204X) is a truncated protein that lacks the activation domain and is associated mainly with AML; the other (R201Q) is associated with FPD and is unable to bind to DNA because of a point mutation. Both proteins contained an HA tag, which allowed us to examine their own DNA binding activities as well. Consistent with the fact that these mutants run in families, the EHT was not affected when mutant proteins were induced before the EHT and progenitors could be formed. When induced in these cells, the R204X protein caused no changes in RUNX1 binding but altered the chromatin landscape, including histone acetylation, and caused a defect in priming chromatin toward the production of common myeloid progenitors. The R201Q protein had the most drastic effect of all tested mutants: Its induction led to a drastic reduction in global endogenous RUNX1 chromatin binding by sequestering CBFβ. The result of RUNX1 inactivation is a defect in setting up chromatin for multilineage differentiation, with the most severe defect in the megakaryocyte lineage, which is in keeping with the FPD phenotype. Importantly, none of the mutant proteins was capable of stable chromatin binding itself as they were undetectable in chromatin immunoprecipitation assays, pointing to gene regulation and chromatin remodeling by RUNX1 being highly dynamic and dependent on the cooperation with other factors. These results are consistent with the idea that the finely balanced interactions that are at the heart of cell fate decisions are disturbed in the presence of aberrant RUNX1 proteins. Over time, and in response to other defects, this disturbance progresses and cell differentiation and cellular function go astray. It is highly likely that other mutant RUNX1 proteins described wield a similar deregulatory influence in individuals carrying such mutations. It is also likely that each class of mutation will have a slightly different biochemical phenotype and reprogram the epigenome in different ways, leading to different disease phenotypes.

CONCLUSIONS AND OUTLOOK

In summary, despite numerous studies in various model systems, it is still unclear precisely how most RUNX1 mutations contribute to disease. Heterogeneity in RUNX1-driven AML is not accounted for

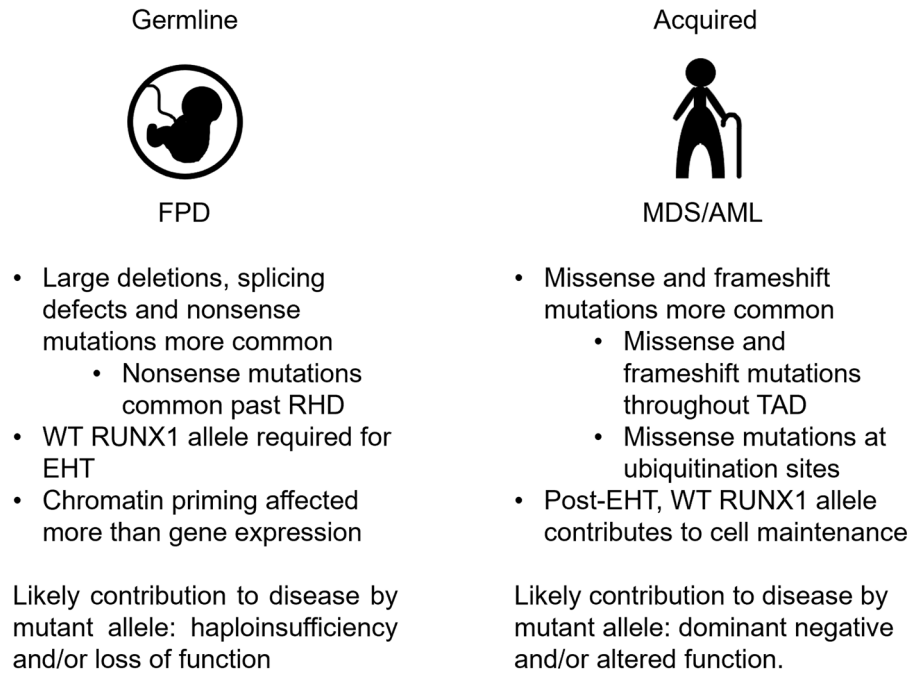

Figure 4 Summary of the different classes of mutations overrepresented in FPD or AML and how these may differentially contribute to disease. AML=acute myeloid leukemia; EHT=endothelial-to-hematopoietic transition; FPD=familial platelet disorder; MDS=myelodysplastic syndromes; RHD=Runt homology domain; TAD=transactivation domain; WT=wild type.

solely by haploinsufficiency of RUNX1 or by co-occurring mutations. We have also identified several mutational hotspots that are specific to either FPD or AML or have a greater proportion of one mutation type in one disease. The altered bias of mutation type between FPD and AML is summarized in Figure 4, along with the likely molecular contribution of this type of mutation to disease. Going forward it will be essential to determine which proteins are expressed in patient cells. Interactions of RUNX1 with other proteins such as transcription factors and chromatin modifiers must also be considered, not just DNA binding capacity, requiring studies in physiologically relevant models.

A key aim for researchers and clinicians working on FPD is to prevent the transformation to AML. This task requires a clear understanding of the mechanism of why this predisposition exists and how transformation takes place. Krutein et al. [123] recently suggested inhibition of RUNX1 degradation as a potential therapy, which they found to be partially effective in an iPSC model containing RUNX1, with a splicing defect resulting in a frameshift and early termination. However, this study also did not determine whether this mutation produced a protein—any protein produced would be truncated without the transactivation domain [123]. Similarly, overexpression of wild-type RUNX1 partially rescues erythroid and megakaryocyte differentiation defects caused by point mutation in the RHD [124]. Restoration of RUNX1 could therefore be useful in cases where the mutation does lead to true haploinsufficiency, to increase RUNX1 from the wild-type allele, but as discussed it is unclear to what extent this is the underlying cause in all cases of FPD. Any rescue strategy will be complicated by the fact that actions of RUNX1 appear to be extremely dosage dependent in ways that have not been fully elucidated [125]; hence only partial rescues have been determined. The recent establishment of a database to collate RUNX1 mutations in FPD will undoubtedly accelerate understanding of the heterogeneity and function of these mutations and inform future research to prevent transformation into AML [33].

In AML caused by mutated RUNX1, Mill et al. [120] determined that knockdown of RUNX1 induced apoptosis and improved survival of RUNX1-mutated AML-engrafted mice. Sensitivity to BET inhibitors associated with reduction in RUNX1 expression was also seen with RUNX1-mutated AML cells [126]. With the advent of drugs to target RUNX1 binding this could offer a specific treatment avenue for RUNX1-mutated AML [127]. We therefore need to resolve how RUNX1 must be modulated in the context of both FPD and AML, with a consideration for the type of mutation, whether a nonfunctional or dominant negative RUNX1 protein is produced and how this therefore affects the various functions of RUNX1.

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