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Brigitte Schlegelberger, Paula G. Heller

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Brigitte Schlegelberger[1**] and Paula G. Heller[2]

[1] Department of Human Genetics, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany

[2] Instituto de Investigaciones Médicas Alfredo Lanari, Universidad de Buenos Aires, IDIM-CONICET, Buenos Aires, Argentina

****Corresponding author:**

Prof. Dr. med. Brigitte Schlegelberger

Department of Human Genetics

Hannover Medical School

Carl-Neuberg-Str. 1

30625 Hannover

Phone: +49-511-532-4522

Fax: +49-511-532-4521

Email: Schlegelberger.brigitte@mh-hannover.de

Abstract

In this review, we discuss disease-causing alterations of RUNT-related transcription factor 1 (*RUNX1*), a master regulator of hematopoietic differentiation. Familial platelet disorder with predisposition to myeloid leukemia (FPDMM) typically present with 1) mild to moderate thrombocytopenia with normal-sized platelets; 2) functional platelets defects leading to prolonged bleeding; and 3) an increased risk to develop MDS, AML or T-ALL. Hematological neoplasms in carriers of a germline *RUNX1* mutation need additional secondary mutations or chromosome aberrations to develop. If a disease-causing mutation is known in the family, it is important to prevent hematopoietic stem cell transplantation from a sibling or other relative carrying the familial mutation. First experiments introducing a wild-type copy of *RUNX1* into iPSC lines from patients with FPDMM appear to demonstrate that by gene correction reversal of the phenotype may be possible.

- **Definition of RUNX1 deficiency/ FPDMM**

RUNT-related transcription factor 1 (RUNX1), previously named core binding factor A2 (CBFA2) and acute myeloid leukemia 1 (AML1), is a master regulator of hematopoiesis [1]. It is involved in the most frequent chromosome translocations in leukemia (i.e. t(12;21)/*RUNX1/ETV6* in pediatric acute lymphoblastic leukemia, t(8;21)/*RUNX1/RUNX1T1* in acute myeloid leukemia (AML), and t(3;21)/*RUNX1/EVI1* in therapy-related AML or chronic myeloid leukemia in blast phase [2, 3]. Moreover, somatic *RUNX1* mutations have recently been identified as recurrent abnormalities in myelodysplastic syndromes (MDS) and AML [4]. These somatic changes are associated with poor prognosis in AML and MDS indicating an increased resistance against exposure to genotoxic agents. *RUNX1* mutations seem to trigger progression into MDS and AML in Fanconi anemia and severe congenital neutropenia [5, 6].

Song et al. (1999) were the first to describe heterozygous germline *RUNX1* mutations in six families, each carrying a different mutation. Individuals carrying germline *RUNX1* may be asymptomatic throughout lifetime or develop familial platelet disorder with myeloid malignancies (i.e. FPDMM, OMIM 601399). Characteristic features are 1) mild to moderate thrombocytopenia; 2) functional platelets defects leading to prolonged bleeding; and 3) an increased risk to develop MDS, AML or T-ALL. There is a great phenotypic heterogeneity. FPDMM is inherited in an autosomal dominant fashion with incomplete penetrance and variable expressivity.

- **Diagnostic criteria to identify persons at risk**

Since the diagnosis of FPDMM in a patient with leukemia carries important critical implications for the patient and also for her/his family, it is important to recognize clinical

features pointing to this genetic predisposition [7]. An important clinical feature is persisting thrombocytopenia or aspirin-like platelet disorder, which are not explained by other reasons.

Thorough pedigree analysis may identify first or second degree relatives with bleeding tendency or hematological neoplasms. It has to be kept in mind that the symptoms may be mild, that hematological neoplasms may present as MDS, AML or T-cell leukemias, and that onset of overt leukemia can be from childhood to adulthood [8]. In case there is a family history of MDS, early onset cancer or a personal history of bleeding tendency, immune deficiency, dysmorphic features and/or intellectual deficits, persons at risk should be transferred to genetic counselling [9, 10].

Comprehensive genetic evaluation and counseling involves a thorough review of an individual's personal medical and family history, including review of somatic cytogenetic and molecular test results and review of medical diagnoses in family members [11]. Through the process of genetic counseling, individuals with leukemia are educated regarding the known hereditary etiologies for hematologic malignancies, provided a personalized risk assessment of the likelihood of a hereditary predisposition within his/her family, and if indicated , offered genetic testing to investigate the possibility of a germline mutation (The University of Chicago Hematopoietic Malignancies Risk Team 2016). As part of genetic counseling, psychosocial assessment and counseling is also provided surrounding psychological concerns unique to hereditary cancer predisposition syndromes, including coping with a diagnosis of cancer, uncertainty, fear of having potentially inherited or passed a cancer predisposition to children [12]. Family members, even those not having inherited a cancer predisposition, may suffer from (survivors') guilt.

In case transplantation from a HLA-matched sibling donor is planned, germline *RUNX1* mutation analysis has to be discussed. It allows to exclude sibling donors, who may be occult carriers of the same genetic syndrome, to prevent adverse outcomes after transplantation like poor stem cell mobilization, delayed engraftment, and increased mortality [13, 14]. Thus, predictive testing of healthy relatives, even of minors, is indicated [15].

Whether there is a distinct subtype of MDS associated with (germline) *RUNX1* mutations needs to be evaluated. Functional analyses show that the absence of RUNX1 results in complete blockage of the differentiation of hematopoietic stem cells [16]. Adult AML with acquired *RUNX1* mutations often presents as immature subtype M0 [17, 18].

In recent years, with the advance of sequencing technologies, many individuals at risk are identified by sequencing large cohorts of patients with MDS or AML and by follow-up of their relatives. Particularly leukemias with homozygous *RUNX1* mutations, bi-allelic *RUNX1* mutations *in trans*, *RUNX1* mutations at a heterozygous allele frequency of 50% and trisomy 21 indicate that the patients are candidates to carry heterozygous germline *RUNX1* mutations causing FPDMM [19, 20].

- **Platelet features**

A personal or family history of low platelet counts and/or bleeding diathesis may be a clue to recognize FPDMM in patients presenting with MDS/AML/T-ALL. Thrombocytopenia is usually mild to moderate and, in some cases, platelet counts may be low-normal or even normal. Platelet size is not affected, similar to other inherited myeloid malignancy syndromes associated with thrombocytopenia, such as *ETV6*- and *ANKRD26*-related thrombocytopenias , which are also characterized by normal-sized platelets [21].

Thrombocytopenia is due to abnormal megakaryocyte maturation and polyploidization and impaired proplatelet formation [22]. Dysmegakaryopoiesis is the most prominent abnormality in bone marrow smears, and is evident even before leukemic transformation [8, 22].

A platelet function defect is present in most, if not all, patients with *RUNX1* germline mutations, leading to abnormal secretion and aggregation. Dense-granule storage pool deficiency represents the most frequent abnormality [8], although other defects, such as partial alpha-granule deficiency, impaired activation of the fibrinogen receptor, GPIIbIIIa, and defective platelet spreading have also been described in some patients [23-25]. The bleeding diathesis is variable within and among families. As some carriers of *RUNX1* mutations may have mild or no bleeding manifestations, the presence of the mutation may go unnoticed and genetic screening is necessary to adequately determine the mutational status.

The finding of platelet abnormalities in patients with FPDMM has revealed the essential role of RUNX1 in the megakaryocytic lineage. RUNX1 acts at different stages of megakaryocyte development by regulating the expression of several molecules relevant to platelet production and function. Reduced expression of RUNX1 target genes, including MPL proto-oncogene, thrombopoietin receptor (*MPL*), non-muscle myosin IIA/myosin heavy chain 9 (*MYH9*) and its regulatory chain MLC2, arachidonate 12-lipoxygenase (*ALOX12*) and *NFE2*, have been shown to underlie the defect in platelet number and function in FPDMM, which involves multiple pathways [22, 24, 26, 27]. In addition, increased levels of non-muscle myosin IIB (MYH10), which is physiologically repressed by RUNX1 during normal megakaryocyte development, contributes to thrombocytopenia by blocking megakaryocyte polyploidization. Persistent expression of *MYH10* in platelets has been proposed as a biomarker of *RUNX1* mutation [28].

- **Functional properties of *RUNX1***

RUNX1 is a master regulator of hematopoietic differentiation. RUNX1 plays an important role in the first wave of hematopoiesis yielding primitive erythroid cells and megakaryocytes [16, 29]. By enhanced expression of CEBPE, it negatively regulates myeloid progenitors and induces granulocytic differentiation [30]. Moreover, RUNX1 binds MLL and methylated H3K4 at PU.1-regulatory regions. RUNX1 mutations impair this interaction resulting in loss of the H3K4me3 mark within PU.1-regulatory regions, and decreased PU.1expression [31].

The granulo/myeloid (G/M) progenitors in *Runx1* knock-out mice have an increased clonogenic potential. *RUNX1* haploinsufficiency coincides with a overexpression of CEBPA and downregulation of CEBPE [30], and with G-CSF hypersensitivity [32]. G/M and megakaryocytic colonies have an immature morphology indicating a shift towards regeneration and an impaired differentiation [33]. Recent data show that RUNX1 also regulates cell adhesion to the bone marrow niche, since Runx1-deficient lineage-committed myeloid progenitors have increased binding to stromal cells [33].

Upon dimerizing with core binding factor beta (CBFB), RUNX1 binds to promotor regions of several transcription factors like PU.1 regulating their expression [1]. Runx1 binding in GMPs correlates with C/ebpa binding and open chromatin [33]. Upon binding of cis-regulatory elements, strong repressors/corepressors, e.g. Gfi1, Sin3a, or protein arginine methyltransferase 6 (PRMT6), may be recruited [34].

Binding to DNA and CBFB occurs in the highly conserved Runt homology domain (RHD) at the N-terminal, transactivation via the transactivation domain at the C-terminal part of RUNX1. Most mutations occur in the N-terminal region. Mutations affecting the

C-terminal part of RUNX1 attenuate the DNA-damage repair response in hematopoietic stem cells [35]. RUNX1 deficiency results in a reduced ribosome biosynthesis, attenuated unfolding protein response, a reduced metabolic profile, lower p53 levels and decreased apoptosis, in line with a model of RUNX1 loss-of-function mutations generating genotoxic stress-resistant hematopoietic stem cells that outcompete normal HSPC [36].

- **Phenotype/ genotype correlation**

Most *RUNX1* mutations lie in the Runt homology domain (RHD). Causative mutations are most often frameshift, nonsense, or in/del mutations that result in premature protein truncation. There are also a number of different missense mutations (Fig. 1A, Fig. 2). Particularly in case of missense mutations, it may be difficult to determine if a genetic variant is a pathogenic disease-causing mutation or a benign variant. Here, functional analyses are needed to better understand the individual consequences. Mutation in RHD, located in the N-terminal part of the protein, impair normal RUNX1 functions through hindered heterodimerization and/or DNA binding [8].

They, as well as mutations in the 5‘ regulatory region, can be summarized as loss-of-function mutations and haploinsufficiency [37]. Missense mutations in the RUNT homology domain, nonsense and frameshift mutations in the C-terminal domain may lead to dominant negative effects [8]. A family with the L472X mutation (according to *RUNX1* transcript 1c, L445X according to *RUNX1* transcript 1b) in the 3‘ region of the gene may predispose to a FPDMM phenotype with severe eczema [38]. Moreover, there may be inherited structural rearrangements involving *RUNX1* [39]. FPDMM can also be due to small deletions involving a few base pairs or single exons of the gene and large deletions leading to loss of the complete coding regions (Fig. 1B). Deletions of large proportions of

the long arm of chromosome 21 cause a contiguous gene syndrome with various clinical signs, e.g. dysmorphisms, mental retardation, thrombocytopenia and increased risk to develop leukemia [9, 10]. Under diagnostic conditions, these large frequently *de novo* deletions can most reliably be detected by arrayCGH/ SNP arrays [8, 10, 15, 40].

There seems to be a higher risk of leukemic transformation in case of dominant-negative point mutations of *RUNX1* as compared to loss of function mutations [8]. While both types of alternations lead to defect megakaryopoiesis and thrombocytopenia, only dominant-negative point mutations enhance the proliferation rate and clonogenic potential of the granulomonocytic population [29]. This correlates with complete inactivation of *RUNX1* and the downregulation of *NR4A3* [41]. Moreover, an increased genetic instability accompanied with a decreased expression of the p53-dependent genes p21 and GADD45A was observed in the expanded granulomonocytic population [29].

However, there is no clear phenotype/ genotype correlation. Within one family, family members carrying the same mutation can present with very different clinical signs and severity. In one family, for example, the father only had mild thrombocytopenia, while three children suffered from myeloid neoplasms [42].

- **Malignant transformation**

The risk of malignant transformation into MDS and AML is estimated to be 30-40% [14]. Patients carrying *RUNX1* mutations with a dominant-negative effect appear to have a higher risk of malignant transformation than patients carrying haploinsufficient *RUNX1* mutations [26, 43]. Different AML FAB subtypes have been reported to occur, while refractory anemia with excess blasts, chronic myelomonocytic leukemia and hypoplastic MDS with myelofibrosis have been described among the cases with MDS and

MPN/MDS. Although malignant transformation most frequently involves the myeloid lineage (MDS/AML), T-cell ALL has also been described, probably related to the role of RUNX1 in T-cell differentiation. Onset of MDS/ AML is at an average of 33 years, with a wide age range, whereas T-cell ALL usually develops at a younger age [11, 19].

During the course of the disease, the second allele may be inactivated, as expected for tumor suppressor genes according to Knudson‘s two-hit hypothesis. As mentioned, there may be second *RUNX1* alterations and duplications of the mutated allele associated with acquired trisomy 21 [20, 39].

Currently there are no definite answers as to what triggers the malignant transformation in carriers of germline *RUNX1* mutations. However, clonal hematopoiesis was identified in more than two thirds of young asymptomatic germline *RUNX1* mutation carriers. The somatic mutations had median variant allele frequencies comparable to MDS/AML patients and included a *DNMT3A* variant, an epigenetic regulator frequently mutated in MDS/ AML. These findings suggest that clonally skewed hematopoiesis frequently precedes development of overt MDS/AML in FPDMM [44].

- **Genetic changes triggering malignant transformation**

Hematological neoplasms in carriers of a germline *RUNX1* mutation need additional secondary mutations to develop. Often myeloid neoplasms display a bi-allelic alteration of *RUNX1*, due to secondary *RUNX1* mutations or trisomy 21 resulting in the duplication of the mutated allele [19, 20, 45]. It appears that germline *RUNX1* mutations are associated with chromosome aberrations typical for MDS and AML, like del(5q), del(7q), +8 or –Y [44, 46]. In adult sporadic AML, *RUNX1* mutations are associated with *MLL* partial tandem duplications, *FLT3-ITD*, *IDH1/2* and *RAS* mutations, and *ETV6* rearrangements.

They often occur in therapy-related AML [17, 18, 47]. *RUNX1* was frequently mutated in myeloid neoplasms in atomic-bomb survivors and in patients with radiation therapy-related myeloid neoplasms [48, 49].

The nature of these secondary mutations may decide when and what kind of malignancy, either MDS, AML or a T-cell neoplasm develops. We reported a family with a father and a daughter carrying the same germline *RUNX1* mutation c.520C>T; p.(Arg174Ter). The father got MDS at the age of 47 years, the daughter at the age of 13 years. In the father, loss of the Y chromosome was found, a cytogenetic abnormality associated with good prognosis. The daughter had a structurally complex karyotype including deletion of 5q, a very poor cytogenetic risk factor in childhood MDS [50]. In this family, the different secondary abnormalities may have had an impact on the age of onset and the clinical severity of the disease [46]. Del(5q) is one of the somatic mutations repeatedly reported to occur in FPDMM [44].

Recently, malignant transformation was reported to be mediated by recurrent somatic mutations in CDC25C gene in up to a half of FPD/AML patients. CDC25C mutations act to enhance mitotic entry and appear to be an early driver event of malignant transformation, followed by acquisition of additional mutations, most notably somatic mutations in GATA2 [51]. Other studies were not able to confirm these findings [19, 44].

Next generation sequencing allows to detect additional mutations in known MDS/AML drivers, e.g. *ASXL1*, *TET2*, *IDH1*, *CEBPD*, *RB1*, *MLL2*, *FLT3-ITD*, *WT1*, and *SRSF2* [19, 44, 45, 52]

- **Clinical management**

Currently MDS and AML in patients carrying a germline mutation is not differently treated. However, if a disease-causing mutation is known in the family, it is important to

prevent hematopoietic stem cell transplantation from a sibling or other relative carrying the familial mutation.

Especially in families with high penetrance regarding leukemia, regular clinical examinations, i.e. differential blood cell counts and annual bone marrow aspirates with morphological, cytogenetic and molecular genetic investigations to detect early signs of leukemic initiation should be discussed with affected individuals [15, 53] reported a patient with increasing thrombocytopenia, who developed a small CD34+ clone with aberrant expression of myeloid markers (CD13 increased, CD33 and CD38 decreased, CD117 and CD123 increased) suggesting that patients with FPDMM, their families and their responsible physicians should be familiar with suspicious clinical symptoms of developing leukemia.

Using new NGS technologies, it is possible to follow-up clonal hematopoiesis. Future studies will show whether serial analysis of clonal hematopoiesis can provide biomarkers for early detection of disease progression in mutation carriers from these high-risk families [44].

- **Outlook**

FPD-iPSCs are a useful tool to investigate mutant RUNX1-mediated molecular processes in hematopoiesis and leukemogenesis [29]. Introducing a wild-type copy of *RUNX1* into iPSC lines from patients with FPDMM reversed most of the hematopoietic differentiation defects and resulted in a significant increase of both the number of megakaryocytic progenitors and of erythroid progenitors. In parallel, the number of granulomonocytic progenitors decreased to control levels indicating that the leukemic potential may also be reduced [54]. Alternative approaches used transcription activator-like effector nucleases

(TALEN) and a plasmid containing wild type *RUNX1* cDNA sequences in patient-derived iPS cells [55]. These experiments appear to demonstrate that by gene correction reversal of the phenotype may be possible.

Conflict of Interest Statement

The authors declare no conflict of interest.

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Figure legends

Figure 1: Detection of a pathogenic RUNX1 mutation. A; Missense mutation c.602G>;p.(Arg201Gln) identified using Next Generation Sequencing , provided by courtesy of Kathrin Thomay and Gudrun Göhring. B; Large deletion leading to the loss of the entire RUNX1 gene identified using array CGH, provided by courtesy of Tim Ripperger and Doris Steinemann.

Figure 2: Schematic representation of germline RUNX1 mutations. RUNX1 mutation nomenclature is based on the reference sequence of transcript variant RUNX1b (Uniprot reference Q01196-1, 453 amino acids). Due to space limitations and for the sake of clarity, one-letter code is used. Frameshift mutations are depicted in blue, missense mutations in green, and nonsense mutations in red. RHD, Runt homology domain (50-177aa); TAD, transactivation domain (242-411aa). [8, 19, 20, 26, 39-41, 43, 45, 46, 52, 56-70]

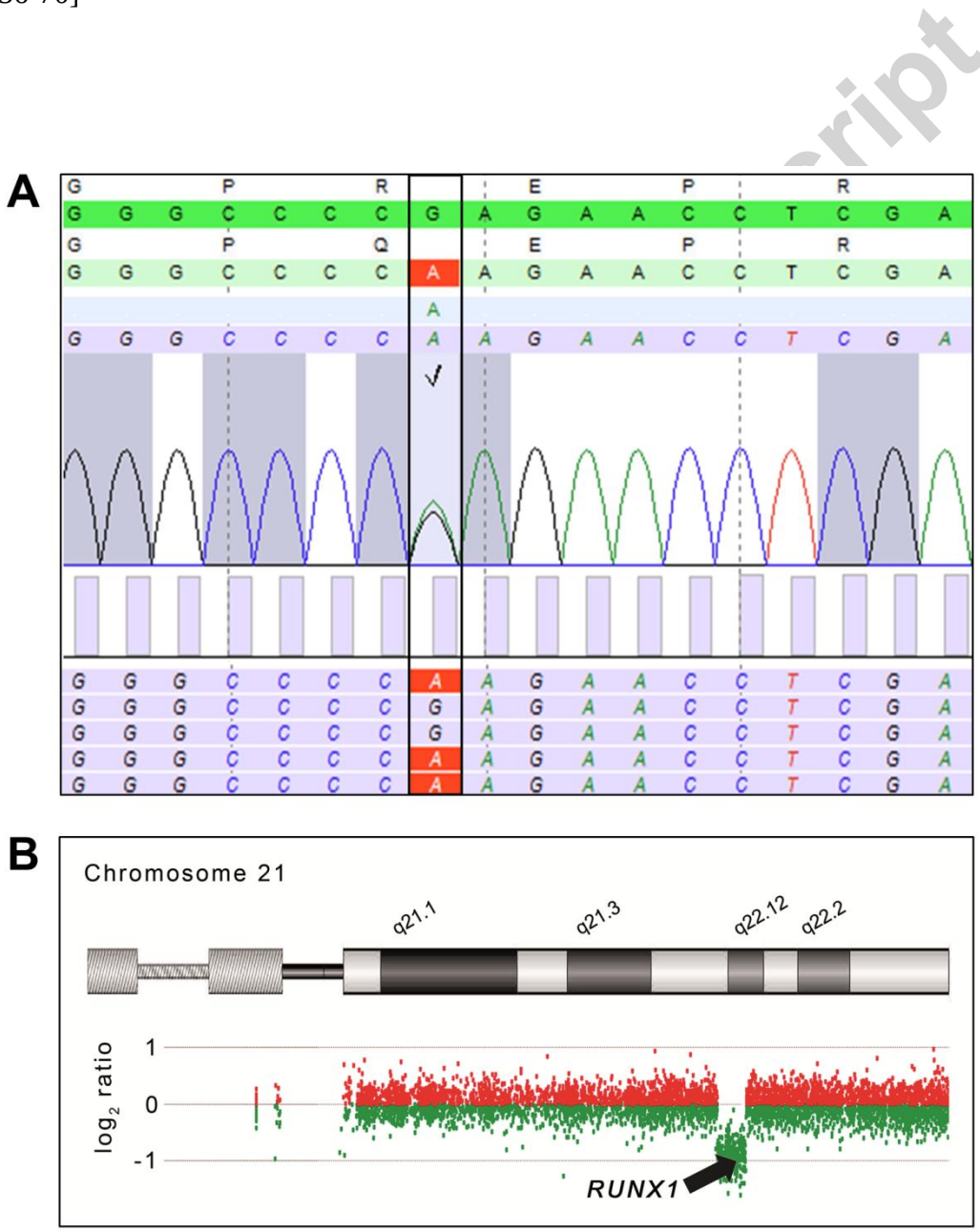

Figure 2