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Severe anemia caused by dominant mutations in *Krüppel-like factor 1* (KLF1)

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Abstract

The etiology and severity of anemia, a common blood disorder, are diverse. Dominant mutations in Krüppel-like factor 1 (KLF1/EKLF) underlie the molecular basis for some of them. KLF1 is a zinc finger transcription factor that plays an essential role in red blood cell proliferation and differentiation. Mutations have been identified in the KLF1 gene that cause hematologic diseases. Two of these alter one allele but generate an extreme phenotype: the mouse Nan mutation (E339D) leads to hemolytic neonatal anemia with hereditary spherocytosis, and the human CDA mutation (E325K) causes congenital dyserythropoietic anemia (CDA) typeIV. These modify functionally important amino acids in the zinc finger DNA-binding domain at positions involved in direct interactions with regulatory elements of KLF1's target genes. Although the two dominant mutations alter the same evolutionarily conserved glutamic acid residue, the substitutions are not equivalent and lead to divergent consequences for the molecular mechanisms underlying activity of these mutants, particularly in recognition and interaction with their unique binding sites. Consequently, the properties of the protein are transformed such that it acquires novel dominant characteristics whose effects may not be limited to the erythroid compartment. KLF1 mutants cause loss-of-function/haploinsufficiency effects on some KLF1 wild-type target genes, while at the same time gain-of-function effects activate ectopic sites and neomorphic gene expression. Such anomalies not only lead to intrinsic red cell problems, but also to expression of non-erythroid genes that systemically disturb organ development.

This review highlights recent molecular, biochemical, and genetic studies of KLF1 mutants, particularly the dramatic consequences that come from just a single amino acid change. The study of these variants provides an important contribution to the overall understanding of the DNA-protein interface of the zinc finger subtype of transcription factors, and the potential clinical consequences of what might appear to be a minor change in sequence.

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Declaration of Competing Interest

The authors declare that there is no conflicts of interest.

Keywords

Krüppel-like factor 1 (KLF1); Transcription factor; Nan – neonatal anemia; Gene expression; Congenital dyserythropoietic anemia; Aberrant transcription

1. Introduction

Anemia is the most common blood disorder, in which decreased amount of circulating red blood cells, hemoglobin, and hematocrit levels are observed. In general, they may be classified into three major groups, anemia caused by: blood loss (a hemorrhage), decreased or defective red blood cell production (inefficient erythropoiesis), or premature destruction of erythrocytes (hemolysis). Many different etiological mechanisms may govern the origin of anemia. Here, we describe one of them that involves an essential erythroid transcription factor - Krüppel-like Factor 1 (KLF1/EKLF). A subset of inefficient erythropoiesis and/or hemolytic anemia occurs as a consequence of mutated KLF1.

KLF1 plays a multifunctional, essential role in virtually all stages of erythrocyte development [1-4]. KLF1 consists of two domains: an N-terminal proline-rich transactivation domain and a C-terminal highly conserved DNA-binding domain comprising three C2H2 zinc fingers that recognize the consensus binding site 5′-NGG-GC/TG-G/TGG-3′ [5-7]. This binding site motif is found in the regulatory regions of many erythroid genes with varied functions; for example, β-globin (*HBB*), the chaperone AHSP, transmembrane, and cytoskeletal protein genes. Heme biosynthesis and blood group antigens also depend on KLF1 activation (reviewed in [3]). At later stages of terminal maturation of erythrocytes, KLF1 activates genes controlling enucleation and cell cycle exit [8-11].

The critical functions of KLF1 during erythropoiesis are supported by genetic ablation studies in mice. KLF1 knockout (KO) leads to defective definitive erythropoiesis and embryonic lethality by day E15 of mouse gestation [1,2]. In addition, mutations that have been identified in the human *KLF1* gene lead to a wide range of phenotypes from benign to pathological [12,13]. In this context, there are two examples of monoallelic, single mutations in KLF1 that generate the dominant phenotype of a severe anemia, manifested even in the presence of one WT-copy allele.

The first case concerns murine severe neonatal anemia (Nan) caused by the Nan-KLF1 mutant. This mouse exhibits hemolytic anemia with many characteristics of hereditary spherocytosis [6,14,15]. The second example concerns human patients with Congenital Dyserthropoietic Anemia type IV (CDA-type IV; OMIM 613673), an autosomal dominant disease. This KLF1 mutant, CDA-KLF1, causes an inherited disorder of red blood cells with hallmarks of morphologic abnormalities of erythroblasts in the bone marrow, and ineffective erythropoiesis together with hemolysis [16,17].

This review will highlight recent studies that help illuminate the similarities and the subtle yet significant differences between these two KLF1 variants, particularly with respect to their dominant effects on gene expression and the resultant pathologies associated with these changes. Given the large background of molecular, biochemical, and genetic studies

of KLF1, of particular interest is to review and compare the mechanisms of action between WT, Nan-KLF1, and CDA-KLF1.

2. The origin and characteristic of KLF1 dominant mutations

The Nan-KLF1 mouse mutant was generated by *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis of a C3H/He male mated to a C3H/He healthy wild type female [18]. The Nan mutation comprises transversion of A1065T, a typically seen type of alteration for ENU-induction [15]. As a result, the codon GAA for glutamic acid in position 339 is changed to GAT encoding aspartic acid (E339D). The Nan defect can be transferred by bone marrow transplantation [14,18] and exhibits a semi-dominant genetic phenotype in terms of inheritance [6].

CDA-KLF1 is a spontaneous mutation in the human *KLF1* gene that involves a transition of GAG to AAG within codon 973 (G973A). As a consequence, glutamic acid in position 325 is substituted to lysine (E325K). So far 8 cases of such a mutation have been described in the literature [16,17,19-30].

Glutamic acid at position 325 is an equivalent to position 339 in mouse KLF1. It is a highly evolutionary conserved amino acid located in the second zinc finger as a part of the “R-E-R” (XYZ) motif that contributes to the transcription factor interface involved in interaction with the 9 bp binding site of KLF1 ‘s target genes (Fig. 1).

Although both substitutions are at the same position in ZnF2, the consequences of the mutations are significantly different. The Nan mutation (E339D) retains the charge (E^- to D^-), whereas the CDA mutation (E325K) alters the charge (E^- to K^+) (Fig. 1). As a result, both mutations acquire new yet distinct features regarding the transcriptional activity of Nan-KLF1 and CDA-KLF1 variants as compared to WT-KLF1. They lead to intrinsic differences in preferences of DNA-binding site recognition and in the repertoire of affected target genes generating pathological outcomes [6,31-33].

3. Pathological consequences of dominant mutations in KLF1

Most studies regarding the consequences of dominant KLF1 mutations have been performed with heterozygotes, as one KLF1 mutant allele is sufficient for the development of severe anemia, and homozygote mutant mice die in the uterus. In humans this is also the case, as no homozygous CDA type IV patients have been reported. Of relevance to this last point, compound heterozygosity of KLF1 mutation in humans can lead to a range of anemias due to the hypomorphic function of each allele, but a lethal mix can arise if each allele is truncated, leading to absence of any KLF1 expression. This likely explains the extreme rarity of any KLF1-null humans (discussed in [12]), where the single only exception in the literature is a hydrops fetalis (severe swelling in the fetus) patient who was transfusion-dependent at birth, was extremely anemic, and developed kernicterus that led to cerebral palsy [34].

The Nan mutation (in Nan/+ mice) leads to severe neonatal anemia that extends throughout adult life as hemolytic anemia that displays many features of hereditary spherocytosis,

including increased osmotic fragility. Splenomegaly, and iron deposition in the kidney, liver, and spleen was also observed [6,14,15]. Embryos of the heterozygotes Nan/+ develop normally, although just after birth they are characterized by skin pallor that follows from hematological features such as reduced numbers of red blood cells, decreased hemoglobin and hematocrit levels, and a significant increase of zinc protoporphyrin levels [14]. Analysis of peripheral blood smears of Nan/+ mice revealed hypochromic erythrocytes with low levels of hemoglobin. Anisopoikilocytosis (erythrocytes of varying different size and shape) and some damaged nucleated red blood cells were reported [6,14]. Closer inspections using scanning electron microscopy have shown that the shape of erythrocytes of Nan/+ mice is more often spherical (spherocytes) than discoidal [14,15]. In addition, examination of erythrocyte ghosts, consisting of only cell membranes without hemoglobin, indicated a global decrease in erythrocyte membrane skeleton proteins [14]. Among them the dramatic reduction of expression of cytoskeleton protein dematin was observed [6]. Such deficiency of the membrane protein contributes to erythrocyte shape and makes them more fragile and prone to hemolysis [14].

Homozygous Nan/Nan mice die in utero at ~E10.5 of gestation [18]. Nan/− heterozygotes, whose single allele of KLF1 is mutated, show total body dysmorphology, with no distinguishable limbs or organs. These observations show clearly how harmful the Nan mutation is at early developmental stages. By comparison, (+/−) heterozygote embryos, which contain only one wild-type copy allele of *KLF1*, are basically not distinguishable from wild type (+/+) KLF1 mice in the embryo or adult [6]. In another comparison, embryos having only one *KLF1* allele with the E339D mutation (Nan/−) die two days earlier than *KLF1* knockout (KO) embryos (−/−), which are simply smaller and pale and do not show any body dysmorphic features [1,2,14]. These comparisons plainly demonstrate how detrimental the single amino acid (E339D) alteration is with respect to development of the whole embryo in a manner not limited to the erythropoietic yolk sac or fetal liver.

The second dominant mutation in the *KLF1* gene is in humans and leads to Congenital Dyserythropoietic Anemia type IV [16,17]. To date, less than 10 patients have been reported and described. Despite this small number, based on phenotypic features and clinical course, these patients can be divided into two groups. The first group is characterized by a mild progression, with transfusion-dependent anemia only in infancy that spontaneously resolves without any treatment [19]. The second group requires more severe treatments in addition to postnatal transfusion dependence, as hydrops fetalis is apparent. Splenectomy improves the quality of life but is not a cure. It has been noted that the severity of symptoms is correlated with the male gender, where intrauterine transfusions were necessary to maintain pregnancy [17,19].

Peripheral blood smears of CDA type IV patients show spherocytes and a very large number of orthochromatic erythroblasts (precursors of erythrocytes) that have not extruded their nuclei [17,19,27]. Observations of the ultrastructure of these cells with an electron microscope revealed a large number of abnormalities, including atypical cytoplasmic inclusions and enlarged nuclear pores [17]. Smears of the bone marrow aspirate have shown erythroid hyperplasia with majority of acidophilic erythroblasts. Closer inspection under an electron microscope revealed abnormal structures of chromatin and chromatin bridges

[35]. Other abnormalities such as peripheral double cytoplasmic membranes, invagination of nuclear membranes with intranuclear precipitated material [27], and euchromatin areas connecting nuclear membrane [29] have been described. In general, patients with the E325K mutation in KLF1 suffer from severe anemia and although they differ in some symptoms, all have increased fetal hemoglobin from 12% to 42% in peripheral blood, which alleviates the anemia to some extent [17,19,27-29]. Other hallmarks of CDA type IV patients is lack of some erythroid cell surface markers, including CD44 and aquaporin 1 (AQP1), in circulating erythrocytes and erythroblasts [17,27].

In addition, some non-erythroid congenital abnormalities were observed in male patients with CDA type IV. The one described by Arnaud and colleagues in 2010 had deceleration of growth, hepatomegaly, hypertrophic cardiomyopathy and several dysmorphic features such as micropenis, hypospadias, wide anterior fontanels and hypertelorism [17]. Ravindranath et al. reported a rare and interesting case of genotypically normal male having internal and external female sex organs [19,30].

4. Molecular mechanism underlying the phenotype of dominant mutations in KLF1

4.1. Identification of the Nan-KLF1 and CDA-KLF1 unique DNA binding sites

Both dominant mutations in KLF1 are located in the second zinc finger (Fig. 1), in position +3 of its α-helix, within the structural motif of the C2H2-type finger that fits into the DNA major groove [5]. This suggested that any substitution of amino acid in this location might interfere with proper, wild-type recognition and binding of KLF1 target genes. The middle 5th position of the 9 bp long binding motif is especially crucial for the interaction with amino acid in position 339/325 of mouse and human KLF1, respectively [36,37].

Indeed, the determined binding motifs for both Nan and CDA-KLF1 mutants differ from the WT motif. Two approaches have been undertaken to identify the consensus binding site for KLF1 variants. First, *in vivo* Genome-Wide ChIP-seq analyses were performed using K1-NanER or K1-CDAER cells derived from a murine Klf1 (−/−) fetal liver cell line transfected with plasmids containing ER (estrogen receptor) fusions with Nan- or CDA-KLF1 [32,38]. Second, the *in vitro* CASTing-seq technique, in which enrichment of complex formation between KLF1-3ZnF and a library of random oligonucleotides, was monitored by a gel retardation assay. The selected oligonucleotides after sequencing enabled identification of the CDA-KLF1 consensus binding site [33]. Using the *in vivo* assay, the following binding motifs presented on G-rich strand were determined: for Nan-KLF1 5′-NGG-G**C/A**N-G/TGG-3′ and for CDA-KLF1 5-NGG-G**A/G**G-GG/AG-3′ [32,38]. The Nan novel binding motif contains C/A in the middle 5th, crucial for interactions with E339, and surprisingly high degeneracy in position 6th, that can bind A, G or T instead of only G, as WT-KLF1 does [32]. Such degeneracy reduces the binding affinity of the motif as observed by EMSA and ChIP-seq data (Fig. 2) [32].

The *in vitro* approach emphasizes different, more broader aspects of the interactions and potential binding abilities, as it is performed irrespective of any particular state of cells such

as proliferation or differentiation. By this technique, the motif 5′NGG-G**G**G/T-G/TG/TG/T-3′ for CDA-KLF1 was identified. It solely contains G in the middle 5th position [33], and differs from the motif obtained *in vivo*, which can have A/G [38]. The sequence degeneracy was directly tested and showed that the CDA-KLF1 mutant binds motifs having only G in the 5th position [33,38]. Another feature of the CDA-KLF1 binding motif obtained *in vitro* is the high degeneracy of the 3′ end of the motif. Specifically, G or T in the 6th position is acceptable, as is any nucleotide in the 7th–9th position if at least one of them is G or T [33]. A summary of the interaction of the KLF1 variants and their DNA binding sites relative to WT-KLF1 transcription factor is shown in Fig. 2.

4.2. Alterations of wild-type target gene activation by mutated KLF1 variants

The Nan- and CDA-KLF1 variants have their own specific, preferentially bound DNA motifs that differ from the WT-KLF1 5′-NGG-G**C/T**G-G/TGG-3′ site. This affects the activation of WT target genes even in the presence of the WT-KLF1 allele. The Nan-KLF1 variant narrows and limits the recognition of WT sites to those having a C in 5th position, but at the same time extends the ability to bind sequences having any nucleotide in the 6th position of the DNA binding motif. Therefore, Nan-KLF1 is limited in recognition of some WT-KLF1 target genes, yet also acquires the ability to activate new, ectopic neomorphic genes [31,32].

In contrast to Nan-KLF1, CDA-KLF1 binding sites are *mutually exclusive* with WT-KLF1 sites; that is, the CDA-KLF1 mutant will not recognize, bind nor activate any of WT-KLF1 targets. However, our recent studies have shown that CDA-KLF1 can appear to activate some WT-KLF1 target genes, but this still follows from the presence of its own binding site, such as occurs when the WT-KLF1 target gene also contains CDA-KLF1 binding sites within its regulatory sequences (for instance, the *Lu/BCAM* gene [33]). The high degeneracy of the 3′-end of the CDA-KLF1 motif may be beneficial in this situation, since it increases the chances of finding such a sequence in the regulatory regions of the WT-KLF1 Target genes (Fig. 2) [33]. This notion is also supported by results obtained from *in vivo* ChIP-seq data that show three-fold more peaks (binding sites) for CDA-KLF1 compared to WT-KLF1 [38].

4.3. Transcriptional consequences of a recognition of novel binding sites by KLF1 variants

The mutated KLF1 transcription factor in Nan/+ or CDA/+ heterozygotes leads to multiple consequences: loss-of-function, haploinsufficiency, and gain-of-function effects at the same time. To gain some insights into the transcriptional consequences of KLF1 mutants, researchers used different approaches and constructed several systems for microarray profiling, transcriptome, 4-thiouridine (4sU)-RNA-seq and qRT-PCR analyses [20,31,32,38,39].

In terms of the mouse mutation, one study analyzed directly acquired Nan/Nan or Nan/+ hematopoietic organs at different stage of development, such that fetal liver or bone marrow, spleen and peripheral blood of adult Nan/+ mice, were used [6,31,32,40,41]. In some studies embryonic stem cells derived from Nan/Nan mice were differentiated into embryoid bodies

and then subjected to analysis of early stages of erythropoiesis [31], while in others the *in vitro* cellular model of the Nan mutation using rescue of a *KLF1* null erythroid cell line (K1) were established and used [32].

In the case of the human mutation, the circumstances were more complicated, mainly due to limited access to patients with CDA type IV. Kohara's group generated induced pluripotent stem cells (iPSCs) from one CDA patient's peripheral blood (CDA-iPSC) in combination with targeted genome editing technology [20]. Other scientists isolated mononuclear cells from a different CDA patient's blood that were cultured for proliferation and differentiation before being studied [39]. In addition, two *in vitro* systems were generated: stable transfected K562 cell lines with dox inducible CDA-KLF1 expression [33] and murine erythrocyte cell lines with tamoxifen-induced CDA-KLF1 expression on a KLF1 (−/−) genetic background [38].

4.3.1. The loss-of-function/haploinsufficiency effect - hypomorphic function

—The mutant CDA-KLF1 and Nan-KLF1 alleles lose all or part of their transcription capacity to activate wild-type KLF1 targets (Fig. 3) and thus generate a haploinsufficient genetic background in heterozygous organisms. There are several KLF1 target genes such as *Lu/BCAM, Bcl11A, HBD, HB2A,* that are highly sensitive to KLF1 expression levels [12,42-44].

Both neonatal anemia and CDA type IV are characterized by impaired globin expression. Fetal and embryonic globin expression are elevated in heterozygous mutants [6,17,19-22,26-29]. This may be due to KLF1 haploinsufficiency in Nan/+ and CDA/+ and reduced level of target *Bcl11A* gene expression. *Bcl11a* encodes a γ-globin repressor and if not correctly expressed, the globin switch is disturbed [45,46]. This provides a possible explanation for the KLF1 haploinsufficiency effect on elevated fetal hemoglobin levels [6,17]. Recent studies revealed yet another possible explanation. The globin switching studies in Nan/+ mice have shown that the E12.5 fetal liver cells display growth and differentiation defects, which may contribute to the delayed appearance of definitive erythrocytes [47]. As a result Nan-KLF1 affects mostly the dynamics of progression from primitive to definitive erythropoiesis during mouse development and not the expression of embryonic globins, which stay low (0.3% [47]).

Lu/BCAM is another KLF1 haploinsufficiency sensitive gene. Usually the presence of the mutation in KLF1 causes a reduction of BCAM level, which leads to the In(Lu) blood group [48]. However, patients with CDA type IV do not develop an In(Lu) disorder due to the ectopic CDA-KLF1 binding site in the *BCAM* regulatory region [33] previously mentioned.

Membrane proteins are yet another group of downregulated proteins in the mutant KLF1 heterozygote. The levels of band3, dematin, β-adducin and protein 55 are reduced in Nan/+ [6]. In addition, reduced levels of protein 4.1 and 4.2 were observed in both Nan/+ and CDA/+ compared to WT. Reduced levels of membrane proteins may explain its instability and may be associated with commonly observed erythrocyte distortion and hemolysis sensitivity [6,20,39].

4.3.2. Gain-of-function - neomorphic function—The hypomorphic model of the biochemical functions of Nan-KLF1 and CDA-KLF1 cannot fully explain the dominant heterozygous phenotype. Given the crucial location of the amino acid change within the zinc finger domain, the unique, specific binding sites of Nan and CDA-KLF1 can activate a new set of genes never normally activated by WT-KLF1. A wide range of transcriptomic approaches and analyses have been used to reveal the ectopic transcriptional consequences of binding the aberrant DNA motifs.

RNA-seq on the K1-NanER cell line showed 735 upregulated and 442 downregulated genes compared to wild type K1-ER. The majority of Differentially Expressed Genes from KLF1 Nan/+ fetal liver were not normally associated with hematopoietic functions, which was consistent with a neomorphic function of the *Nan* mutation [32]. Expression of *Dusp7*, *Sh3bp1* and *Zfp36* gave the highest scores for the neomorphic targets of Nan-KLF1. *Sh3bp1* is a RhoGAP protein normally expressed by neural tissues. *Dusp7* the dual specificity phosphatase that upregulation could lead to downregulation of p38-MAP kinase signaling and *Zfp36* could induce a signal to proliferate (Fig. 3). All together they might be involved in erythroid cell toxicity and the semi-dominant anemia [32].

Data obtained from RNA-seq analysis of Nan/+ E13.5 fetal liver cells compared with their wild-type littermates revealed ~80 most highly increased ectopically expressed genes in Nan/+. Two of them encode for genes that lead to expression of secreted proteins: hepcidin and interferon regulatory factor 7 (IRF7) (Fig. 3). They were selected for further analysis because neither hepcidin nor IRF7 are expressed within the wild-type erythroid compartment and they could systemically affect normal erythropoiesis *via* the circulation [31]. *Hamp* encoding hepcidin is a regulator of cellular iron use by virtue of its interaction with ferroportin, and is primarily expressed in the adult liver. IRF7 is a transcription factor primarily expressed by macrophages that mediates inflammation by virtue of its activation of interferon β (IFNβ) [31]. Misexpression of hepcidin and deficient expression of erythroferrone lead to a decrease in iron availability and lower red cell numbers. Misexpression of IRF7 induces IFNβ expression, which has a repressive effect on erythroid development [31]. Such aberrant activation of genes encoding secreted factors that exert a negative effect on erythropoiesis and iron use together with intrinsic hypomorphic effects of Nan-KLF1 within erythroid compartment contribute to exacerbation of the severity of neonatal anemia [6,14,15,31].

Similar approaches have been used to decipher the mechanism underling the contribution of CDA-KLF1 in dyserythropoietic anemia. For example, RNA-seq on the K1-CDA-ER inducible cell line was performed. In total, 244 genes were significantly upregulated and 19 genes were significantly down-regulated in response to KLF1-CDA-ER [38]. Analysis of the pathways most affected by the ectopic expression of KLF1-CDA-ER revealed a number of altered categories: aldosterone-regulated sodium reabsorption, EGFR tyrosine kinase inhibitor resistance, TGFβ signaling pathway, relaxin signaling pathway, and colorectal cancer [38]. Although these pathways are functionally distinct from each other and engaged in separate tissues, in combination they might be involved in disrupting the normal erythroid transcriptional program in CDA/+ cells [38].

Transcriptome studies were performed on erythroid cells expanded from peripheral blood of a patient with CDA type IV [39]. The presence of CDA-KLF1, which recognizes and binds new specific DNA sites, leads to ectopic misactivation of non-erythroid genes in the CDA erythroid cell. These targets include *CCL13*, *LTC4S*, *PDPN*, and *IL17RB* (Fig. 3), genes coding for molecules that play varied roles in inflammation and respiratory issues and that are not typically expressed in the erythroid cell [39]. Expression of these genes was only possible due to the expression of CDA-KLF1, which activates aberrant DNA sites. For example, a potential site in the IL17RB promoter binds CDA-KLF1, as shown by quantitative *in vivo* analysis carried out in stable K562 cell lines with induced expression of CDA-KLF1 [33].

So far, given all the neomorphic genes for CDA-KLF1, it remains difficult to find any correlation with non-erythroid features seen in many CDA type IV patients, such as short stature and gonadal dysgenesis. Further RNA expression profiles of other patients are needed. It is also likely that at least some of the characteristic might be secondary due to severe anemia in utero.

In CDA (and similar to Nan), the gain-of-function effects may not depend entirely on the expression of neomorphic target genes. This may also apply to the expression of some wild-type target genes that are activated at the wrong time/location or in an uncontrolled amount due to the occurrence of WT and aberrant binding sites in close proximity. Genes that control the cell cycle belong to this group. Increased expression of negative regulators such as CDKN2C (p18) and CDKN2A (p16, p14 arf) was observed in cell cycle dysregulation in iPS cells derived from a patient with CDA type IV, which caused G1 phase cell cycle arrest in CDA erythroid cells [20]. E2F2 and E2F4 levels are decreased when analyzed in CDA type IV patient red cells [39]. Similarly in Nan, disrupted expression patterns are complex, as both E2F2 and CDKN2C are affected [6]. Moreover, CDKN2C (p18) and CDKN1B (p27) are KLF1 targets that are involved not only in regulation of cell cycle exit but also in enucleation of murine erythroid cells [10]. Consistent with this late stage role of KLF1, dramatic enucleation problems are seen in all CDA type IV patients [*e.g.* [27]]. Recent RT-qPCR analysis has confirmed that the CDKN1B gene contains binding sites specific for CDA-KLF1 in its regulatory regions that are able to activate p27 transcription [33].

5. Future prospects

The KLF1 gene was identified decades ago, as was its role in the control of β-globin expression [1,2,36,49]. However, the relationship between KLF1 mutations and hematologic disease has only recently been established [12,13]. As discussed in the present review, it is now clear that the Nan- and CDA-KLF1 mutations are associated with the disruption of transcription control of erythroid cell regulators and, together with other abnormalities in the erythroid membrane and enzymes, leads to dyserythropoiesis and its associated morphological changes and pathology of the red cell. The result in both mouse and man is lifelong anemia that can be transfusion-dependent, extramedullary erythropoiesis that can result in changes such as splenomegaly, neomorphic genetic changes that alter red cell identity, and organ damage that follows from lysis of red cells and iron deposition. As a result, the prevalence of patients with CDA type IV may well be quite underestimated,

complicated also by the potential association with hydrops fetalis [34] and loss of late stage pregnancies or death of newborns without investigation of the cause (discussed in [12]).

The success of future analyses for KLF1 mutations, in particular those that relate to CDA type IV, will rely on a number of coordinated approaches. First, directed genetic sequence analysis of KLF1 (promoter, intron, exon) should be routinely included in cases of aberrant hematologic parameters (discussed in [12,50]). Although this is important on an individual patient basis, on a global scale this also led to the statistically robust and dramatic link in prevalence of KLF1 mutations with incidence of β-thalassemia in southern China [51]. Whole exome sequencing can aid in the identification of coinherited mutations [19].

A corollary of this is that genetic analysis that corresponds to the clinical diagnosis is required, specifically categorization consistent with properties of all CDA type IV patients: a dominant disease with the specific KLF1 E325K mutation in one allele [52,53]. This should not be confused with compound heterozygous KLF1 mutations (e.g. [54,55]), particularly those leading to complex hemolytic anemias (discussed in [12]).

A third avenue to pursue will be to develop accessible means for analysis of more samples. This is required by the paucity of CDA type IV patients, which only number 8 so far. Specifically, establishment of iPS cells [20], or use of a model cell system into which the mutation is incorporated (*e.g.*, BEL-A cells [56]) will provide a ready and large-scale source of material for in-depth genetic and biochemical analyses.

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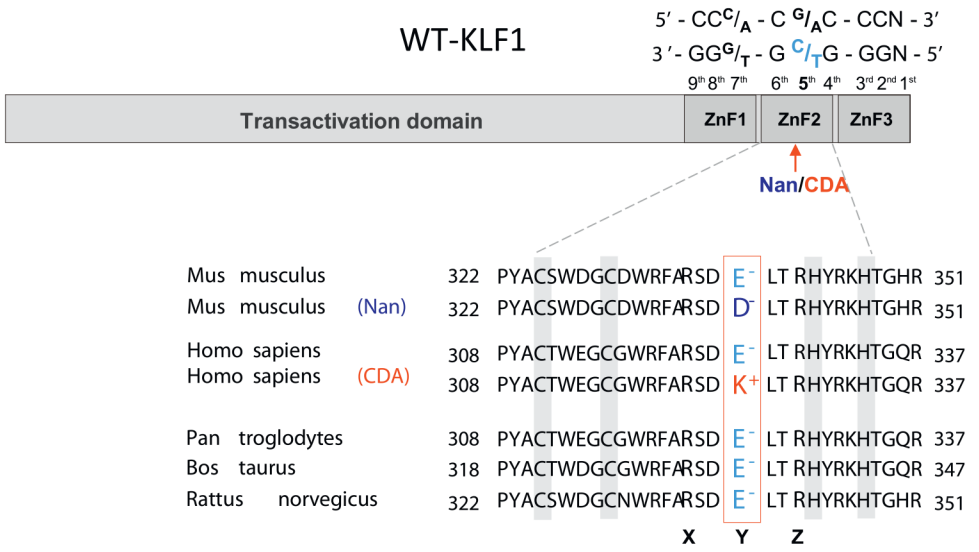

Fig. 1. KLF1 domain structure with location of dominant mutations Nan and CDA.

(Top) A schematic of KLF1 transcription factor domain structure, showing the transactivation and the DNA-binding domains, the latter of which consists of three zinc fingers (ZnF). Dominant mutations located in ZnF2 are marked by a red arrow. The 9 bp WT-KLF1 binding site with position numbering is shown above the ZnFs. (Bottom) An alignment of amino acids sequences of ZnF2 from various mammalian species. The mutations map to the middle amino acid of the R-E-R motif (assigned as X, Y, Z) [5] critical for DNA interactions. The evolutionarily conserved glutamic acid (E) interacting with the 5th position of 9 bp binding site (shown at the top of KLF1 schematic) is marked in light blue. The mouse Nan mutation (E^- to D^-) is marked in dark blue and the human CDA mutation (E^- to K^+) is marked in red. The alteration in charge of substituted amino acid is emphasized. Cysteines and histidines involved in zinc coordination are shadowed.

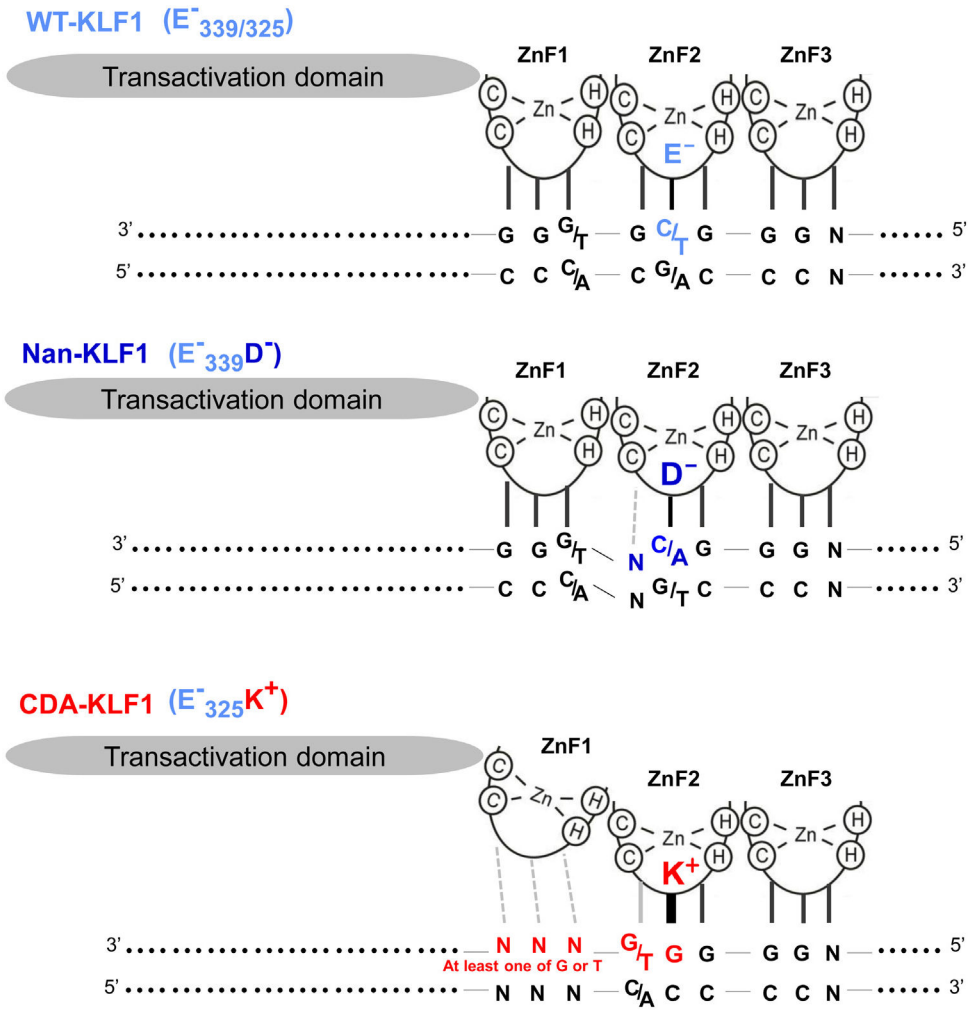

Fig. 2. A scheme summarizing interface between KLF1s (WT and mutants) and their DNA consensus binding sites.

WT-KLF1 recognizes and binds DNA binding sites with C or T in the 5th position (marked in light blue), which directly contacts glutamic acid (pos. E339/325) [6,7]. The Nan-KLF1 (E339D) mutant alters specificity in the 5th position to C or A and loses specificity in the 6th position by accepting any nucleotide (marked in dark blue and bulge) [32]. The CDA-KLF1 (E325 K) mutant recognizes and binds the sites that are mutually exclusive with the WT-KLF1 and Nan-KLF1 sites. CDA-KLF1 interacts with G in the 5th position. CDA-KLF1 accepts higher degeneracy at the 3′ end of the binding sequence, namely: G or T in the 6th position, and any nucleotides in the 7th-9th positions, if at least one of them is G or T (marked in red) [33].

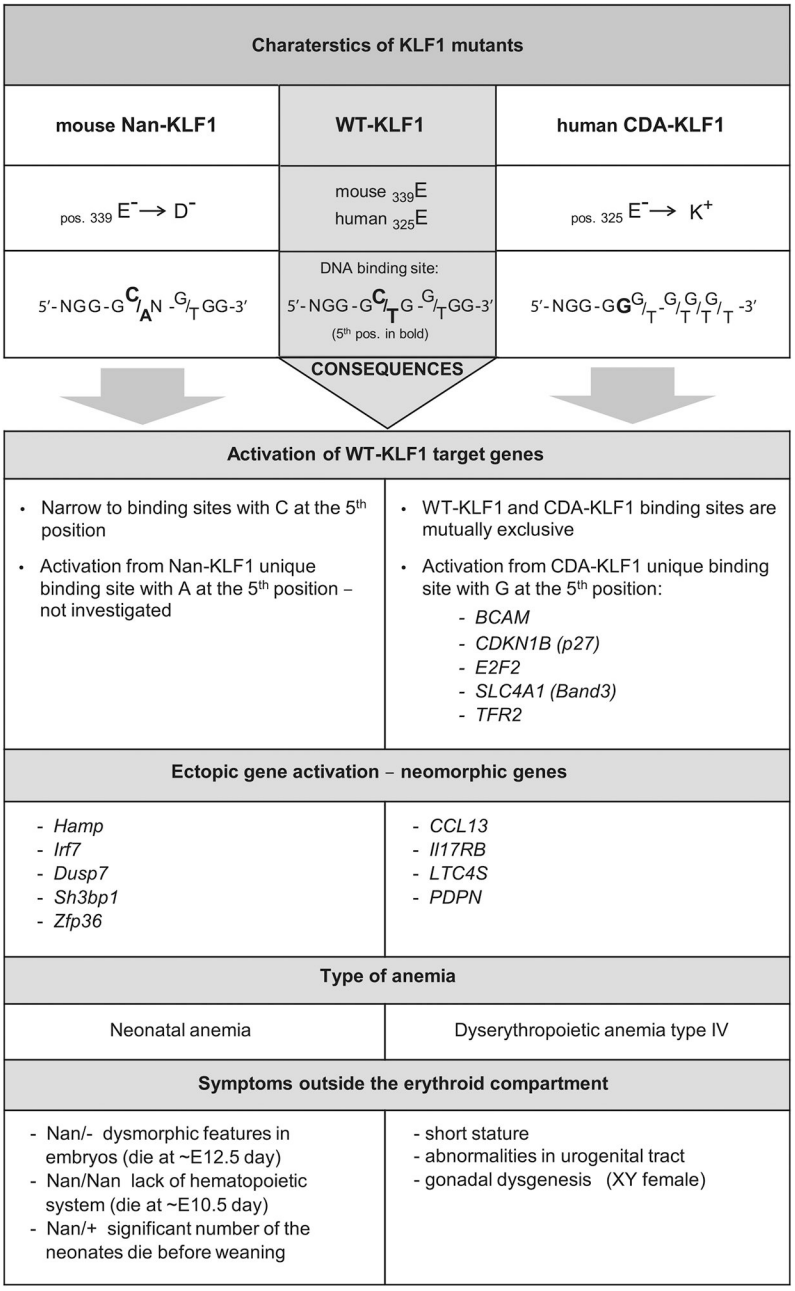

Fig. 3.

The comparison of clinical and molecular characteristics between Nan-KLF1 and CDA-KLF1 dominant mutants in comparison with WT-KLF1.