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Sequence-selective disruption of EKLF zinc finger-DNA interactions: a unique mechanism of severe anemia in the *Nan* mutant mouse

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Studies of mouse models of anemia have long provided fundamental insights into red blood cell formation and function. Here we show that the semi-dominant mouse mutation, *Nan* (neonatal anemia), carries a single amino acid change (E339D) within the second zinc finger of the erythroid Krüppel-like factor, EKLF, a critical erythroid regulatory transcription factor. The mutation alters the DNA binding specificity of EKLF such that it no longer binds promoters of a subset of its DNA targets. Remarkably, even when mutant *Nan* and wild-type EKLF alleles are expressed at equivalent levels, the mutant form selectively interferes with expression of EKLF target genes whose promoter elements it no longer binds. This yields a distorted genetic output and selective protein deficiencies that differ from those seen in EKLF-heterozygous and EKLF-null red cells, and presents a unique and unexpected mechanism of inherited disease.

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Introduction

Hereditary spherocytosis (HS) is the most common cause of inherited hemolytic anemia in Northern Europeans, with an estimated frequency of approximately 1/5000 (1). Molecular defects in the structural components of the red cell membrane skeleton comprising vertical interactions with the overlying lipid bilayer (spectrin, ankyrin, protein 4.2, band 3) underlie HS, which follows from mechanically weakened red cells that lose membrane surface area, become increasingly spheroidal, and are removed by the spleen (1, 2).

Spontaneous and targeted mutations in membrane skeleton genes in mice have been instrumental in defining the components, interactions, and functions of membrane skeleton proteins (3-11). The mouse mutation *Nan* (neonatal anemia) is an ethylnitrosourea (ENU)-induced semi-dominant hemolytic anemia first described by Mary Lyon over 25 years ago (12). *Nan* homozygotes die at E10-11 from a "severe lack of haemopoiesis;" with no overt, non-erythroid defects evident. Heterozygotes (*Nan*/+) survive with a life-long, severe hemolytic anemia that displays many features of HS including increased osmotic fragility, splenomegaly, and iron deposition in the kidney, liver, and spleen (12). *Nan* is transferable through hematopoietic stem cells and localizes to mouse Chromosome (Chr) 8 (13). Here, we describe positional cloning of *Nan*. Unlike other models of HS, *Nan* does not carry a primary defect in a membrane skeleton gene. Rather, *Nan* is caused by a mutation in the *Klf1* (Krüppel-like factor 1) gene encoding Erythroid Krüppel-like Factor (EKLF) (14). The mutation causes HS by a heretofore undescribed mechanism that is unique not only to HS, but to genetic disease in general.

EKLF, the founding member of the KLF family of transcription factors (14, 15), is a hematopoietic C2H2 zinc finger transcriptional factor that plays a global role in erythroid gene expression by regulating expression of heme biosynthetic, red cell membrane, globin stabilizing, and cell cycle proteins (16-18). EKLF expression is stringently restricted during development to erythropoietic sites - embryonic yolk sac, fetal liver, and adult bone marrow and spleen (19). During hematopoiesis, EKLF is first expressed at low levels in common myeloid progenitors, but achieves a high level of expression solely within the megakaryocyte-erythroid progenitor, remaining elevated selectively in its red cell progeny (20). EKLF levels directly influence this bipotential lineage decision by repressing megakaryopoiesis while accentuating erythropoiesis (20-24).

The activation function of EKLF has been most extensively analyzed at the ß-globin locus, where its plays critical roles in genetic regulation of the adult ß-globin promoter (25, 26). First, specific amino acids within its three C2H2 zinc fingers interact with guanosine residues at its cognate DNA binding site, leading to precise and high affinity binding (14, 27). Second, it integrates chromatin remodeling and transcriptional activities via critical protein-protein interactions (28-31). Consequently, EKLF plays a central role in establishing the precise three-dimensional looping of the distant LCR (locus control region) with the proximal ß-globin promoter in adult erythroid cells (32), resulting in formation of an "active chromatin hub". This property extends beyond the ß-like globin region, as it also facilitates and coordinates recruitment of more distant loci into transcription factories in the erythroid nucleus (33). Third, EKLF is a regulator of ß-like globin switching (34). Although EKLF-heterozygotes are phenotypically normal, in the total absence of EKLF the active chromatin hub is not properly formed (32) and adult ß-globin is not expressed, leading to embryonic lethality by E14.5 due to a profound ß-thalassemia (35, 36). At the same time, murine embryonic ßh1-globin and (transgenic) human fetal γ-globin genes are not switched off properly and remain expressed at higher levels and for a longer time during development (37-39). In humans, point mutations in the EKLF cognate binding site lead to ß-thalassemia (27); these patients have markedly elevated fetal hemoglobin levels (discussed in (40)).

We have identified a single amino acid substitution (E339D) in the second zinc finger of EKLF in *Nan* mutants. The earlier embryonic lethality of *Nan* homozygotes and severe anemia in *Nan* heterozygotes distinguish *Nan* mutants from targeted EKLF knockout strains and led us to investigate the molecular nature of the EKLF mutation in detail. We find that the E339D change in EKLF results in failure to bind and transactivate a subset of downstream targets in *Nan* erythroid cells in a manner dependent upon the cognate EKLF DNA binding sequence, where a single nucleotide makes the difference between recognition by both wild type (WT)- and Nan-EKLF versus recognition by WT-EKLF alone. An EKLF-DNA interaction model provides a structural basis for the failure of Nan-EKLF to bind selective recognition sites. Moreover, the Nan-EKLF variant distorts the expression pattern of EKLF target genes even in the presence of WT-EKLF. Quite unexpectedly, genes deficient in expression in *Nan* heterozygotes are those whose EKLF binding elements are not recognized by Nan-EKLF, such as the membrane protein dematin, the cell cycle regulator E2F2, and the BKLF transcription factor. Such target-selective effects lead to a distorted genetic readout and account for the severe anemia in *Nan*/+ mice. Our

findings have general implications for the structural parameters involved in DNA recognition by KLF proteins and the unanticipated selective effects on target gene regulation that can arise after mutation.

Results

Phenotyping and mapping of *Nan.* In agreement with original reports (12, 13), we find that *Nan* homozygotes die *in utero* at ~E10-11, while heterozygotes are severely anemic at birth and throughout life. Loss of heterozygotes *in utero* occurs; reciprocal *Nan*/+ x WT matings produce only 34% affected offspring. The red cell count, hemoglobin, and hematocrit are significantly decreased in *Nan*/+ adults versus WT (Supplemental Table 1). The percent circulating reticulocytes and spleen weights are dramatically increased (Supplemental Table 2), indicating a compensatory acceleration of erythropoiesis. *Nan*/+ peripheral blood smears (Fig. 1A) reveal extensive anisocytosis with many microcytes, spherocytes, and hypochromic cells, consistent with the increased red cell distribution width (RDW) and hemoglobin distribution width (HDW). Platelet levels are slightly, but significantly, increased (Supplemental Table 1).

Nan/+ bilirubin and iron levels do not differ significantly from controls, but zinc protoporphyrin (ZnPP) is dramatically increased (Supplemental Table 2). Increased ZnPP suggests that a heme biosynthesis defect is unlikely (41). Consistent with severe hemolysis, iron accumulates in *Nan*/+ kidney (Fig. 1B), liver (Fig. 1C), and spleen (Fig. 1D). Erythropoietic foci are observed in the mutant liver (Fig. 1C, arrow).

Examination of membrane skeleton proteins by SDS-PAGE (Fig. 1E) and by western blotting (Fig. 1F) quantitated with scanning densitometry (Supplemental Table 3) reveal that band 3, proteins 4.1 and 4.2, dematin (protein 4.9), β-adducin, and p55 are decreased in *Nan*/+ red cell ghost membranes compared to WT. Consistent with western blotting results, p55 mRNA expression is markedly decreased in peripheral blood reticulocytes and in the spleen (Supplemental Figure 1). These changes are in contrast to the global deficiencies in *Nan* membrane skeleton proteins reported by White *et al.* (42), which may reflect differences in genetic background. Notably, as has been consistently observed in studies of adducin deficiency (10, 43), marked up-regulation (> 400%) of the actin capping protein CapZα (EcapZ) occurs in the red cell membrane and likely compensates for loss of adducin in actin capping (Fig. 1F, Supplemental Table 3).

We fine-mapped *Nan* to a 1.3 megabase (Mb) interval between *D8Mit78* (86.93 Mb) and *D8Mit79* (88.19 Mb) on Chr 8 (Ensemble, http://www.ensembl.org) containing 46 known or predicted genes (Mouse Genome Informatics, http://www.informatics.jax.org). More than 600 kb of sequence were obtained, including all exons and intron-exon boundaries. Only *Klf1* showed a sequence change in *Nan*; an A to T transversion at base pair 1064 (reference sequence NM_010635) changes glutamic acid to aspartic acid in exon 3 (E339D) within the second zinc finger required for DNA binding (14). The change creates a *Mbo*I restriction site that segregates 100% with the *Nan* mutation. Sequencing of *Klf1* in E9.5 WT, *Nan*/+, and *Nan/Nan* DNA showed the expected sequence change (Fig. 1G).

E339D selectively alters target gene binding. Each EKLF zinc finger interacts primarily with the G-rich strand of its DNA target via critical basic amino acid/guanosine interactions that yield a highly specific consensus interaction sequence (14) (Fig. 2A). Mutations in this sequence lead to ß+ thalassemia in humans (HbVar: http://globin.bx.psu.edu/cgi-bin/hbvar/query_vars3). The E339D mutation is located in the second zinc finger of EKLF in position +3 of its α-helix, within the structural motif of the C2H2 type finger that fits into the DNA major groove (27). Based on structural data from other C2H2 zinc finger proteins (44, 45), this position is involved in interactions with the middle nucleotide of the consensus EKLF binding motif 5'NGG G**N**G NGG3' (Fig. 2A, B). E339 is absolutely conserved across the entire murine and human KLF family and across EKLF proteins from different species (15). Based on this critical location, we tested whether the E339D mutation affects binding to EKLF targets (Fig. 2C, D).

WT-EKLF forms complexes with all tested oligos comprising EKLF binding motifs from target genes known to be bound by EKLF in vivo, regardless of whether the nucleotide in the middle position of the motif (as presented on the G-rich strand) is cytidine or thymidine (Fig. 2C, E). However, Nan-EKLF is able to generate complexes efficiently only when cytidine is present; for example, with sites derived from p21 (*Cdkn1a*, cyclin-dependent kinase inhibitor 1A), p18 (*Cdkn2c*, cyclin-dependent kinase inhibitor 2C), and AHSP (*Ahsp*, alpha hemoglobin stabilizing protein) (Fig. 2C, E). Based on these results we can distinguish categories of EKLF binding site recognition specificities (Fig. 2E). The first category comprises sites that contain cytidine in the crucial middle position of the EKLF binding motif; these are recognized and bound by both WT- and Nan-EKLF. The second category comprises sites with thymidine in the middle position; these are recognized only by WT-EKLF and include genes encoding ß-globin (*Hbb*), E2F2

(*E2f2*), and BKLF1b (*Klf3*). Interestingly, use of an oligonucleotide that contains a guanosine in this critical position is not recognized by either WT- or Nan-EKLF (Fig. 2C), providing a third recognition category (Fig. 2E). We conclude that the single amino acid change, E339D, yields an EKLF protein that, unlike wild type, discriminates pyrimidine binding sites in the middle position and specifically fails to bind those containing thymidine.

Structural basis for weakened Nan-EKLF category II interactions. The crystal structure of Zif268 (EGR1, early growth response 1) bound to DNA served as the prototype model for EKLF's second zinc finger bound to the ß-globin promoter (27) (Fig. 3A). The carboxyl group of E339 generates Van der Waals interactions with the methyl group of thymidine in position 5. In addition, the neighboring R342 is involved in a salt bridge with E339. Also important, the electrostatic repulsion between the carboxyl group of E339 and the nearest phosphates on the DNA backbone (located between nucleotides T5-G4 and G4-G3) is reduced by the carboxyl group being turned away from those phosphates. This results in the stabilization of the WT-EKLF and DNA complex structure (Fig. 3B).

In the case of Nan-EKLF (Fig. 3C) the carboxyl group of D339 is positioned too close to the methyl group of thymidine, causing a steric clash and resulting in repulsion. Similarly, the phosphate groups are repulsed by the shorter distance to the aspartate carboxyl group. R342 is located too far away to generate a stable salt bridge with D339. As a consequence of these structural changes, the stability of the complex between zinc finger 2 of Nan-EKLF and DNA is reduced, explaining its inability to interact with the ß-globin element or any binding site containing a thymidine in the critical center position (category II sites).

Nan-EKLF fails to activate reporter genes whose expression is dependent on category II sites. We next performed functional analyses for transactivation by using luciferase reporter assays in K562 erythroleukemia cells that do not express endogenous EKLF (23, 34). For these analyses we chose three reporter genes, two driven by promoters with category II sites [ß-globin-Luc and BKLF(1b)-Luc] and one with a category I element (p21-Luc). All three reporters tested are efficiently activated by increasing amounts of WT-EKLF (Fig. 4). However, Nan-EKLF is only able to activate the p21 promoter (Fig. 4C); activation of ß-globin and BKLF(1b) is insignificant (Fig. 4A, B). We conclude that inefficient complex formation between Nan-EKLF and category II elements results in failure to activate linked reporters.

Target expression levels in EKLF and *Nan* heterozygotes *in vivo*. Gene-targeted EKLF heterozygotes (hereafter designated EKLF +/-, or simply +/- to distinguish from *Nan*/+) are phenotypically indistinguishable from WT mice (35, 36) and thus are haplosufficient. As this is in direct contrast to *Nan* heterozygotes, we addressed whether there is any genotypic influence on the relative transcription level of endogenous EKLF targets by the E339D *Nan* allele in the presence of the WT EKLF allele. To do this, we quantitatively analyzed and compared mRNA from *Nan*/+ and EKLF +/- fetal liver cells. To enable a suitable assessment across samples, individual transcript levels were normalized to those derived from homozygous WT (+/+) littermates.

After normalization to their respective +/+ controls, both heterozygotes show a similar 50% drop in EKLF expression (Fig. 5A, blue vs grey, or red vs grey). This result was expected for EKLF +/- cells carrying one null allele; however, *Nan* heterozygotes contain two intact EKLF alleles, and we expected its expression to be similar to +/+. To ensure that transcription from both *Nan* EKLF alleles contributes to the final *Nan* phenotype, we sequenced EKLF cDNAs from three +/+ and three *Nan*/+ fetal livers. Indeed, the sequence data reveal the presence of mRNA in approximately equal levels encoding both codons GAA and GAT that can be translated to E or D in mRNA from the *Nan*/+ (Fig. 5B). Western blots verify the two-fold decrease in EKLF protein in these samples (Fig. 5C).

We next compared expression levels in the +/- and *Nan*/+ fetal livers for a variety of genes, including erythroid EKLF targets (16-18). Genes encoding ß-globin (*Hbb*), erythroid aminolevulinic acid synthase (*Alas2*), porphrobilinogen deaminase (Pbgd; *Hmbs*, hydroxymethylbilane synthase), alpha hemoglobin stabilizing protein (*Ahsp*), dematin (*Epb4.9*), calcium-activated potassium channel (*Kcnn4*), transcription factors *Gata1* and *Klf3*, and cell cycle regulators (p21, p18, E2F2) were examined. All these genes show dramatically reduced or no expression in EKLF-null fetal liver cells. Comparing transcript levels in EKLF +/- to +/+ cells, however, shows that expression of all of these EKLF targets is unchanged or only moderately affected in these heterozygotes (Fig. 5D, blue vs grey). Hence, one intact EKLF allele is sufficient to maintain normal, or near normal, expression levels of erythroid targets, explaining the normal phenotype of EKLF +/- mice.

The situation differs for *Nan* heterozygotes. Comparison of *Nan*/+ to +/+ reveals several significantly divergent transcripts, an observation most readily assessed by comparing +/- to *Nan*/+. For some targets (e.g., AHSP, Alas2, Pbgd, Kcnn4, GATA1, p21) the difference in relative expression between +/- and *Nan*/+ is insignificant to moderate (Fig. 5D, blue vs red). On

the other hand, the relative mRNA level for ß-globin and p18 mRNA in *Nan*/+ is significantly (≥50%) lower compared to the EKLF +/- (Fig. 5D, blue vs red). The most dramatic relative divergence in expression is observed for dematin, which is reduced 25-fold (Fig. 5D, blue vs red; verified by western blot analysis in Fig. 5C), for the E2F2 cell cycle transcription factor, decreased 7.5-fold (Fig. 5D, blue vs red), and the BKLF transcription factor (transcribed from the erythroid 1b promoter), down 8-fold (Fig. 5D, blue vs red).

These effects do not follow from an erythroid proliferation failure, as non-EKLF targets such as *Pcna* (proliferating cell nuclear antigen), *Myc*, and *Ccnd1* (cyclin D1) are not significantly altered (Supplemental Fig. 2). Rather, whether expression of an EKLF target gene is affected by the presence of the *Nan* allele depends upon whether it has a category I or category II binding site, as defined by our in vitro analyses. That is, expression from the AHSP and p21 promoters containing category I binding sites is unaffected; however, expression from the ß-globin and E2F2 promoters, whose category II cognate sites are not recognized by Nan-EKLF, are significantly depressed. Given the dramatic decrease in expression from the dematin promoter in *Nan*/+ cells, we tabulated binding sites occupied by EKLF (17) (Supplement Table 4) and find that they are all category II sites. The significant reduction in *Nan/+* p18 expression suggests that this gene may possess both categories of cognate sites. We selected and analyzed class I p18 promoter sites that are known to bind EKLF; however, there may be unknown intronic or downstream sites that are also critical for final output (as recently shown for p21 (46)), and these sites could be category II.

Collectively these surprising data reveal that Nan-EKLF is selectively detrimental to expression of a subset of EKLF target genes even in the presence of WT-EKLF; remarkably, it is the subset whose cognate binding elements are not recognized by Nan-EKLF (category II).

Altered hemoglobin switching in *Nan*/+ mice. EKLF is critical for proper developmental switching of the ß-like globin gene cluster. Adult ß-globin is not expressed in its absence (35, 36), while the embryonic genes are expressed at a higher level and for a longer time (37-39). As observed for ß-globin, α-globin expression is decreased by 50% in *Nan*/+ compared to the EKLF heterozygote (Fig. 5E, blue vs red), likely explaining the absence of globin chain imbalance in the *Nan*/+ mouse (Fig. 5E). Concomitant with the decrease in adult ß-globin, embryonic ßh1 levels are augmented 2-fold in the +/- heterozygotes compared to +/+ (Fig. 5E, blue vs grey); this is further accentuated in the *Nan*/+ cells, where ßh1 levels are increased an additional 2-fold (Fig.

5E, blue vs red). In the adult spleen, ßh1 accounts for virtually all ß-like globin expression (Fig. 5F). We do not detect any differences in εy expression. These data indicate that the genetic switch in ß-like globins is incompletely controlled in the *Nan*/+ mouse, and also demonstrate that the effect of Nan-EKLF is not limited to decreasing erythroid gene expression.

Discussion

We have localized the neonatal anemia (*Nan*) gene to a point mutation within the second zinc finger of EKLF transcription factor, a change that alters a critical amino acid important for DNA recognition. Although this is a conservative substitution (glutamic to aspartic acid), the effects are dramatic on EKLF DNA target recognition and the resultant genetic readout. These changes explain the unexpectedly more severe phenotype exhibited by the *Nan* mouse mutation compared to the EKLF-null mouse and has structural and novel genetic implications.

Novel insights from the *Nan* phenotype. The *Nan* mutation maps to the middle of the critical "XYZ" amino acids (44) within the EKLF C2H2 finger structure. The change from the highly conserved glutamic to aspartic acid at EKLF amino acid 339 in *Nan* is extremely detrimental. Based on structural data, the "Y" amino acid is located at the +3 position in the alpha helix of EKLF zinc finger 2 (27). An earlier proposal based on phage selection suggested that aspartic acid at that position would preferentially interact with cytosine (45), a postulate consistent with our own observations of Nan-EKLF's ability to interact with the 5'G**C**G, but not with the 5'G**T**G triplet in its recognition sequence.

Our results also show that WT-EKLF can interact with either pyrimidine in this triplet. Our model for EKLF zinc finger interaction with its DNA target (27) provides a structural rationale for the dramatic effect of a seemingly subtle change from E339 to D339 in *Nan* EKLF that results in a more restricted recognition. Furthermore, although not modeled here, it is easy to see from this structure how the presence of a large purine at the triplet site (i.e., 5'G**G**G) could be completely detrimental to EKLF interaction and explain the inability of both WT-and Nan-EKLF to interact with the category III (E2F2-1) site. Accordingly, we suggest that the WT-EKLF recognition sequence be refined to 5'NGGG**Y**GNGG, and that the Nan-EKLF mutant limits recognition to 5'NGGG**C**GNGG.

The absolute precision of C2H2 zinc finger interaction with DNA is known to be critical for proper genetic readout. Single point mutations at critical guanosine residues within the EKLF

recognition sequence lead to dramatic decreases in affinity in vitro (27) and correlate with point mutations at this site that lead to ß-thalassemia. Our results present a strikingly more phenotypically complex scenario that follows from a mutation in the protein that alters its recognition code, resulting in an altered specificity variant that produces a cell with a radically different output than the EKLF-null, -heterozygous, or -WT red cell.

The *Nan* phenotype is unusual in that the mouse exhibits homozygous lethality significantly earlier (E10-11) than EKLF-null embryos (E14.5) (35, 36), and exhibits severe anemia when heterozygous, an effect not observed in EKLF heterozygotes (35, 36). Expression patterns of selected EKLF targets show that some are dramatically affected by the *Nan* EKLF mutant even in the presence of the WT EKLF allele. Altering expression patterns of structural and/or transcriptional proteins can account for the dramatic phenotype seen in *Nan* heterozygotes. For example, dematin and adducin are critical components of the red cell membrane skeleton. Deficiency of dematin, α- or β-adducin alone leads to mild hemolytic anemia (4, 8, 10). Notably, combined deficiency of dematin and β-adducin, as occurs in *Nan*, produces a much more severe hemolytic anemia (47). The E2F2 transcription factor is important for cell cycle control during terminal erythroid differentiation and for maintaining proper red cell volume (48, 49). Genetic mis-expression is further supported by the abnormal residual embryonic (βh1) globin expression in *Nan*. This new phenotype is not equivalent to that of the EKLF-null mouse, as not all EKLF targets are equally affected, but instead only those with a “T” (category II) rather than a “C” (category I) within their EKLF zinc finger 2 promoter recognition motif.

Genetic distortion by Nan-EKLF. These considerations suggest that, although the *Nan* mutation exhibits a semi-dominant genetic phenotype in terms of inheritance, it is unlikely that the molecular explanation is that of a dominant negative/interfering effect by the mutant EKLF protein (50). A basic tenet of this model is not satisfied, as EKLF is not known to dimerize, and its target DNA binding motif is not symmetrical or adjacently repeated; hence, poisoning of a homodimer-dependent activity is not likely. In addition, this conclusion is based on our observations that, in some cases, the *Nan* heterozygote is behaving as an EKLF heterozygote; that is, target expression is unaffected by the *Nan* mutation in the presence of one copy of the wild type allele, which is inconsistent with a dominant negative effect. On the other hand, some genes are dramatically negatively affected in the *Nan* heterozygote and mimic the EKLF-null expression pattern. In this latter case, it might be envisioned that the inability of mutant E339D

EKLF to bind its cognate category II sequence frees it to bind limiting amounts of cofactors and exert a dominant effect on activity of the wild type EKLF protein, thus preventing it from efficiently forming a proper chromatin remodeling and transcription initiation complex (28-31, 51). However, a selective dominant effect on a subset of genes would need to be invoked for this to be true. In addition, in vivo competition is most effective when the effector is in excess (50, 52), a situation not observed in *Nan* heterozygotes.

Although a simpler explanation is that some EKLF target genes are just more sensitive to haploinsufficiency than others, such a passive hypothesis is not satisfying given the dramatic effects of the monoallelic *Nan* mutation on select targets [e.g., dematin, E2F2, and BKLF(1b)] that are more profound than seen in the EKLF heterozygotes. Rather, our observations suggest that Nan-EKLF is a novel sequence-selective variant whose altered binding specificity directly distorts the genetic readout within *Nan* red blood cells by a unique and previously unencountered mechanism.

Relevance of the Nan mutation to human disease. A recent genome-wide association analysis (53) identified the chromosome 19p13.13 locus, where human *KLF1* (*EKLF*) resides, as significantly associated with clinically important hematologic traits, particularly decreased MCV and MCH, as seen in *Nan*/+ (Supplemental Table 1). In another study, the human In(Lu) phenotype was mapped to mutations in one allele of *KLF1* (54). These persons exhibit virtually undetectable levels of the Lutheran antigen on their red cells; however, other red cell EKLF target genes are more subtly affected, and their phenotype is benign with no evidence of pathology. Although there is some parallel with our own analysis, the In(Lu) mutant is more akin to an EKLF heterozygote as the mutations lead to loss-of-function of the affected *KLF1* allele only.

A patient presenting with congenital dyserythropoietic anemia carrying a *KLF1/EKLF* allele mutated at the equivalent site (human E325K) as *Nan* was recently described (55). Studies of this transfusion-dependent patient in the 1990's revealed severe anemia with high HbF and reticulocyte levels, membrane abnormalities, altered cell cycle parameters, deficient protein synthesis (56, 57) and increased embryonic ζ- and ε-globin expression (58, 59). These effects are consistent with our observations of the *Nan* mutant mouse and demonstrate that carrying a single *KLF1* allele mutated at this site can lead to a human disease phenotype.

Materials and Methods

Mice were maintained at The Jackson Laboratory in climate-controlled rooms (12-hour light cycle) with free access to acidified water and chow (NIH 5K52). The *Nan* mutant was a female offspring of an N-ethyl-N-nitrosourea (ENU)-mutagenized C3H/101 F1 male and a "PT" female at the Medical Research Council, Harwell, UK (12, 60). The PT stock was a mixed background mutation testing stock. The anemic female was mated to an untreated C3H/101 F1 male, and the mutation propagated by intercrossing offspring. Offspring have been intercrossed for many more than 20 generations; hence the original Harwell *Nan* stock used in this study is fully inbred. In accordance with the International Committee on Standardized Genetic Nomenclature for Mice **(**http://www.informatics.jax.org/mgihome/nomen/strains.shtml) the official genetic designation for *Nan* is $Klf1^{Nan}$.

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Figure Legends

Fig. 1. Characterization and positional cloning of *Nan*/+.

(A) Peripheral blood smears from adult wild type (+/+) and *Nan* heterozygotes (*Nan*/+). Arrow, spherocyte; Bar, 10 μM.

(B-D) Prussian blue staining for the presence of iron in tissues indicated. Bars, 50 μM. Arrow, hematopoiesis in adult *Nan*/+ liver.

(E) SDS-PAGE and (F) Western blotting showing the major red cell membrane skeleton proteins present in adult red cell ghost preparations. Non-adjacent lanes are separated with a line (protein 4.1 and EcapZ).

(G) Sequencing chromatograms showing A → T transversion in *Klf1* in E9.5 fetuses (arrow).

Fig. 2. DNA binding/recognition specificity of WT-EKLF versus Nan-EKLF.

(A) Schematic representation of the EKLF domains and consensus DNA binding motif recognized by particular zinc fingers (ZnF), with each ZnF recognizing a triplet DNA sequence (14). The E339D mutation within the second zinc finger is indicated, as is the location of the base interaction within the target middle DNA triplet ("N" in red).

(B) Alignment of the EKLF zinc fingers according to the position of Cys and His amino acids (shaded) involved in Zn coordination, and the secondary structure depicted above (arrows are ß-sheets, cylinder is α-helix). The amino acids (positions -1, +3 and +6 of the α-helix) important for nucleotide sequence recognition within each finger are shaded in green; the E339D change is highlighted in red.

(C) Gel shift assays comparing complex formation between WT- or Nan-EKLF in extracts from transfected cells (as indicated) and radiolabeled double-stranded oligonucleotides comprising EKLF binding sites from the following known EKLF targets: β-globin (14); p21 (sites 3 and 4 (46)); p18 (site A (61)); E2F2 (sites 1-3 (62)); AHSP (63); BKLF (erythroid promoter site 1b (64)). Asterisk: nonspecific band.

(D) EKLF western blots of extracts used for the gel shift analysis; 2 μl were used in (C).

(E) Three categories of binding sites within EKLF target genes that are differentially recognized by WT-EKLF or Nan-EKLF. Note that AHSP also contains a non-consensus thymine (underlined) that accounts for its inherent low binding affinity to WT-EKLF; yet this site is still well bound by Nan-EKLF.

Fig. 3. Molecular modeling of the structural changes of protein-DNA interactions introduced by the E339D mutation in EKLF.

(A) The sequence of the seven amino acids of EKLF ZnF2 surrounding E339D, along with the nine base pair ß-globin promoter interaction sequence, are shown above the 3D model (27) of the interaction between EKLF ZnF 2 (purple ribbon) and its β-globin promoter binding site (yellow backbone); stick structures highlight the region of interest. Boxed region is expanded in (B, C).

(B) The model structure (27) and scheme depicting the distances between E339 and neighboring residues involved in stabilizing the interactions between WT-EKLF and the β-globin promoter binding site. Green arrows indicate positive interactions; red arrows indicate the weak repulsive interactions.

(C) The structural consequences of E339 to D mutation is demonstrated by changes in calculated distances between crucial residues that destabilize the interactions between Nan-EKLF and the β-globin element. Red arrows indicate strong repulsive interactions or steric interference; yellow line is the weakened positive interaction.

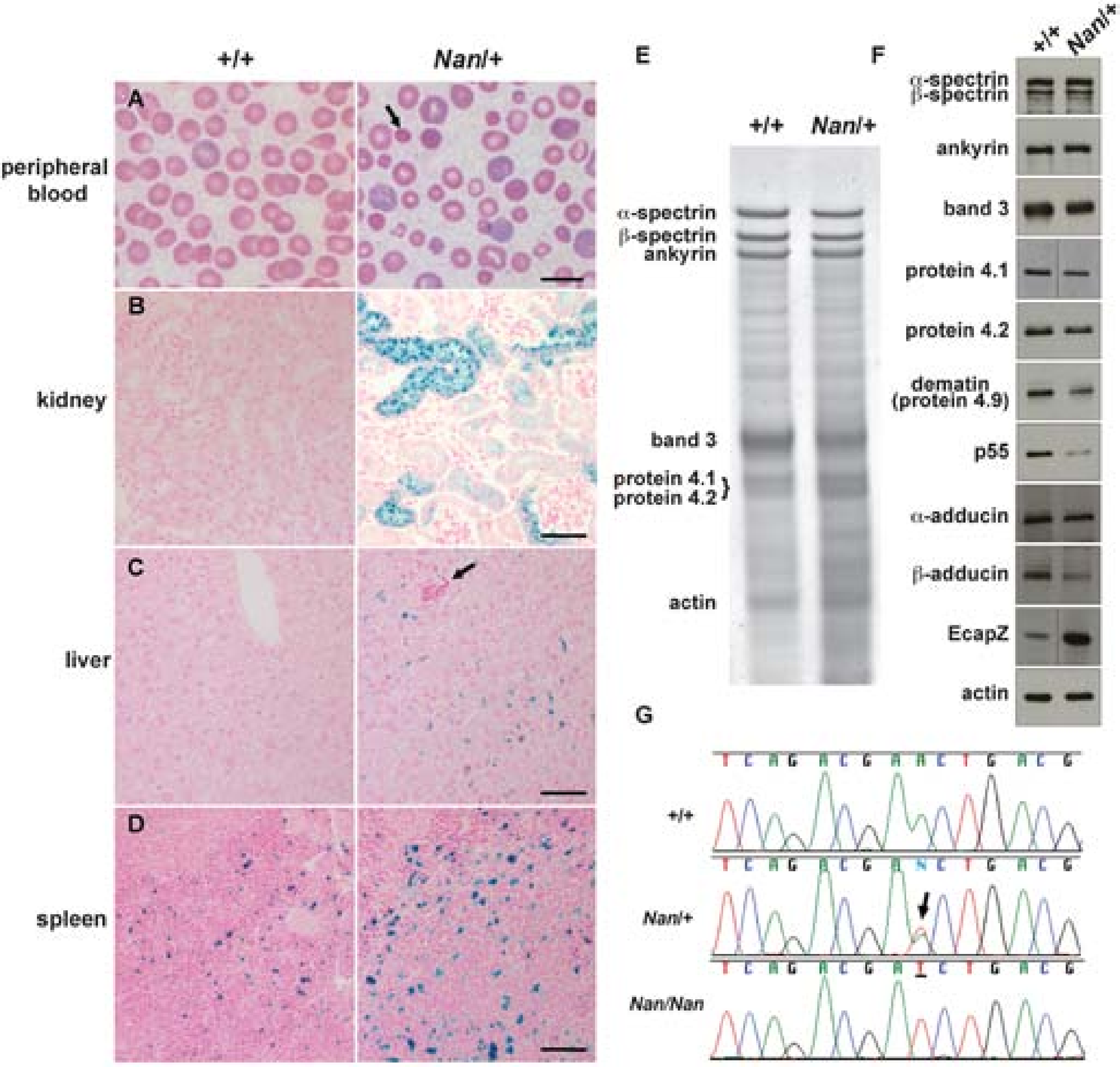
Fig. 4. Comparison of the transcriptional abilities of WT-EKLF and Nan-EKLF.

K562 erythroleukemia cells that do not express endogenous EKLF (23, 34) were cotransfected with plasmids expressing the luciferase reporter gene under control of β-globin (A), BKLF-1b (B), or p21 (C) promoters along with increasing amounts of WT-EKLF or Nan-EKLF. The nine base pair EKLF target sequence in each promoter is shown below; the critical thymidine or cytidine residue in the middle triplet is highlighted. The western blots below show the levels of EKLF expression in extracts from the same experiment; the top band (asterisk) is a non-specific signal that serves as a loading control. A *Renilla* reporter construct was included as a normalization control for transfection efficiency. An average of triplicates is shown (arithmetic mean ± SD).

Fig. 5. Quantitative analysis of the *in vivo* expression levels of EKLF target genes in fetal liver cells of heterozygous +/- and *Nan*/+ embryos and their homozygous +/+ littermates, and in adult spleens.

(A) Comparison of EKLF mRNA levels in +/- and *Nan*/+ heterozygous fetal liver cells normalized to their respective littermate +/+ homozygous level (color coded as indicated).

(B) cDNA sequence chromatograms confirming transcription of both *Nan*/+ alleles [codons GAA (glu: E) and GAT (asp: D)] compared to +/+ [codon GAA (glu: E) only] in the three fetal liver samples used for analysis.
(C) Western blot analysis of the EKLF and dematin protein levels in fetal liver total cell extracts from three *Nan*/+ and +/+ littermates. Actin is used as a loading control.
(D) Quantitative RT-PCR analysis of EKLF target mRNA expression in heterozygous +/- and *Nan*/+ fetal livers normalized to their respective +/+ homozygous littermates [color coded as in (A)].
(E) Analysis of the mRNA levels of embryonic/adult globins in heterozygous +/- and *Nan*/+ fetal livers normalized to their respective +/+ homozygous littermates [color coded as in (A)]. A western blot analysis of α and β-globin chains expressed in heterozygous +/- and *Nan*/+ fetal livers is shown (right).
(F) Analysis of the mRNA levels of embryonic/adult globins in *Nan*/+ adult spleen normalized to that from its +/+ littermate [color coded as indicated].


+/+
Nan/+
A
peripheral blood
B
kidney
C
liver
D
spleen
E
+/+
Nan/+
α-spectrin
β-spectrin
ankyrin
band 3
protein 4.1
protein 4.2
actin
F
+/+
Nan/+
α-spectrin
β-spectrin
ankyrin
band 3
protein 4.1
protein 4.2
dematin (protein 4.9)
p55
α-adducin
β-adducin
EcapZ
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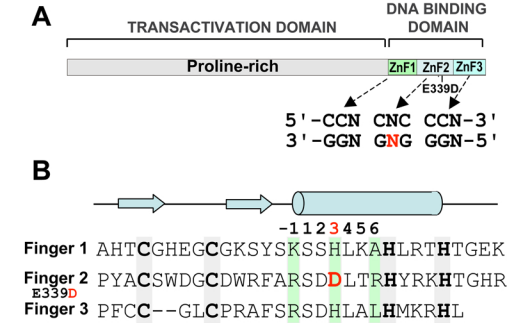
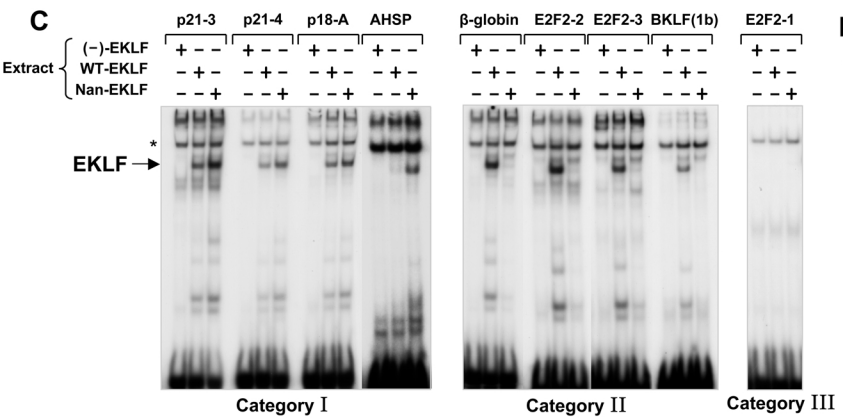
G
+/+
Nan/+
Nan/Nan

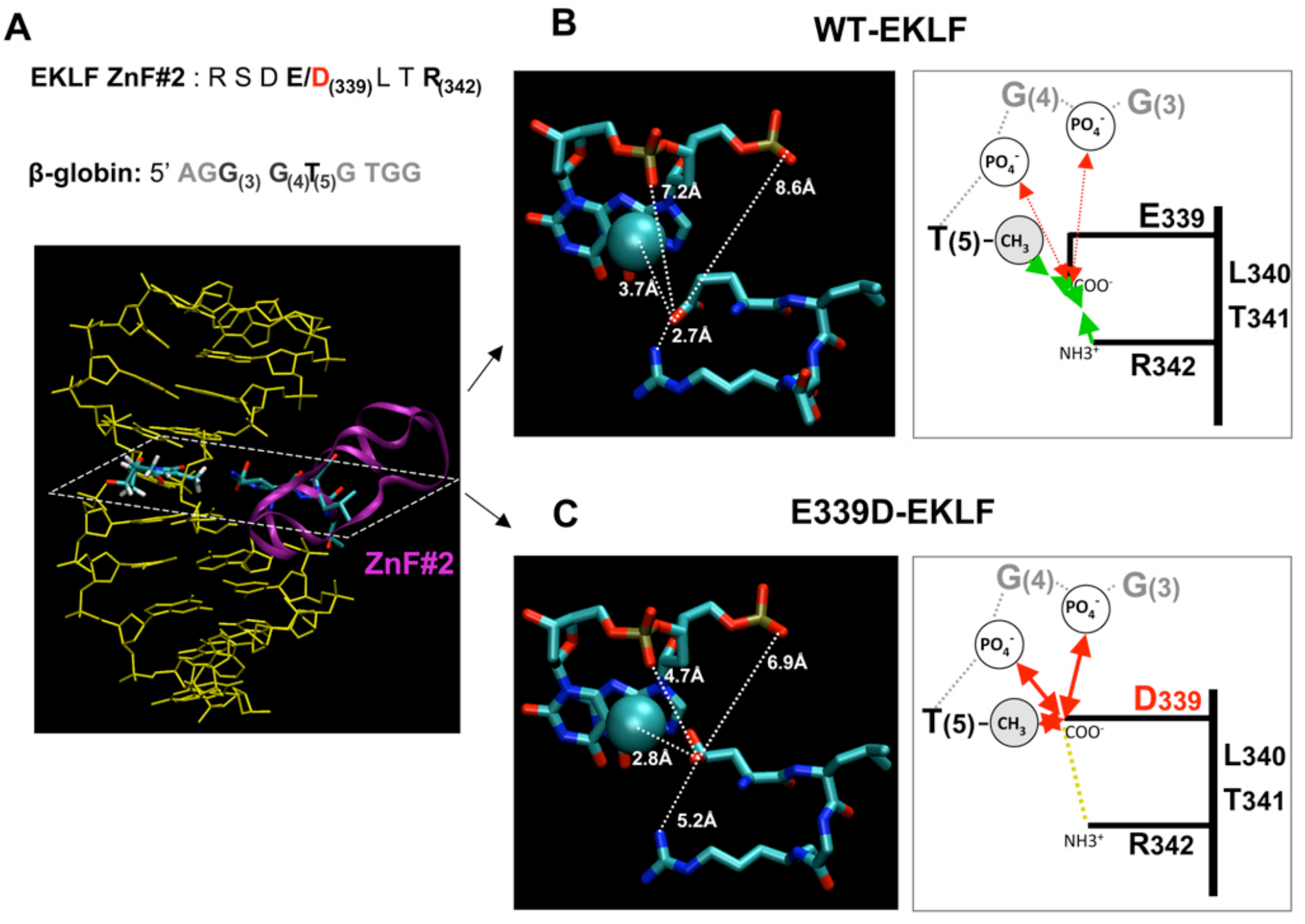


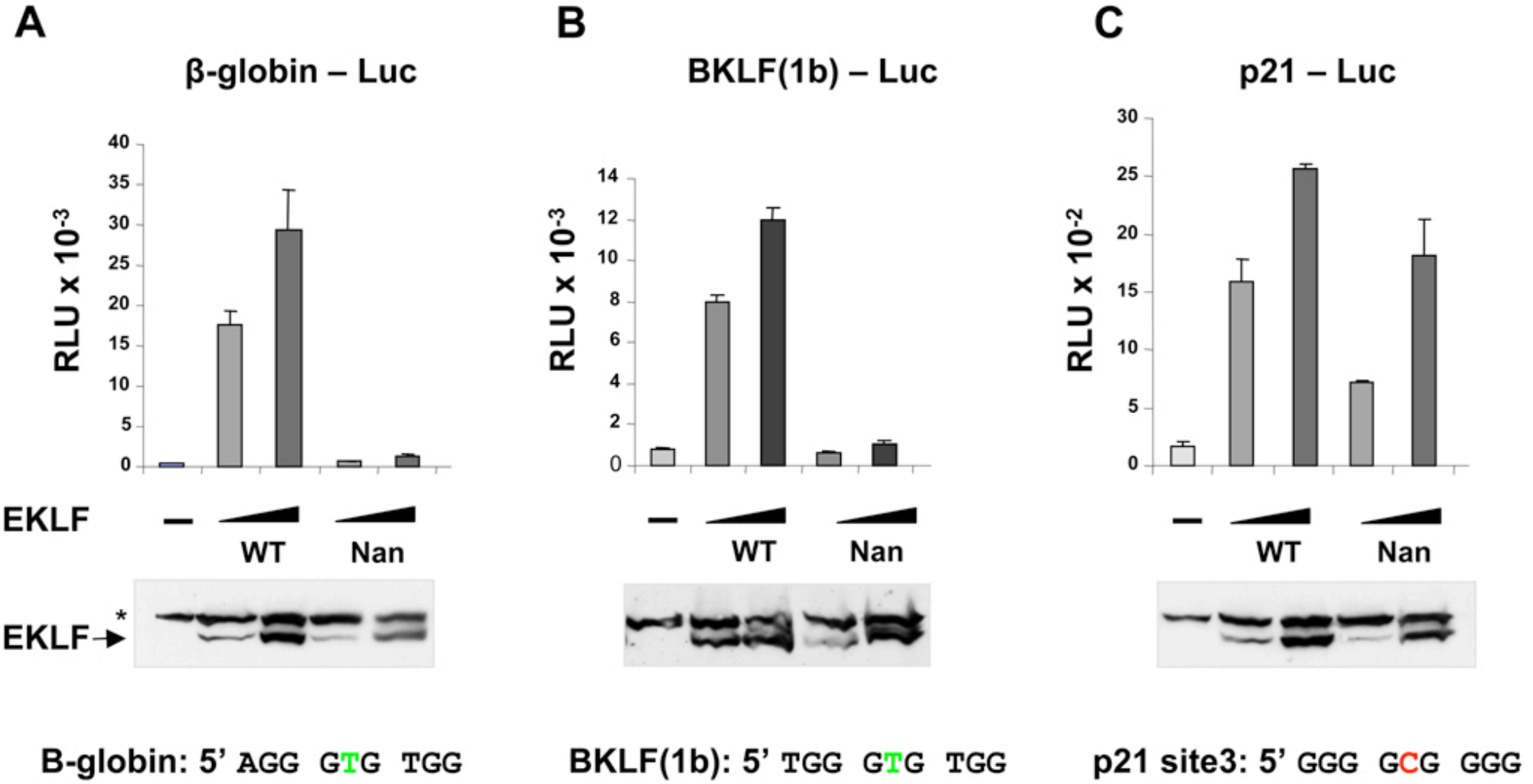
E

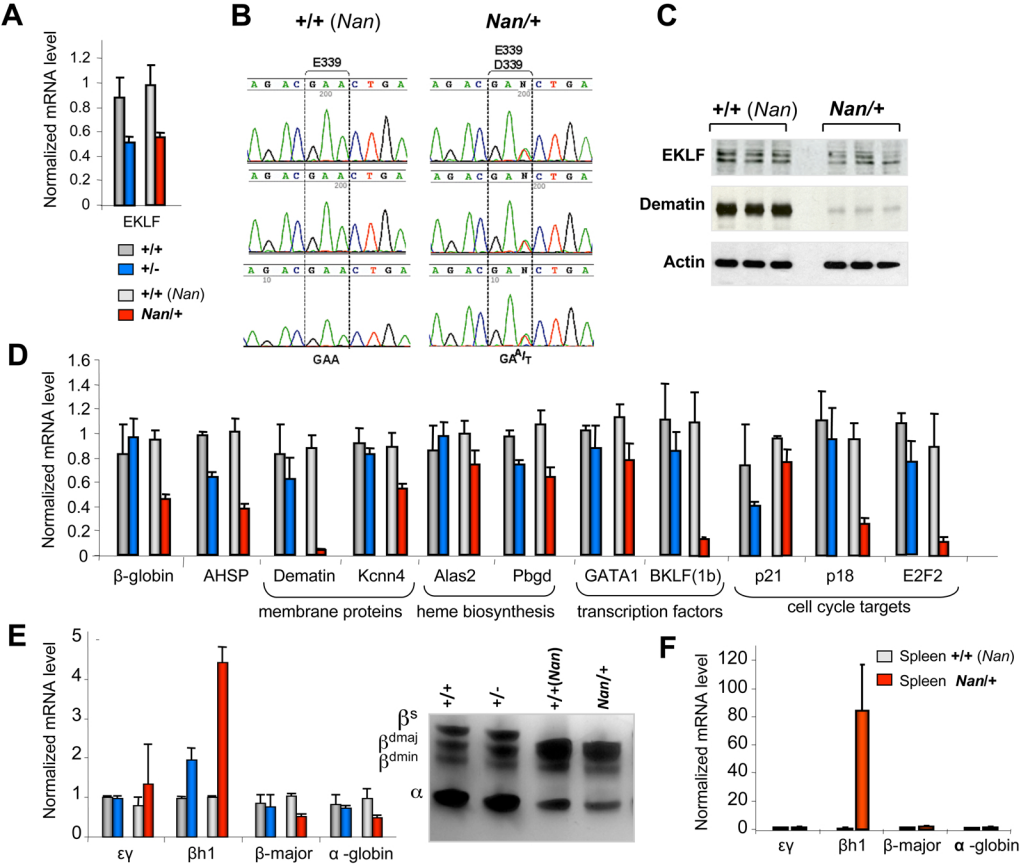
EKLF target *	ds oligo binding motif 5' to 3'	WT-EKLF	Nan-EKLF	Category
p21 site 3	GGG GCG GGG	+	+	I
p21 site 4	TGG GCG TGG	+	+	
p18 - A	GGG GCG TGG	+	+	
AHSP	TGG GCG GTG	+	+	
β-globin	AGG GTG TGG	+	–	II
E2F2-2	GGG GTG TGG	+	–	
E2F2-3	AGG GTG GGG	+	–	
BKLF (1b)	TGG GTG TGG	+	–	
E2F2-1	TGG GGG TGG	–	–	III

* binding site within EKLF target gene




A
EKLF ZnF#2 : R S D E/D(339) L T R(342)
β-globin: 5' AGG(3) G(4)T(5)G TGG
ZnF#2
B
WT-EKLF
7.2Å
8.6Å
3.7Å
2.7Å
G(4)
G(3)
PO4-
PO4-
T(5)-CH3
E339
COO-
L340
T341
NH3+
R342
C
E339D-EKLF
4.7Å
6.9Å
2.8Å
5.2Å
G(4)
G(3)
PO4-
PO4-
T(5)-CH3
D339
COO-
L340
T341
NH3+
R342


A
β-globin – Luc
RLU x 10-3
40
35
30
25
20
15
10
5
0
EKLF
–
WT
Nan
EKLF
*
B
BKLF(1b) – Luc
RLU x 10-3
14
12
10
8
6
4
2
0
–
WT
Nan
C
p21 – Luc
RLU x 10-2
30
25
20
15
10
5
0
–
WT
Nan
B-globin: 5’ AGG GTG TGG
BKLF(1b): 5’ TGG GTG TGG
p21 site3: 5’ GGG GCG GGG


A
Normalized mRNA level
1.2
1
0.8
0.6
0.4
0.2
0
EKLF
+/+
+/-
+/+ (Nan)
Nan/+
B
+/+ (Nan)
Nan/+
E339
E339
D339
A G A C G A A C T G A
A G A C G A A C T G A
A G A C G A N C T G A
A G A C G A N C T G A
GAA
GAA/T
C
+/+ (Nan)
Nan/+
EKLF
Dematin
Actin
D
Normalized mRNA level
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
β-globin
AHSP
Dematin
Kcnn4
Alas2
Pbgd
GATA1
BKLF(1b)
p21
p18
E2F2
membrane proteins
heme biosynthesis
transcription factors
cell cycle targets
E
Normalized mRNA level
5
4
3
2
1
0
εγ
βh1
β-major
α -globin
+/+
+/-
+/+(Nan)
Nan/+
βs
βdmaj
βdmin
α
F
Normalized mRNA level
120
100
80
60
40
20
0
Spleen +/+ (Nan)
Spleen Nan/+
εγ
βh1
β-major
α -globin