Severe anemia in the *Nan* mutant mouse caused by sequence-selective disruption of erythroid Krüppel-like factor

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Edited by Mark T. Groudine, Fred Hutchinson Cancer Research Center, Seattle, WA, and approved July 13, 2010 (received for review April 13, 2010)

Studies of mouse models of anemia have long provided fundamental insights into red blood cell formation and function. Here we show that the semidominant 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 so 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 interference yields a distorted genetic output and selective protein deficiencies that differ from those seen in EKLF-heterozygous and EKLF-null red blood cells and presents a unique and unexpected mechanism of inherited disease.

mouse model | neonatal anemia | red blood cells | zinc finger mutation

Hereditary spherocytosis (HS) is the most common cause of inherited hemolytic anemia in Northern Europeans, with an estimated frequency of ∼1/5,000 (1). Defects in the structural components of the red blood 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 blood cells that lose membrane surface area, become increasingly spheroidal, and are removed by the spleen (1).

Spontaneous and targeted mutations in membrane skeleton genes in mice have been instrumental in defining the components, interactions, and functions of membrane skeleton proteins (2). The mouse mutation *Nan* (for "neonatal anemia") is an ethylnitrosourea (ENU)-induced semidominant hemolytic anemia first described by Mary Lyon more than 25 y ago (3). *Nan* homozygotes die at embryonic day (E)10–11 from a "severe lack of haemopoiesis;" with no overt, nonerythroid 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 (4). 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 Krüppel-like factor 1 (*Klf1*) gene encoding erythroid Krüppel-like factor (EKLF) (14). The mutation causes HS by a 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 (5, 6), is a hematopoietic C2H2 zinc finger transcriptional factor that plays a global role in erythroid gene expression by regulating expression of heme biosynthetic, red blood cell membrane, globin stabilizing, and cell-cycle proteins (7–9). During hematopoiesis, EKLF first is 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 blood cell progeny (10). EKLF levels directly influence this bipotential lineage decision by repressing megakaryopoiesis while accentuating erythropoiesis (10–12).

The activation function of EKLF has been analyzed most extensively at the β-globin locus, where its plays critical roles in genetic regulation of the adult β-globin promoter (13, 14). 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 (5, 15). Second, it integrates chromatin remodeling and transcriptional activities via critical protein–protein interactions (16–19). Consequently, EKLF plays a central role in establishing the precise 3D looping of the distant locus control region with the proximal β-globin promoter in adult erythroid cells (20), resulting in formation of an active chromatin hub. Third, EKLF is a regulator of β-like globin switching (21). Although EKLF heterozygotes are phenotypically normal, in the total absence of EKLF the active chromatin hub is not properly formed (20), and adult β-globin is not expressed, leading to embryonic lethality by E14.5 caused by profound β-thalassemia (22, 23). 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 (24–26). In humans, point mutations in the EKLF cognate binding site lead to β-thalassemia (15) with markedly elevated fetal hemoglobin levels (27).

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 on the cognate EKLF DNA binding sequence, in which a single nucleotide makes the difference between recognition by both 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, the genes deficient in expression in *Nan* heterozygotes are those whose EKLF binding elements are not recognized by Nan-EKLF. Such target-selective effects lead to a distorted genetic readout and account for the severe anemia in *Nan*/+ mice. Our findings have

Author contributions: M.S., J.J.B., and L.L.P. designed research; M.S., K.E.S., S.G.A., and M.M. performed research; M.S., J.J.B., and L.L.P. analyzed data; and M.S., J.J.B., and L.L.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1004996107/-/DCSupplemental.

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 (3, 4), we find that *Nan* homozygotes die in utero at ~E10–11. Heterozygotes are severely anemic at birth and throughout life. Loss of heterozygotes in utero occurs also; reciprocal *Nan*/+ × WT matings produce only 34% affected offspring. The red blood cell count, hemoglobin, and hematocrit are decreased significantly in *Nan*/+ adults as compared with WT (Table S1). Circulating reticulocytes and spleen weights are increased dramatically (Table S2) Peripheral blood smears (Fig. 1*A*) reveal extensive anisocytosis with many microcytes, spherocytes, and hypochromic cells, consistent with the increased red blood cell distribution width and hemoglobin distribution width. Platelet levels are slightly but significantly increased (Table S1).

Nan/+ bilirubin and iron levels do not differ significantly from controls, but zinc protoporphyrin is dramatically increased (Table S2), suggesting that a heme biosynthesis defect is unlikely (28). Consistent with severe hemolysis, iron accumulates in *Nan*/+ kidney (Fig. 1*B*), liver (Fig. 1*C*), and spleen (Fig. 1*D*). Erythropoietic foci are observed in the mutant liver (Fig. 1*C*, arrow).

Examination of membrane skeleton proteins by SDS/PAGE (Fig. 1*E*) and by Western blotting (Fig. 1*F*) quantitated with scanning densitometry (Table S3) reveal decreased band 3, proteins 4.1 and 4.2, dematin (protein 4.9), β-adducin, and p55 in *Nan*/+ red blood cell ghost membranes compared with WT. Consistent with Western blotting results, p55 mRNA expression is markedly decreased in erythroid tissues (Fig. S1). These changes are in contrast to the global deficiencies in *Nan* membrane skeleton proteins reported by White et al. (29), perhaps reflecting differences in genetic background. Up-regulation of the actin-capping protein CapZα (EcapZ) occurs in the red blood cell membrane and probably compensates for loss of adducin in actin capping (Fig. 1*F* and Table S3) (30).

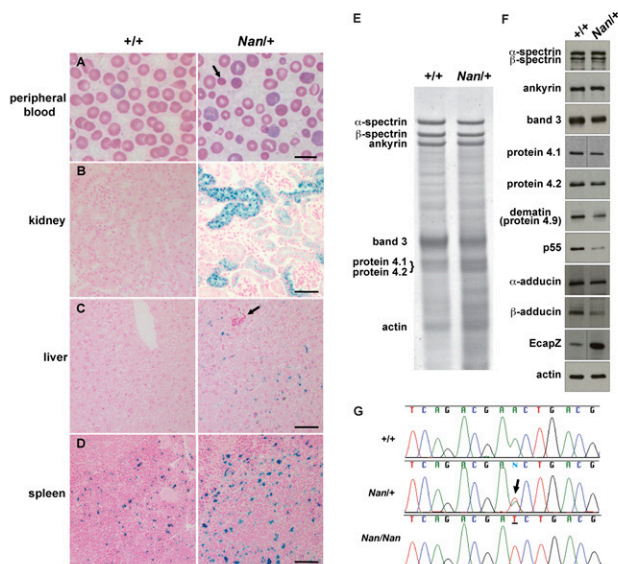


Fig. 1. Characterization and positional cloning of *Nan*/+. (*A*) Peripheral blood smears from adult WT (+/+) and *Nan* heterozygotes (*Nan*/+). Arrow indicates spherocyte. (Scale bar, 10 μm.) (*B*–*D*) Prussian blue staining for iron in tissues. Arrow indicates hematopoiesis in adult *Nan*/+ liver. (Scale bars, 50 μm.) (*E*) SDS/PAGE and (*F*) Western blotting showing the major membrane skeleton proteins in adult red blood cell ghost preparations. Nonadjacent lanes (protein 4.1 and EcapZ) were repositioned for display. (*G*) Sequencing chromatograms showing A → T transversion in *Klf1* in E9.5 fetuses (arrow).

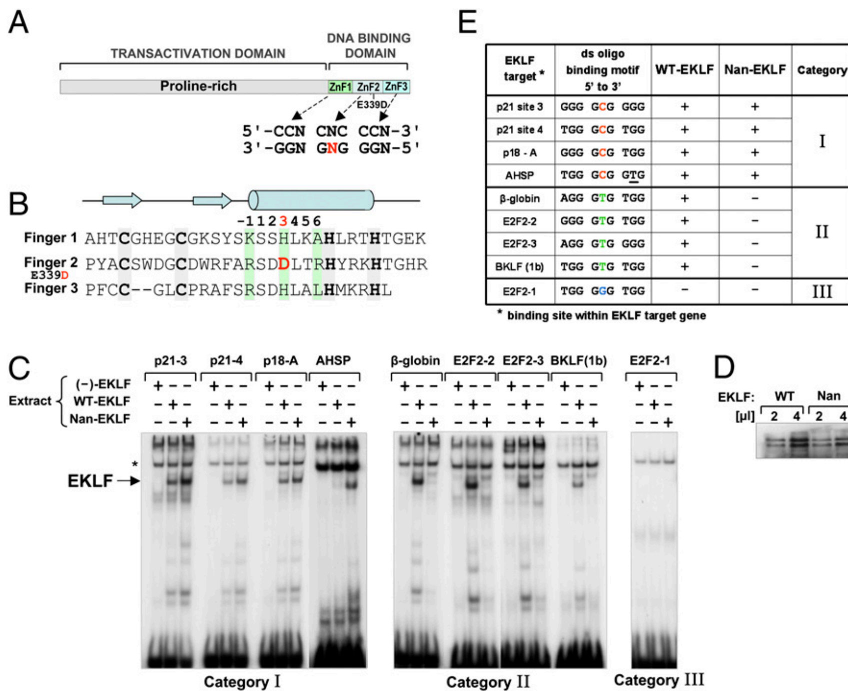
We fine-mapped *Nan* to a 1.3-megabase interval between *D8Mit78* and *D8Mit79* on Chr 8 (Ensemble, http://www.ensembl.org) containing 46 known or predicted genes (Mouse Genome Informatics, http://www.informatics.jax.org). More than 600 kb of sequence were obtained, including all exons and intron–exon 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 an *Mbo*I 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. 1*G*).

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 (5) (Fig. 2*A*). Mutations in this sequence lead to β-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 (15). Based on structural data from other C2H2 zinc finger proteins (31, 32), this position is involved in interactions with the middle nucleotide of the consensus EKLF binding motif 5′ NGG GNG NGG 3′ (Fig. 2 *A* and *B*). E339 is absolutely conserved across the entire murine and human KLF family and across EKLF proteins from different species (6). Based on this critical location, we tested whether the E339D mutation affects binding to EKLF targets (Fig. 2 *C* and *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. 2 *C* and *E*). However, Nan-EKLF is able to generate complexes efficiently only when cytidine is present [e.g., with sites derived from p21 cyclin-dependent kinase inhibitor 1A, p18 cyclin-dependent kinase inhibitor 2C, and α-hemoglobin stabilizing protein (*Ahsp*)] (Fig. 2 *C* and *E*). Based on these results, we can distinguish categories of EKLF binding site recognition specificities (Fig. 2*E*). The first category comprises sites that contain cytidine in the crucial middle position of the EKLF binding motif; these sites are recognized and bound by both WT and Nan-EKLF. The second category comprises sites with thymidine in the middle position; these sites are recognized only by WT EKLF and include genes encoding β-globin (*Hbb*), E2F2 (*E2f2*), and BKLF1b (*Klf3*). An oligonucleotide that contains a guanosine in this critical position is not recognized by either WT or Nan-EKLF (Fig. 2*C*), providing a third recognition category (Fig. 2*E*). We conclude that the single amino acid change, E339D, yields an EKLF protein that 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 bound to DNA served as the prototype model for EKLF's second zinc finger bound to the β-globin promoter (15) (Fig. 3*A*). 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's being turned away from those phosphates, resulting in the stabilization of the WT EKLF and DNA complex structure (Fig. 3*B*).

In the case of Nan-EKLF (Fig. 3*C*), 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 car-



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

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 zinc finger recognizing a triplet DNA sequence (5). The E339D mutation within the second zinc finger is indicated, as is the location of the base interaction within the target middle DNA triplet (red "N"). (*B*) Alignment of the EKLF zinc fingers according to the position of Cys and His amino acids (shaded) involved in zinc coordination. (*Upper*) In the depiction of the secondary structure, arrows indicate β-sheets, and the cylinder indicates the α-helix. (*Lower*) 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 and 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 (5); p21 sites 3 and 4 (33); p18 site A (47); E2F2 sites 1–3 (48); AHSP (49); and BKLF (50). *Nonspecific band. (*D*) EKLF Western blots of extracts used for the gel shift analysis (2 μL) 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 nonconsensus thymine (underlined) that accounts for its inherent low binding affinity to WT EKLF; however, this site still is well bound by Nan-EKLF.

boxyl group. R342 is located too far away to generate a stable salt bridge with D339. As a consequence, the stability of the complex between zinc finger 2 of Nan-EKLF and DNA is reduced, explaining its inability to interact with the β-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 (21, 33). We chose three reporter genes, two driven by promoters with category II sites [β-globin-Luc and BKLF(1b)-Luc] and one with a category I element (p21-Luc). All three reporters tested are activated efficiently by increasing amounts of WT EKLF (Fig. 4). However, Nan-EKLF only activates the p21 promoter (Fig. 4*C*); activation of β-globin and BKLF(1b) is insignificant (Fig. 4 *A* and *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 them from *Nan*/+ heterozygotes) are phenotypically indistinguishable from WT mice (22, 23) and thus are haplosufficient. Because this haplosufficiency is in direct contrast to *Nan* heterozygotes, we investigated 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. For this investigation, 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. 5*A*, blue vs. gray, or red vs. gray). 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. 5*B*). Western blots verify the 2-fold decrease in EKLF protein in these samples (Fig. 5*C*).

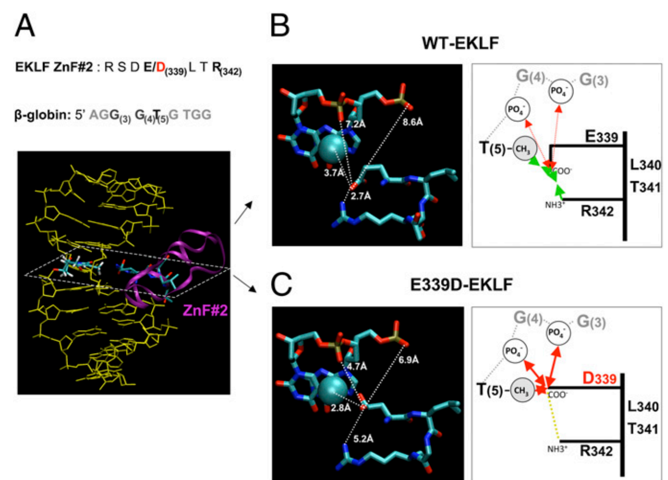


Fig. 3. Molecular modeling of the structural changes of protein–DNA interactions introduced by the E339D mutation in EKLF. (*A*) The sequence of the seven amino acids of EKLF zinc finger 2 surrounding E339D and the nine-bp β-globin promoter interaction sequence (*Upper*) are shown above the 3D model (*Lower*) (15) of the interaction between EKLF zinc finger 2 (purple ribbon) and its β-globin promoter binding site (yellow backbone); stick structures highlight the region of interest. The boxed region is expanded in *B* and *C*. (*B*) The model structure (*Left*) (15) 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 (*Right*). Green arrows indicate positive interactions; red arrows indicate the weak repulsive interactions. (*C*) The structural consequences of E339-to-D mutation (*Left*) are demonstrated by changes in calculated distances between crucial residues that destabilize the interactions between Nan-EKLF and the β-globin element (*Right*). Red arrows indicate strong repulsive interactions or steric interference; the yellow line shows the weakened positive interaction.

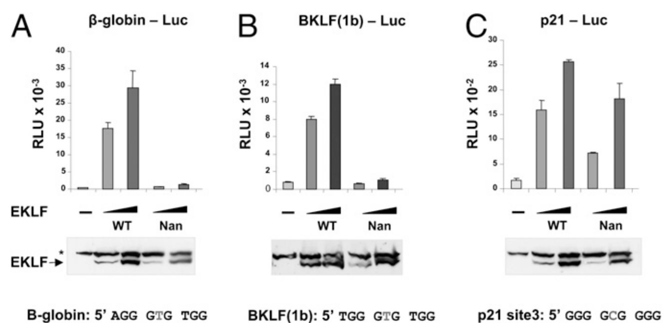


Fig. 4. Comparison of the transcriptional abilities of WT EKLF and Nan-EKLF. K562 erythroleukemia cells that do not express endogenous EKLF (21, 33) were cotransfected with plasmids expressing the luciferase reporter gene under control of β-globin (*A*), BKLF-1b (*B*), or p21 (*C*) promoters along with increasing amounts of WT EKLF or Nan-EKLF. The nine-bp EKLF target sequence in each promoter is shown at the bottom of each panel. The Western blots in the middle of each panel show the levels of EKLF expression in extracts from the same experiment; the top band (*) is a nonspecific signal that serves as a loading control. A *Renilla* reporter construct was included as a normalization control for transfection efficiency. An average of triplicate experiments (arithmetic mean ± SD) is shown.

We next compared expression levels in the +/– and *Nan*/+ fetal livers for a variety of genes, including erythroid EKLF targets (7–9). Genes encoding β-globin (*Hbb*), erythroid aminolevulinic acid synthase (*Alas2*), porphrobilinogen deaminase (Pbgd), α-hemoglobin stabilizing protein (*Ahsp*), dematin (*Epb4.9*), calcium-activated potassium channel (*Kcnn4*), transcription factors *Gata1* and *Klf3*, and cell-cycle regulators (p21, p18, and E2F2) were examined. Some of these genes show dramatically reduced or no expression in EKLF-null fetal liver cells. Comparing transcript levels in EKLF+/– and +/+ cells, however, shows that expression of all these EKLF targets is unchanged or is only moderately affected in these heterozygotes (Fig. 5*D*, blue vs. gray). 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*/+ and +/+ reveals several significantly divergent transcripts, an observation most readily assessed by comparing +/– and *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. 5*D*, blue vs. red). On the other hand, the relative mRNA level for β-globin and p18 mRNA in *Nan*/+ is significantly (≥50%) lower than in EKLF+/– (Fig. 5*D*, blue vs. red). The most dramatic relative divergence in expression is observed for dematin, which is reduced 25-fold (Fig. 5*D*, blue vs. red; verified by Western blot analysis in Fig. 5*C*), for the E2F2 cell-cycle transcription factor, which is decreased 7.5-fold (Fig. 5*D*, blue vs. red), and the BKLF transcription factor (transcribed from the erythroid 1b promoter), which is decreased 8-fold (Fig. 5*D*, blue vs. red).

These effects do not follow from an erythroid proliferation failure, because non-EKLF targets such as proliferating cell nuclear antigen (*Pcna*), *Myc*, and cyclin D1 (*Ccnd1*) are not altered significantly (Fig. S2). Rather, whether expression of an EKLF target gene is affected by the presence of the *Nan* allele depends on 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; how-

Fig. 5. Quantitative analysis of the in vivo expression levels of EKLF target genes in fetal liver cells of heterozygous (+/–) and *Nan*/+ embryos, in 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 with +/+ [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*) (*Left*) 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*). (*Right*) Western blot analysis of α- and β-globin chains expressed in heterozygous +/– and *Nan*/+ fetal livers. (*F*) Analysis of the mRNA levels of embryonic/adult globins in *Nan*/+ adult spleen normalized to that from its +/+ littermate (color coded as indicated).

ever, expression from the β-globin and E2F2 promoters, whose category II cognate sites are not recognized by Nan-EKLF, are depressed significantly. Given the dramatic decrease in expression from the dematin promoter in *Nan*/+ cells, we tabulated binding sites occupied by EKLF (8) (Table S4) and found 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 also are critical for final output [as recently shown for p21 (33)], and these sites could be category II sites.

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, the affected subset 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 β-like globin gene cluster. Adult β-globin is not expressed in the absence of EKLF (22, 23), but the embryonic genes are expressed at a higher level and for a longer time (24–26). As observed for β-globin, α-globin expression is decreased by 50% in *Nan*/+ compared with the EKLF heterozygote (Fig. 5*E*, blue vs. red), probably explaining the absence of globin-chain imbalance in the *Nan*/+ mouse (Fig. 5*E*). Concomitant with the decrease in adult β-globin, embryonic ßh1 levels are augmented 2-fold in the +/− heterozygotes compared with +/+ homozygotes (Fig. 5*E*, blue vs. gray); this augmentation is accentuated further in the *Nan*/+ cells, where ßh1 levels are increased an additional 2-fold (Fig. 5*E*, blue vs. red). In the adult spleen, ßh1 accounts for virtually all β-like globin expression (Fig. 5*F*). We do not detect any differences in εy expression. These data indicate that the genetic switch in β-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 localized *Nan* to a point mutation within the second zinc finger of the EKLF transcription factor that alters a critical amino acid important for DNA recognition. Although a conservative substitution (glutamic to aspartic acid), the effects on EKLF DNA target recognition and the resultant genetic readout are dramatic. These changes explain the unexpectedly more severe phenotype exhibited by *Nan* compared with the EKLF-null mouse and have structural and genetic implications.

Insights from the *Nan* Phenotype. The *Nan* mutation maps to the middle of the critical "XYZ" amino acids (31) within the EKLF C2H2 finger structure. Based on structural data, the "Y" amino acid is located at the +3 position in the α-helix of EKLF zinc finger 2 (15). An earlier proposal based on phage selection suggested that aspartic acid at that position would interact preferentially with cytosine (32), a postulate consistent with our observation 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 (15) provides a structural rationale for the dramatic effect of a seemingly subtle change from E339 to D339 in *Nan*-EKLF that results in 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 (15) and correlate with point mutations at this site that lead to β-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 blood cell.

The *Nan* phenotype is unusual in that the mouse exhibits homozygous lethality significantly earlier (E10–11) than EKLF-null embryos (E14.5) (22, 23) and exhibits severe anemia when heterozygous, an effect not observed in EKLF heterozygotes (22, 23). Expression patterns of selected EKLF targets show that some are affected dramatically 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 blood cell membrane skeleton. Deficiency of dematin or α- or β-adducin alone leads to mild hemolytic anemia (30). Notably, combined deficiency of dematin and β-adducin, as occurs in *Nan*, produces a much more severe hemolytic anemia (34). The E2F2 transcription factor is important for cell-cycle control during terminal erythroid differentiation and for maintaining proper red blood cell volume (35, 36). Genetic misexpression is supported further by the abnormal residual embryonic (βh1) globin expression in *Nan*. This new phenotype is not equivalent to that of the EKLF-null mouse, because not all EKLF targets are affected equally; instead, only those with a "T" (category II) rather than a "C" (category I) within their EKLF zinc finger 2 promoter recognition motif are affected.

Genetic Distortion by Nan-EKLF. Although the *Nan* mutation exhibits a semidominant 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 (37). A basic tenet of this model is not satisfied, because 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. Moreover, in some cases the *Nan* heterozygote behaves as an EKLF heterozygote; that is, target expression is unaffected by the *Nan* mutation in the presence of one copy of the WT allele, inconsistent with a dominant negative effect. On the other hand, some genes are negatively affected dramatically in the *Nan* heterozygote and mimic the EKLF-null expression pattern. In this latter case, the inability of mutant E339D EKLF to bind its cognate category II sequence might free it to bind limiting amounts of cofactors and exert a dominant effect on activity of the WT EKLF protein, thus preventing it from efficiently forming a proper chromatin-remodeling and transcription-initiation complex (16–19). However, a selective dominant effect on a subset of genes would be required. In addition, in vivo competition is most effective when the effector is in excess (37, 38), a situation not observed in *Nan* heterozygotes.

Although a simpler explanation is that some EKLF target genes are 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)]; these effects are more profound than seen in the EKLF heterozygotes. Rather, our observations suggest that Nan-EKLF is a unique sequence-selective variant whose altered binding specificity directly distorts the genetic readout within *Nan* red blood cells by a previously unencountered mechanism.

Relevance of the Nan Mutation to Human Disease. A recent genome-wide association analysis (39) identified the chromosome 19p13.13 locus, where human *KLF1* (*EKLF*) resides, as significantly associated

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with clinically important hematologic traits, particularly decreased mean corpuscular volume and mean corpuscular hemoglobin, as seen in *Nan*/+. In another study, the human In(Lu) phenotype was mapped to mutations in one allele of *KLF1* (40). These persons exhibit virtually undetectable levels of the Lutheran antigen on their red blood cells; however, other red blood cell EKLF target genes are affected more subtly, 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, because the mutations lead to loss of function only of the affected *KLF1* allele.

A patient presenting with congenital dyserythropoietic anemia carrying a *KLF1*/*EKLF* allele mutated at the equivalent site (human E325K) as *Nan* was described recently (41). Studies of this transfusion-dependent patient in the 1990s revealed severe anemia with high hemoglobin F and reticulocyte levels, membrane abnormalities, altered cell-cycle parameters, deficient protein synthesis (42, 43), and increased embryonic ζ- and ε-globin expression (44, 45). 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-h light/dark cycle) with free access to acidified water and chow (NIH 5K52; Purina Mills). The *Nan* mutant was a female offspring of an ENU–mutagenized C3H/101 F1 male and a "PT" female at the Medical Research Council, Harwell, U.K. (3, 46). 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 was 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 $Klf1^{Nan}$.

ACKNOWLEDGMENTS. We thank Dr. X. Li (Mount Sinai School of Medicine) for discussion and Drs. P. Wassarman and P. Soriano (Mount Sinai School of Medicine) and M. T. Davisson and L. R. Donahue (The Jackson Laboratory) for critical review of the manuscript. We thank M. Lyon (Medical Research Council, Harwell, UK) for providing *Nan* mice for breeding at The Jackson Laboratory. This work was supported by National Institutes of Health Public Health Service Grants HL088468 (to L.L.P.) and DK46865 and DK77822 (to J.J. B.). The Quantitative PCR Shared Research Facility is supported by Mount Sinai School of Medicine.

1. Grace RF, Lux SE (2009) Disorders of the red cell membrane. *Hematology of Infancy and Childhood*, eds Orkin SH, et al. (Saunders Elsevier, Philadelphia), 7th Ed, pp 659–837.
2. Peters LL, Barker JE (2001) Spontaneous and targeted mutations in erythrocyte membrane skeleton genes: Mouse models of hereditary spherocytosis. *Hematopoiesis: A Developmental Approach*, ed Zon LI (Oxford Univ Press, New York), pp 582–608.
3. Lyon MF (1983) Dominant haemolytic anaemia. *Mouse News Letter* 68:68.
4. Lyon MF (1986) Position of neonatal anaemia (Nan) on chromosome 8. *Mouse News Letter* 74:95.
5. Miller IJ, Bieker JJ (1993) A novel, erythroid cell-specific murine transcription factor that binds to the CACCC element and is related to the Krüppel family of nuclear proteins. *Mol Cell Biol* 13:2776–2786.
6. Chen Z, et al. (2010) Porcine KLF gene family: Structure, mapping, and phylogenetic analysis. *Genomics* 95:111–119.
7. Drissen R, et al. (2005) The erythroid phenotype of EKLF-null mice: Defects in hemoglobin metabolism and membrane stability. *Mol Cell Biol* 25:5205–5214.
8. Hodge D, et al. (2006) A global role for EKLF in definitive and primitive erythropoiesis. *Blood* 107:3359–3370.
9. Pilon AM, et al. (2006) Alterations in expression and chromatin configuration of the alpha hemoglobin-stabilizing protein gene in erythroid Kruppel-like factor-deficient mice. *Mol Cell Biol* 26:4368–4377.
10. Frontelo P, et al. (2007) Novel role for EKLF in megakaryocyte lineage commitment. *Blood* 110:3871–3880.
11. Bouilloux F, et al. (2008) EKLF restricts megakaryocytic differentiation at the benefit of erythrocytic differentiation. *Blood* 112:576–584.
12. Tallack MR, Perkins AC (2010) Megakaryocyte-erythroid lineage promiscuity in EKLF null mouse blood. *Haematologica* 95:144–147.
13. Bieker JJ (2000) EKLF and the development of the erythroid lineage. *Transcription Factors: Normal and Malignant Development of Blood Cells*, eds Ravid K, Licht JD (Wiley-Liss, New York), pp 71–84.
14. Perkins A (1999) Erythroid Kruppel like factor: From fishing expedition to gourmet meal. *Int J Biochem Cell Biol* 31:1175–1192.
15. Feng WC, Southwood CM, Bieker JJ (1994) Analyses of β-thalassemia mutant DNA interactions with erythroid Krüppel-like factor (EKLF), an erythroid cell-specific transcription factor. *J Biol Chem* 269:1493–1500.
16. Zhang W, Kadam S, Emerson BM, Bieker JJ (2001) Site-specific acetylation by p300 or CREB binding protein regulates erythroid Krüppel-like factor transcriptional activity via its interaction with the SWI-SNF complex. *Mol Cell Biol* 21:2413–2422.
17. Bottardi S, Ross J, Pierre-Charles N, Blank V, Milot E (2006) Lineage-specific activators affect beta-globin locus chromatin in multipotent hematopoietic progenitors. *EMBO J* 25:3586–3595.
18. Sengupta T, Chen K, Milot E, Bieker JJ (2008) Acetylation of EKLF is essential for epigenetic modification and transcriptional activation of the beta-globin locus. *Mol Cell Biol* 28:6160–6170.
19. Sengupta T, Cohet N, Morlé F, Bieker JJ (2009) Distinct modes of gene regulation by a cell-specific transcriptional activator. *Proc Natl Acad Sci USA* 106:4213–4218.
20. Drissen R, et al. (2004) The active spatial organization of the beta-globin locus requires the transcription factor EKLF. *Genes Dev* 18:2485–2490.
21. Donze D, Townes TM, Bieker JJ (1995) Role of erythroid Kruppel-like factor in human γ- to β-globin gene switching. *J Biol Chem* 270:1955–1959.
22. Nuez B, Michalovich D, Bygrave A, Ploemacher R, Grosveld F (1995) Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene. *Nature* 375:316–318.
23. Perkins AC, Sharpe AH, Orkin SH (1995) Lethal β-thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLF. *Nature* 375:318–322.
24. Lim SK, Bieker JJ, Lin CS, Costantini F (1997) A shortened life span of EKLF-/- adult erythrocytes, due to a deficiency of β-globin chains, is ameliorated by human γ-globin chains. *Blood* 90:1291–1299.
25. Perkins AC, Gaensler KML, Orkin SH (1996) Silencing of human fetal globin expression is impaired in the absence of the adult β-globin gene activator protein EKLF. *Proc Natl Acad Sci USA* 93:12267–12271.
26. Wijgerde M, et al. (1996) The role of EKLF in human β-globin gene competition. *Genes Dev* 10:2894–2902.
27. Manwani D, Galdass M, Bieker JJ (2007) Altered regulation of beta-like globin genes by a redesigned erythroid transcription factor. *Exp Hematol* 35:39–47.
28. Ohgami RS, et al. (2005) nm1054: A spontaneous, recessive, hypochromic, microcytic anemia mutation in the mouse. *Blood* 106:3625–3631.
29. White RA, et al. (2009) Hematologic characterization and chromosomal localization of the novel dominantly inherited mouse hemolytic anemia, neonatal anemia (Nan). *Blood Cells Mol Dis* 43:141–148.
30. Robledo RF, et al. (2008) Targeted deletion of alpha-adducin results in absent beta- and gamma-adducin, compensated hemolytic anemia, and lethal hydrocephalus in mice. *Blood* 112:4298–4307.
31. Klevit RE (1991) Recognition of DNA by Cys2, His2 zinc fingers. *Science* 253:1367–1395.
32. Pabo CO, Peisach E, Grant RA (2001) Design and selection of novel Cys2His2 zinc finger proteins. *Annu Rev Biochem* 70:313–340.
33. Siatecka M, Lohmann F, Bao S, Bieker JJ (2010) EKLF directly activates the p21WAF1/CIP1 gene by proximal promoter and novel intronic regulatory regions during erythroid differentiation. *Mol Cell Biol* 30:2811–2822.
34. Chen H, et al. (2007) Combined deletion of mouse dematin-headpiece and beta-adducin exerts a novel effect on the spectrin-actin junctions leading to erythrocyte fragility and hemolytic anemia. *J Biol Chem* 282:4124–4135.
35. Dirlam A, Spike BT, Macleod KF (2007) Deregulated E2f-2 underlies cell cycle and maturation defects in retinoblastoma null erythroblasts. *Mol Cell Biol* 27:8713–8728.
36. Li FX, Zhu JW, Hogan CJ, DeGregori J (2003) Defective gene expression, S phase progression, and maturation during hematopoiesis in E2F1/E2F2 mutant mice. *Mol Cell Biol* 23:3607–3622.
37. Veitia RA (2009) Dominant negative factors in health and disease. *J Pathol* 218:409–418.
38. Veitia RA (2007) Exploring the molecular etiology of dominant-negative mutations. *Plant Cell* 19:3843–3851.
39. Ganesh SK, et al. (2009) Multiple loci influence erythrocyte phenotypes in the CHARGE Consortium. *Nat Genet* 41:1191–1198.
40. Singleton BK, Burton NM, Green C, Brady RL, Anstee DJ (2008) Mutations in EKLF/KLF1 form the molecular basis of the rare blood group In(Lu) phenotype. *Blood* 112:2081–2088.
41. Singleton BK, et al. (2009) A novel EKLF mutation in a patient with dyserythropoietic anemia: The first association of EKLF with disease in man. *Blood* 114:A162.
42. Parsons SF, et al. (1994) A novel form of congenital dyserythropoietic anemia associated with deficiency of erythroid CD44 and a unique blood group phenotype [In (a-b-), Co(a-b-)]. *Blood* 83:860–868.
43. Wickramasinghe SN, Illum N, Wimberley PD (1991) Congenital dyserythropoietic anaemia with novel intra-erythroblastic and intra-erythrocytic inclusions. *Br J Haematol* 79:322–330.
44. Agre P, et al. (1994) Human red cell Aquaporin CHIP. II. Expression during normal fetal development and in a novel form of congenital dyserythropoietic anemia. *J Clin Invest* 94:1050–1058.
45. Tang W, et al. (1993) Expression of embryonic zeta-globin and epsilon-globin chains in a 10-year-old girl with congenital anemia. *Blood* 81:1636–1640.
46. West JD, Peters J, Lyon MF (1984) Genetic differences between two substrains of the inbred 101 mouse strain. *Genet Res* 44:343–346.
47. Tallack MR, Keys JR, Perkins AC (2007) Erythroid Kruppel-like factor regulates the G1 cyclin dependent kinase inhibitor p18INK4c. *J Mol Biol* 369:313–321.
48. Tallack MR, Keys JR, Humbert PO, Perkins AC (2009) EKLF/KLF1 controls cell cycle entry via direct regulation of E2f2. *J Biol Chem* 284:20966–20974.
49. Keys JR, et al. (2007) Genomic organisation and regulation of murine alpha haemoglobin stabilising protein by erythroid Kruppel-like factor. *Br J Haematol* 136:150–157.
50. Funnell AP, et al. (2007) Erythroid Krüppel-like factor directly activates the basic Krüppel-like factor gene in erythroid cells. *Mol Cell Biol* 27:2777–2790.