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Mutation in Erythroid Specific Transcription Factor KLF1 Causes Hereditary Spherocytosis in the *Nan* Hemolytic Anemia Mouse Model

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Abstract

KLF1 regulates definitive erythropoiesis of red blood cells by facilitating transcription through high affinity binding to CACCC elements within its erythroid-specific target genes including those encoding erythrocyte membrane skeleton (EMS) proteins. Deficiencies of EMS proteins in humans lead to the hemolytic anemia Hereditary Spherocytosis (HS) which includes a subpopulation with no known genetic defect. Here we report that a mutation, E339D, in the second zinc finger domain of KLF1 is responsible for HS in the mouse model *Nan*. The causative nature of this mutation was verified with an allelic test cross between *Nan*/+ and heterozygous $Klf1^{+/-}$ knockout mice. Homology modeling predicted *Nan* KLF1 binds CACCC elements more tightly, suggesting that *Nan* KLF1 is a competitive inhibitor of wild type KLF1. This is the first association of a KLF1 mutation with a disease state in adult mammals and also presents the possibility of being another causative gene for HS in humans.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version.

Keywords

Hereditary Spherocytosis; Anemia; KLF1; EKLF; Mouse Mutant

Introduction

Erythroid Krüppel-like factor (EKLF, KLF1) is the founding member of a family of transcriptional activators and repressors whose defining feature is their DNA binding motif [1]. Three highly conserved C2H2 zinc finger domains at the carboxyl terminus regulate transcription by binding to consensus CACCC elements [2]. The zinc finger domains are involved in protein-protein interactions that modulate transcriptional specificity of KLF proteins [3]. The N-terminal region of KLF proteins is less conserved and contains transcriptional activators and/or repressor domains that function through interactions with additional transcription factors and chromatin remodelers [4,5].

KLF1 is an essential regulator of erythropoiesis [1,6–9], and as such, its expression is limited to erythroid cells of yolk sac, fetal liver, spleen and bone marrow. KLF1 activates a diverse set of genes associated with erythropoiesis [10,11], including the β-globin gene (*Hbb*) [12,13] and genes encoding the erythrocyte membrane skeleton (EMS) [14–16]. Gene targeting of *Klf1* in mice provides insight into the essential role of KLF1 during erythropoiesis [6,7,16]. The complete loss of KLF1 in homozygous $Klf1^{-/-}$ embryos results in death by 15 days gestation due to defective definitive erythropoiesis [6,7,16]. However, heterozygous $Klf1^{+/-}$ knockout mice are haplo-sufficient as they survive to adulthood with no deleterious phenotype. A significant finding was that transcription of EMS genes in fetal liver from KLF1-null embryos is decreased dramatically [16].

The EMS confers properties of stability, durability and deformability to red blood cells. Many studies have shown that qualitative or quantitative disruption of the EMS components or its protein-protein interactions leads to inherited hemolytic anemia in mouse and man [17,18]. The major proteins of the EMS are α and β spectrin, ankyrin, protein band 3, protein 4.1R and protein 4.2. Deficiencies of EMS proteins lead to the hemolytic anemia Hereditary Spherocytosis (HS) which is the most common hereditary anemia in persons of Northern European descent with an incidence of 1/2,000 [17,18]. Mutations in the EMS genes *SPTA1* (α-spectrin), *SPTB* (β-spectrin) and *ANK1* (ankyrin), comprise approximately 70% of the mutations causing HS, while mutations in *SLC4A1* (Band 3) and *EPB42* (Protein 4.2) [18,19] constitute another 20% of the patient population. Approximately 10% of HS patients have no known genetic defect in EMS genes [19].

HS causing genes have been identified using mouse models [20–22] which have been invaluable for elucidating the function of the EMS. The Neonatal anemia mouse (gene symbol *Nan*) [23] provides a unique model to identify a new gene for HS. The hematological presentation of the *Nan* mouse mimics that of human HS in that the erythrocytes from adult *Nan*/+ mice exhibit spherocytosis and osmotic fragility [24]. The anemia phenotype of *Nan*/+ mice also includes decreased red blood cell number, decreased hematocrit, decreased hemoglobin and elevated zinc protoporphyrin [24]. Characterization of *Nan*/+ red blood cell ghost proteins show a substantially decreased amount of almost all

EMS proteins [24]. We have previously mapped the *Nan* locus to a critical region on Chromosome 8 that contains *Klf1* [24]. There are 31 functional genes in this region, none of which encode EMS proteins, leaving *Klf1* as the most promising candidate gene for *Nan*. We now describe the identification and characterization of a mutation in *Klf1* as the defective gene in *Nan* mice. This is the first association of a KLF1 mutation with a disease state in adult mammals and also presents the possibility that *KLF1* is a causative gene for HS in humans.

Results

Phenotypic and Hematologic Characterization of Nan Mice

The *Nan* mutation was originally generated by *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis on the C3H101H strain [23] and transferred to C57BL/6J (B6) and WB/ReJ (WB) [24]. In this study, we transferred the *Nan* mutation to the FVB/NJ (FVB) strain. FVB *Nan*/+ pups can be recognized at birth due to their pallor, and their anemia persists into adulthood. Erythrocytes from adult FVB *Nan*/+ mice exhibit spherocytosis (Supplementary Fig. S1) and osmotic fragility characteristic of HS. Adult FVB *Nan*/+ mice present with decreased red blood cell number, decreased hematocrit, decreased hemoglobin and elevated zinc protoporphyrin (Supplementary Table S1). Thus, the strain background does not affect the anemia phenotype caused by the *Nan* mutation.

Klf1 is Mutated in Nan Mice

We had mapped previously the *Nan* locus to a 648 Kb region on Chromosome 8 which includes 31 genes as determined by the sequence available in GenBank contig NT078575 [24]. There were no known EMS genes in the *Nan* critical region. However, *Klf1* was present and is known to regulate the expression of the EMS genes *Ank1*, *Slc4a1* and *Spnb1* [16], leaving *Klf1* as the most promising candidate gene for *Nan*. DNA sequence analysis of the *Klf*1 gene in *Nan/+* mice (B6, WB, and FVB *Nan* congenic lines) revealed an A to T transversion (A1065T) in exon 3 (Fig. 1), resulting in an amino acid substitution of glutamic acid to aspartic acid at amino acid position 339 (E339D) in the second zinc finger domain of KLF1 (Fig. 2). An A to T transversion is typically seen for ENU-induced mutations. To verify that this SNP was unique to *Nan*, we sequenced this region from 21 different inbred mouse strains including the founder strain for *Nan* mice (C3H101H), 4 other sublines of the C3H strain (C3H/HeJ, C3HeB/FeJ, C3H/HeSnJ, C3H/HeOuJ) and the 101H parental strain (Supplementary Table S2). Analysis of the *Klf1* nucleotide sequence in these different strains demonstrates that the A1065T mutation is found only in *Nan* mice, indicating the mutation is specific to *Nan*.

The remaining 30 genes in the *Nan* critical genomic region were sequenced, including the exons along with the flanking intron boundaries, and no causative gene mutations for *Nan* were found. Only common polymorphisms were found in the remaining 30 genes in the *Nan* critical genomic region. To determine if there was a regulatory gene defect in any of the 30 genes, RTPCR of bone marrow RNA from *Nan* and wild type mice showed that there was no change in expression (including evidence of alternative splicing) for all 30 genes.

The amino acid sequence for the three zinc finger domains of all KLF1 proteins indicates that the glutamic acid is highly conserved (Fig. 2). The E339D alteration disrupts the arginine - glutamic acid - arginine (R_1--E--R_2) protein motif (Fig. 2) required for interactions of zinc finger 2 (ZF2) with its DNA targets [25], and the impact of this change is therefore significant. Interestingly, the glutamic acid at this position in ZF2 is conserved in all mammalian members of the KLF protein family (*Klf1* through *Klf17*), revealing a strong evolutionary selection for this residue in the function of KLF proteins.

Allelic Test Cross Confirms Klf1 is Nan

To document further that the E339D KLF1 mutation in *Nan* is causative, we established an allelic test cross between *Nan*/+ mice and heterozygous $Klf1^{+/-}$ knockout mice. Adult *Nan*/+ mice exhibit lifelong hemolytic anemia [24], whereas *Nan*/*Nan* embryos die *in utero* at 12.5 days gestation [23]. Since both *Nan*/*Nan* and KLF1-null homozygote knockout mice ($Klf1^{-/-}$) die *in utero*, we predicted that if *Nan* and *Klf1* were the same gene, then no *Nan* mice with the *Klf1* knockout would be born from this allelic test cross. The result of the allelic test cross showed that of 91 mice analyzed at birth, 32 *Nan* mice without the *Klf1* knockout (*Nan*/+) were generated whereas no *Nan* mice carrying the *Klf1* knockout (*Nan*/-) were detected (Fig. 3a). The probability of exclusively producing 32 *Nan*/+ mice by chance, with no *Nan*/-mice generated from this test cross, was extremely small ($p = 2.3 \times 10^{-10}$). Thus the allelic test cross results provide strong evidence of the candidacy of *Klf1* being the defective gene for *Nan*. Some animals generated from these crosses were allowed to develop into adulthood and hematological characterization by the one step osmotic fragility test confirmed the HS phenotype of the *Nan*/+ mice. In contrast, $Klf1^{+/+}$ and $Klf1^{+/-}$ mice were normal.

The fate of *Nan*/-embryos was established by examination of fetuses at different gestational ages from timed matings of *Nan*/+ × $Klf1^{+/-}$ mice. At 14.5 days gestation, *Nan*/- embryos had developed abnormally and died *in utero*, while the presence of pale *Nan*/+ and normally developing embryos ($Klf1^{+/+}$ and $Klf1^{+/-}$) were observed (Fig. 3b). These findings were generated by genotyping embryos from this mating for the wild type or A1065T *Nan* mutation of *Klf1* by DNA sequencing and for the *Klf1* knockout allele by electrophoretic analysis of an established PCR reaction (http://jaxmice.jax.org/protocolsdb/). The phenotypically normal embryos were genotyped as either $Klf1^{+/+}$ or $Klf1^{+/-}$. The results of this test cross provide substantial evidence that *Klf1* is the mutated *Nan* gene. In addition, 12.5 day *Nan*/-embryos from timed allelic crosses were also found to be dysmorphic implicating the allelic nature of the compound heterozygote (Fig. 3c). The dysmorphic features in *Nan*/-embryos are in contrast to the phenotype observed for $Klf1^{-/-}$ embryos which exhibit severe anemia without dysmorphology suggesting that the expression of the abnormal *Nan* KLF1 protein manifests itself in a profound phenotype in comparison to $Klf1^{-/-}$ embryos [6,7].

Protein Homology Modeling Predicts Altered DNA Binding in Nan KLF1

Since the KLF1 E339D mutation is in a highly conserved zinc finger domain involved in DNA binding, we predicted the DNA binding interactions of *Nan* KLF1 would be altered. To test this hypothesis, we examined the impact of the E339D mutation on KLF1 by

molecular homology modeling of interactions of wild type and *Nan* KLF1 proteins with the β-globin (*Hbb*) CACCC DNA element 5' TTCCACACCCT 3' [1,7], in which zinc finger 1 (ZF1) binds CCA, ZF2 binds CAC, and zinc finger 3 (ZF3) binds CCT. Homology modeling of KLF1 indicates that the E339D mutation locates in ZF2, where the side chains of the R_1--E--R_2 (wild type) and R_1--D--R_2 (*Nan*) motifs face inwards towards the DNA major groove (Fig. 4a). Consistent with previous models of C2H2 ZF domains [25,26], wild type R_1 (R336) binds the DNA by hydrogen bonding with C_1 in the common CACCC binding motif and E339 stabilizes this interaction by hydrogen bonding to NH(ε) in the R_1 side chain (Fig. 4b, Supplementary Video S1 and S2). Molecular dynamics simulation (10 picoseconds) supports this structural model and also shows the effects of disrupting this stabilizing interaction with the E339D mutation in *Nan* KLF1. In the mutant protein, D339 maintains only sporadic interaction with the R_1 side chain, forming alternate hydrogen bonds with both NH(ε) and the terminal NH_2 groups, leaving the R_1 side chain free to hydrogen bond with the pairing Guanine to C_1 in the CACCC motif (Fig. 5c, Supplementary Video S3 and S4). The impact of the amino acid change in *Nan* KLF1 is a 3-dimensional steric change with the concomitant suggestion that *Nan* KLF1 binds tighter to target DNA.

Discussion

HS is a common anemia in persons of Northern European descent and as such has received much attention in the last two decades during which HS causing genes have been identified [17–19]. However, there remains a sub-population of 10% of HS patients for whom no known genetic defect has yet been identified [19]. The identification of the gene causing the *Nan* mutation is significant because the *Nan* mutant has allowed discovery of a new HS gene which may also cause this disease in humans.

Nan mice exhibit an E339D KLF1 mutation and the collective results of the (i) allelic test cross, (ii) the anemia of *Nan*/+ embryos, (iii) the dysmorphology coupled with embryonic lethality of *Nan*/- mice and (iv) protein modeling showing the impact of the amino acid change on KLF1 binding, support the causative nature of this mutation. Since both the wild type and mutant forms of KLF1 are present in *Nan* mice, we hypothesize that *Nan* KLF1 functions as a competitive inhibitor of wild type KLF1. This leads to ineffective production of EMS proteins and concomitant hemolytic anemia. Competitive inhibition also helps explain the apparent disconnect between the finding that *Nan*/+ heterozygous mice are anemic, whereas $Klf1^{+/-}$ heterozygous mice are normal and haplo-sufficient. The dysmorphology observed in *Nan*/- embryos demonstrates further the severe dominant-negative effect of the *Nan* mutation in homozygote embryos. The dysmorphic phenotype differs from the pale phenotype of $Klf1^{-/-}$ embryos [6,7] suggesting the *Nan* E339D KLF1 mutation has a profound effect that alters gene expression and development in the absence of wild-type KLF1.

We speculate that the putative dominant/negative competitive inhibition that results from the alteration in DNA binding of *Nan* KLF1 impairs the function of wild type KLF1-associated transcription complexes that occur normally in the production of β-globin and EMS proteins. The recent finding that KLF1-regulated genes are coordinately transcribed at transcription factories in erythroid cells [27] provides an intriguing mechanism to speculate

how *Nan* KLF1 may alter transcription of EMS genes. The clustering of KLF1-regulated genes at transcription factories is KLF1-dependent, and this coordinated organization of transcriptional complexes promotes reinitiation of transcription and boosts the output and production of erythroid proteins [27]. The transcriptional complexes may be impaired because the mutated *Nan* KLF1 protein binds tightly to the EMS target genes and blocks the activity of wild type KLF1. Furthermore, this model predicts that any mutation in KLF1 that alters specificity of DNA binding and/or disrupts interactions with proteins in transcriptional complexes may suppress wild type KLF1 activity.

Conclusion

The *Nan* mutation is the first direct association of a mutation in KLF1 with a disease state in adult mammals and the exact mechanism of its effect warrants further investigation. Furthermore, we propose that mutations in human *KLF1* that suppress the expression of EMS genes will also lead to HS and may explain the subpopulation of HS cases with no known genetic defect. The putative dominant/negative competitive inhibition of the *Nan* mutation makes the *Nan* mouse an excellent model system to study the function of KLF1.

Materials and Methods

Mice

All mice were maintained at the University of Missouri-Kansas City (UMKC) Lab Animal Center with animal usage guidelines and procedures approved by the UMKC Institutional Animal Care and Use Committee. C57BL/6J (B6), WB/ReJ (WB), and FVB/NJ (FVB) mice, along with (B6 × 129S4)F_1-*Klf1*^{tm1Sho} knockout mice (JAX stock number 002474) were obtained from The Jackson Laboratory (Bar Harbor, ME). Genomic DNAs for mouse strains were also obtained from The Jackson Laboratory and the 101H strain sample for DNA extraction was a gift from the Medical Research Council Harwell (Oxfordshire, England).

Genotyping

Nan mice were recognized by their pallor at birth and were genotyped using either a single-step osmotic fragility test or testing for zinc protoporphyrin levels. Genomic DNA from mouse tissue (tail, spleen, and embryonic) was prepared from samples as described previously [28,29]. The presence of the E339D KLF1 *Nan* mutation was detected by PCR amplification and DNA sequence analysis of the third exon of *Klf*1 (*Klf*1-ex3F 5’ TTGCTTGGAGGGTGGTACTT 3’; *Klf*1-ex3R 5’ GGAGGTGTGTGTACAGGTAAGTG 3’). *Klf1* knockout mice were genotyped using a separated PCR assay which detected the presence or absence of the neomycin resistance cassette within the *Klf*1 gene, according to the procedures developed by The Jackson Laboratory Animal Resource Department (http://jaxmice.jax.org/protocolsdb/).

DNA sequence analyses

Genomic PCR was performed using primers (Oligo Etc., Wilsonville, OR; IDT Coralville, IA) derived from sequences flanking the exons in each gene from the *Nan* critical region.

Gene sequences were obtained from Genbank (http://www.ncbi.nlm.gov) and Ensembl. (http://www.ensembl.org). Primer design, PCR conditions, and DNA sequence analyses were completed as described previously [29]. The primer sequences utilized for these assays are available upon request. SNPs were characterized utilizing the Single Nucleotide Polymorphism database (http://www.ncbi.nlm.nih.gov/projects/SNP/). cDNA sequences were used to number the nucleotide position of the SNPs in *Nan* mice. The accession code for the gene sequences in the *Nan* critical region is GenBank contig NT078575.

Molecular Modeling

Homology models of wild type KLF1 and *Nan* KLF1 proteins were created with MODELLER [30] using Zif268 (PDB:1AAY) as the template structure. The bound DNA in 1AAY was mutated manually with 3DNA [31] to include the common KLF1 binding motif CACCC (5'-TCCA**CACCC**T-3'). Instead of optimizing these structures for static analysis, molecular dynamics simulations were performed with GROMACS [32] as follows on the initial models to examine the full effect of the *Nan* mutation on the protein-DNA interaction. First, the models (protein and bound DNA) were enriched with hydrogen atoms not present in the original homology model using the "Add hydrogens" tool at WHATIF [33] and the "Add hydrogens" tool at MolProbity [34] and reformatted to the appropriate labeling schema for GROMACS input. Second, each model was used to generate a unique topology file with the GROMACS *pdb2gmx* tool and GROMOS96 53a5 force field [35]. These structures were then energy-minimized with the GROMACS *grompp* and *mdrun* tools and placed into theoretical boxes (GROMACS *editconf*), which were subsequently solvated with water molecules (GROMACS *genbox*). At this point, a 10 picosecond simulation of the protein DNA interaction was performed, again using the GROMACS *grompp* and *mdrun* tools. Analysis of the simulations was performed by visualization of the final structure and trajectory files in VMD [36] and high-resolution figures were constructed in PyMol (www.pymol.org).

Statistical analyses

Hematological assays were performed on five animals. Differences between groups were determined by Student's *t*-test using SPSS Statistics 17.0 software (Chicago, IL). Differences were considered significant when $P < 0.05$. The probability (p) of producing 32 *Nan*/+ mice and no *Nan*/-mice was calculated using the product rule; $p = (1/2)^{32}$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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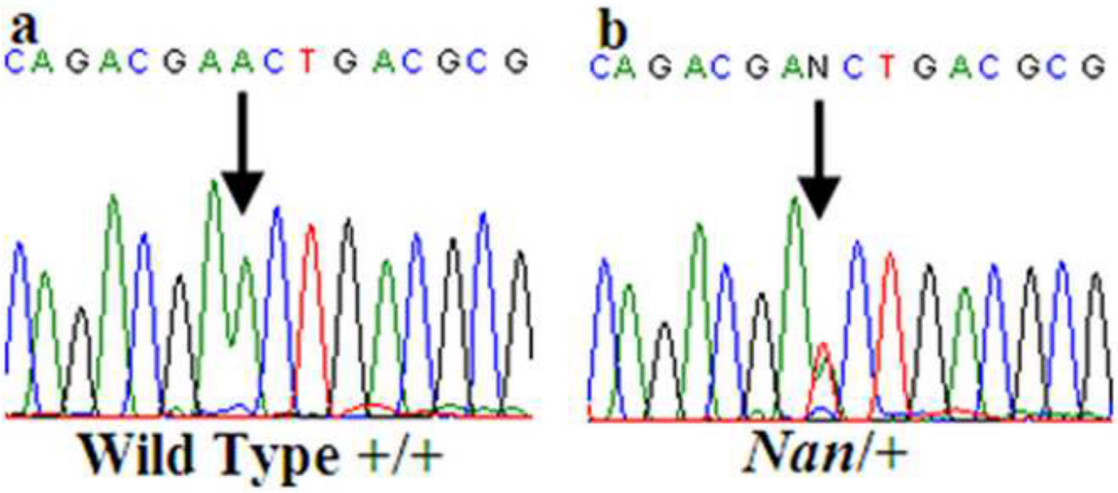

Fig. 1.

Identification and characterization of the *Klf1* mutation in *Nan* mice. (**a**) Wild type $Klf1^{+/+}$ sequence corresponding to the GAA codon for glutamic acid at position 339 of the KLF1 protein. The single peak of the A dinucleotide involved in the mutation is identified with the arrow. (**b**) Corresponding *Klf1* sequence from a heterozygous *Nan*/+ mouse showing the double peak of the A and T dinucleotides, identified with an arrow, at the third position of the codon representing the presence of both the mutant *Nan* and wild type alleles. The codon change from GAA to GAT results in the substitution of aspartic acid for glutamic acid.

Fig. 2.

Amino acid sequence alignment of the three zinc finger domains from mammalian KLF1 proteins. This alignment shows the high conservation of these domains, especially the R_1-E-R_2, DNA binding motif in zinc finger 2. The R_1-D-R_2 motif is found only in *Nan* mice.

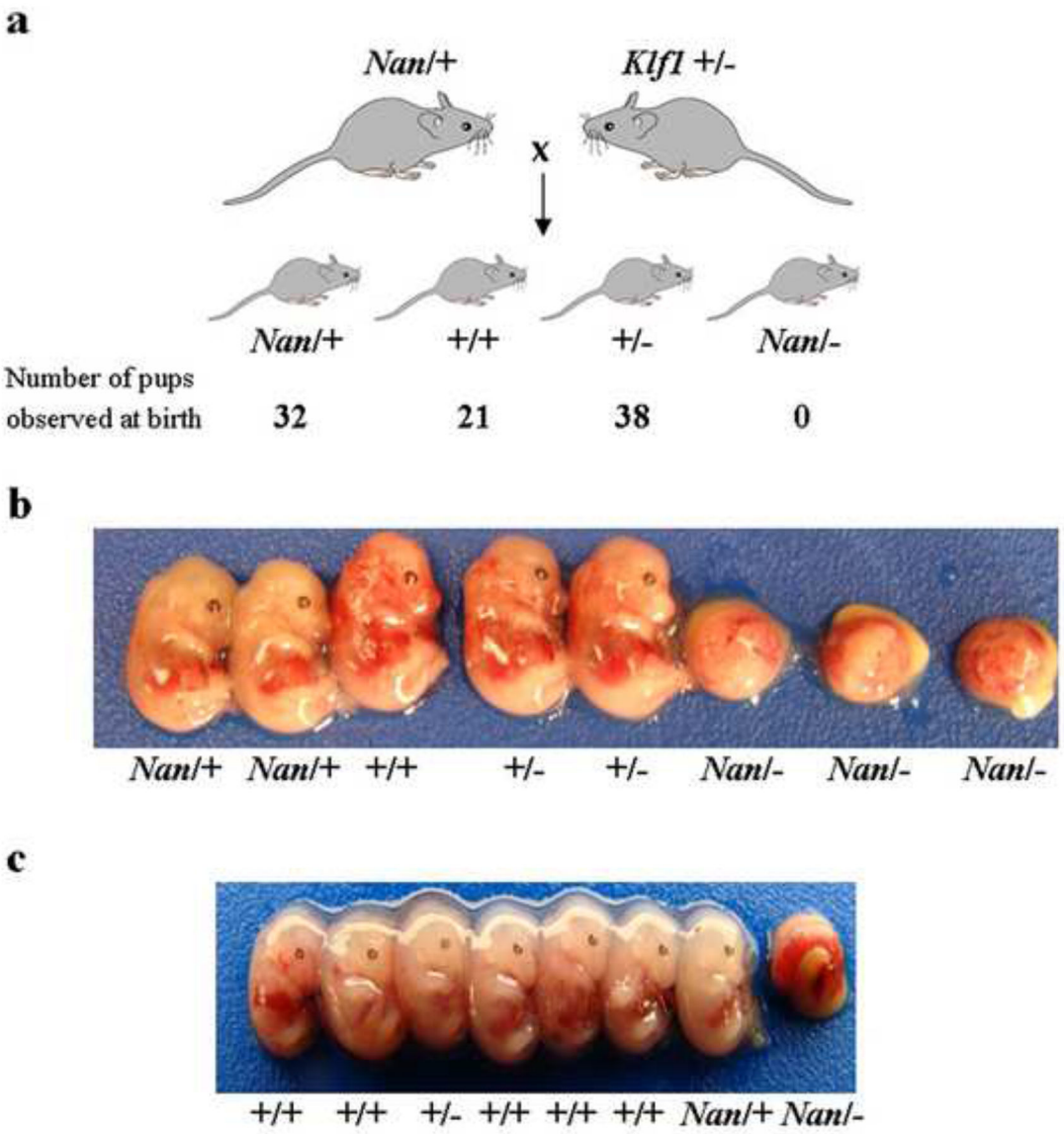

Fig. 3.

Allelic test cross of *Nan*/+ mice with *Klf1*$^{+/-}$ knockout mice. (**a**) The *Nan*/+ mice could be distinguished phenotypically from the *Klf1*$^{+/+}$ and *Klf1*$^{+/-}$ mice by their pallor at birth. At 1 to 2 days the pups were sacrificed and the *Nan* genotype was determined by measuring zinc protoporphyrin levels. The *Nan Klf1* mutation was verified by PCR amplification and DNA sequence analyses of the third exon of *Klf1* to detect the A1065T mutation. The *Klf1* knockout genotype was determined by a separated PCR assay which detects the neomycin resistance cassette present in the *Klf1* knockout mice. No *Nan*/*Klf1*$^{-}$ mice were observed at birth. (**b**) E14.5 embryos from a C57BL/6J *Nan*/+ × (B6 × 129S4)F_1-*Klf1*$^{+/-}$ mating were genotyped as above and digitally photographed. *Nan*/+ embryos were pale but otherwise appeared to be developing normally. *Klf1*$^{+/+}$ and *Klf1*$^{+/-}$ embryos were phenotypically identical. The *Nan*/-embryos present with dysmorphology in contrast to either *Klf1*$^{-/-}$ or *Nan*/*Nan* embryos as previously described [6, 7, 23]. (**c**) E12.5 embryos from a C57BL/6J

Nan/+ mice × (B6 × 129S4)F_1-*Klf1*(+/−) mating display the same phenotype as E14.5 embryos.

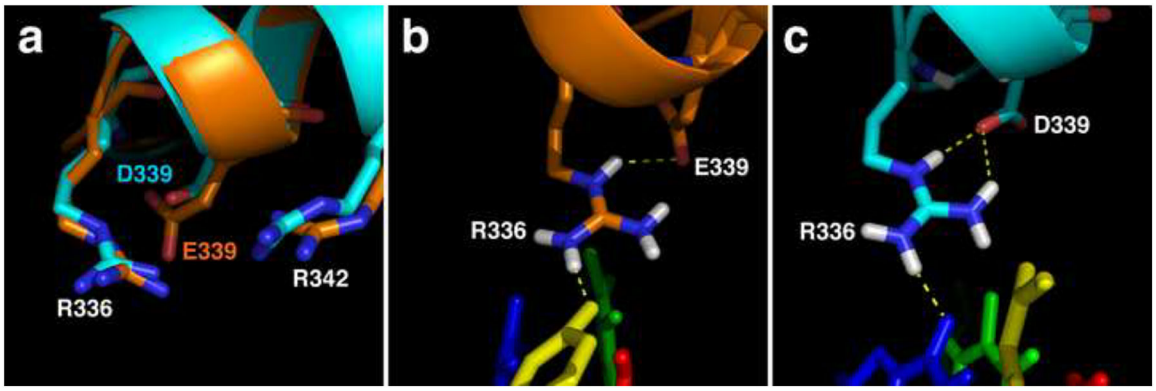

Fig. 4.

Computational modeling and dynamic simulation of wild type and *Nan* KLF1 protein structures with bound DNA. (a) Alignment of computationally generated homology models of wild type (orange) and *Nan* (cyan) KLF1 structures reveals a subtle change in the *Nan* mutant. (b) Analysis of molecular dynamics simulations of the KLF1-DNA interaction indicate the formation of a stabilizing hydrogen bond (dashed yellow line) between E339 and R336 in the wild type KLF1 protein, facilitating interaction of R336 with a cytosine base (yellow) in the major groove of the consensus DNA binding motif (5'-**C**ACCC-3'). (c) According to the simulation model, the mutation of wild type E339 to an aspartic acid (D339) in *Nan* KLF1 results in an alteration of the side chain hydrogen bond between the mutated residue and R336, which then interacts with a guanine base (blue) rather than the cytosine of the wild type model.