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RESEARCH ARTICLE

Neomorphic effects of the *neonatal anemia* (*Nan-Eklf*) mutation contribute to deficits throughout development

Antanas Planutis[1], Li Xue[1], Cecelia D. Trainor[2], Mohan Dangeti[1], Kevin Gillinder[3], Miroslawa Siatecka[1,4], Danitza Nebor[5], Luanne L. Peters[5], Andrew C. Perkins[3,6] and James J. Bieker[1,7,8,9,*]

ABSTRACT

Transcription factor control of cell-specific downstream targets can be significantly altered when the controlling factor is mutated. We show that the semi-dominant neonatal anemia (*Nan*) mutation in the EKLF/KLF1 transcription factor leads to ectopic expression of proteins that are not normally expressed in the red blood cell, leading to systemic effects that exacerbate the intrinsic anemia in the adult and alter correct development in the early embryo. Even when expressed as a heterozygote, the Nan-EKLF protein accomplishes this by direct binding and aberrant activation of genes encoding secreted factors that exert a negative effect on erythropoiesis and iron use. Our data form the basis for a novel mechanism of physiological deficiency that is relevant to human dyserythropoietic anemia and likely other disease states.

KEY WORDS: Erythropoiesis, Transcription factor, Anemia, Monoallelic mutation, Cytokine effects, Mouse

INTRODUCTION

Direct activation of tissue-restricted gene expression relies on transcription factors that faithfully recognize their cognate binding sequence in DNA and influence the transcriptional and epigenetic outcome (Novershtern et al., 2011). Of special importance for erythropoiesis is EKLF/KLF1, which exerts a global role in tissue-specific gene regulation by virtue of its three-finger recognition of the 9 bp 5′CCMCRCCCN sequence at target gene promoters and enhancers (reviewed by Siatecka and Bieker, 2011; Tallack and Perkins, 2010; Yien and Bieker, 2013). Its effects are circumscribed owing to its tissue-restricted expression in erythroid cells and its bipotent myeloid progenitor. Genetic ablation studies in the mouse show that EKLF is absolutely essential because death results from a profound β-thalassemia and the low to virtually non-existent expression of global erythroid genes of all categories.

Monoallelic mutation of *KLF1* in humans can lead to a benign outcome that is nonetheless phenotypically important (Borg et al., 2011; Helias et al., 2013; Singleton et al., 2012; Tallack and Perkins, 2013; Waye and Eng, 2015), as haploinsufficient levels of KLF1 lead to altered β-globin switching, which can be advantageous in individuals with β-thalassemia (Liu et al., 2014). However, some mutations lead to anemias (Arnaud et al., 2010; Huang et al., 2015; Jaffray et al., 2013; Singleton et al., 2011; Viprakasit et al., 2014; reviewed by Perkins et al., 2016). The human KLF1 mutation (E325K) in congenital dyserythropoietic anemia (CDA) (Arnaud et al., 2010; Jaffray et al., 2013; Singleton et al., 2011) is at the same amino acid as that seen in the mouse *Nan* mutant (Heruth et al., 2010; Siatecka et al., 2010b), albeit a different substitution. *Nan* is inherited in a semi-dominant fashion: homozygotes die *in utero* at E10-11, while heterozygous *Nan*/+ mice exhibit lifelong severe anemia that is characterized by reticulocytosis, splenomegaly, altered globin expression and cell membrane defects (Lyon, 1983; Siatecka et al., 2010b; White et al., 2009). Transfer of *Nan* mouse anemic hematological properties after bone marrow transplantation of *Nan*/+ cells to a wild-type recipient shows the dominant, cell-autonomous nature of the effect (Lyon, 1983; White et al., 2009).

The mutation in zinc finger 2 at E339D, although conservative, is at a universally conserved amino acid and leads to altered recognition properties such that Nan-KLF1 recognizes only the 5′CCMCGCCCN subset of wild-type sequences and leads to a distorted genetic output (Siatecka et al., 2010b). Thus, there are two types of binding sites: those able to bind both wild-type KLF1 and Nan-KLF1 (category I) and those able to bind only wild-type KLF1 (category II) (Siatecka et al., 2010b). Paradoxically, the mutant Nan-KLF1 form interferes with the expression of target genes with category II sites, to which it can no longer bind. The molecular explanation behind this remains unclear. However, the change also confers recognition of an altered sequence that is not normally associated with KLF1 recognition (Gillinder et al., 2016), such that genes that are never seen in the erythroid cell are ectopically expressed in the *Nan*/+ mutant.

An incompletely explained aspect of the *Nan*/+ mouse is that its anemia is wide-ranging during development in both the embryo and the adult, and may not be completely accounted for by the distortion in the intrinsic pattern of expression in the *Nan*/+ erythroid cell. This is directly relevant to the humans with CDA, who also exhibit a range of effects that appear extrinsic to those caused by a tissue-restricted factor such as KLF1. We have addressed these issues by testing the hypothesis that ectopic expression of a specific subset of genes leads to effects that are superimposed on the red cell defects and thus plays a significant contributory role to the *Nan* anemia phenotype.

RESULTS

Altered recognition sequences of Nan-EKLF lead to circumscribed and ectopic/neomorphic gene expression in the *Nan*/+ mouse fetal liver erythroid cell

Inspection of globally expressed genes whose levels are altered in *Nan*/+ E13.5 fetal livers compared with their wild-type littermates

[1]Department of Developmental and Regenerative Biology, Mount Sinai School of Medicine, New York, NY 10029, USA. [2]Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD 20892, USA. [3]Mater Research Institute, University of Queensland, Woolloongabba QLD 4102, Queensland, Australia. [4]Department of Genetics, University of Adam Mickiewicz, Poznan 61-614, Poland. [5]Jackson Laboratory, Bar Harbor, ME 04609, USA. [6]Princess Alexandra Hospital, Brisbane QLD 4102, Queensland, Australia. [7]Black Family Stem Cell Institute, Mount Sinai School of Medicine, New York, NY 10029, USA. [8]Tisch Cancer Institute, Mount Sinai School of Medicine, New York, NY 10029, USA. [9]Mindich Child Health and Development Institute, Mount Sinai School of Medicine, New York, NY 10029, USA.

*Author for correspondence (james.bieker@mssm.edu)

J.J.B., 0000-0001-5128-7476

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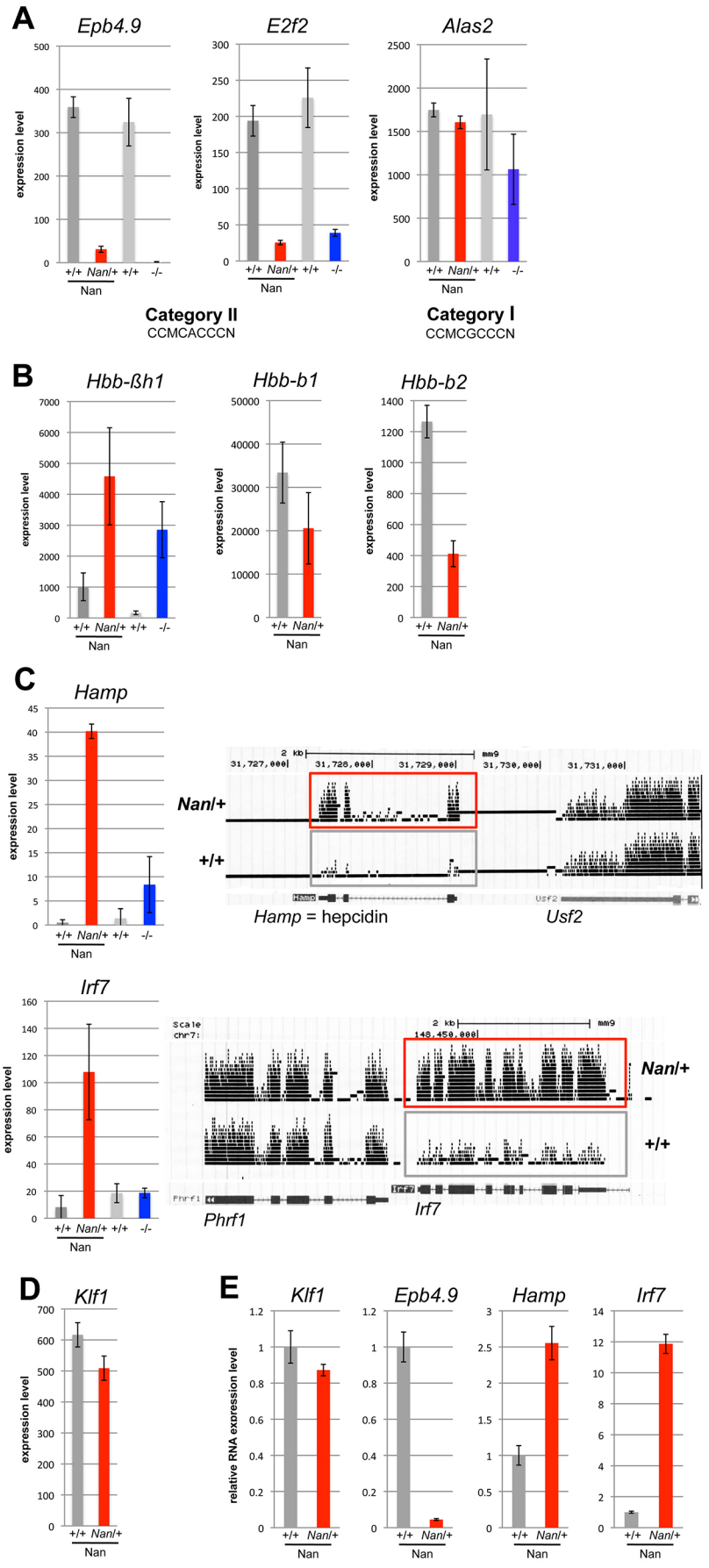

Fig. 1. Expression of EKLF (KLF1) targets in fetal liver samples. (A-C) RNA-seq expression data of E13.5 fetal liver cells from wild-type (+/+) (dark gray) and Nan/+ (red) littermates ('Nan'), or from wild-type (+/+) (light gray) and EKLF-null (–/–) (blue) littermates were analyzed for expression of target genes. Each is an average of biological triplicate samples. (A) Genes containing category II (*Ebp4.9*, *E2f2*) or category I (*Alas2*) target sites (as defined by Siatecka et al., 2010b; examples are shown in Table S1) were analyzed for expression. (B) Expression of embryonic βh1 globin (*Hbb-bh1*) and adult β globins (*Hbb-b1* and *Hbb-b2*) show target gene dysregulation. (C) Expression level of novel targets hepcidin (*Hamp*) and *Irf7*, which are most dramatically increased in the Nan/+ samples, are shown next to a representative browser expression profile from one sample each [wild type (+/+) (dark gray) and Nan/+ (red)]. In each case, expression of the adjacent genes is unaffected by Nan-EKLF expression status. (D) Expression of total EKLF (*Klf1*) in the Nan littermates is shown for comparison; this analysis does not differentiate between wild-type and Nan-EKLF RNA. (E) Total RNA from adherent cell-depleted and expanded erythroid cells isolated from wild-type (+/+) or Nan/+ littermates ('Nan') were monitored by q-RT-PCR for expression of EKLF (*Klf1*), *Ebp4.9*, hepcidin (*Hamp*) or *Irf7*. Results are the average of three biological replicates each performed in triplicate.

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(Gillinder et al., 2016) support the initial genotypic analysis of the *Nan* mouse (Siatecka et al., 2010b), showing a dysregulated erythroid transcript pattern in spite of the heterozygosity of the *KLF1* mutation. For example (Fig. 1A), expression of dematin (*Epb4.9*, also known as *Dmtn*) and *E2f2* are virtually absent in KLF1 knockout cells, and this is mimicked in *Nan*/+ cells; these contain category II elements that are affected by the presence of Nan-KLF1. As a control, *Alas2*, which contains a category I-binding element, is not altered in *Nan*/+ cells compared with wild type. Finally, hemoglobin switching is altered, as embryonic *Hbb-bh1* globin expression is retained at high levels in both knockout and *Nan*/+ E13.5 fetal liver, whereas adult *Hbb-b1* and *Hbb-b2* globin expression are 50-70% less (Fig. 1B), as previously observed (Siatecka et al., 2010b). Although many of these changes arise from the more-limited target DNA sequence recognized by Nan-KLF1 protein (i.e. 5′CCMC*G*CCCN) compared with wild type (i.e. 5′CCMC*R*CCCN) (Siatecka et al., 2010b), our recent studies show that there is a second consequence of the E339D mutation in Nan-KLF1, namely that a different recognition sequence (5′CCMNGCCCN) is newly recognized (Gillinder et al., 2016). We considered whether this might play an additional role in the anemia and the phenotypic properties observed throughout development in *Nan*/+ embryos and adults, specifically whether the new targets would give rise to aberrant expression of unusual/unexpected proteins in *Nan* erythroid cells that yield deleterious effects.

We began by focusing on the relatively small number (~80) of genes that are newly and most highly expressed in *Nan*/+ cells compared with wild type (Gillinder et al., 2016), and further parsed this to genes that are secreted and thus could systemically affect normal erythropoiesis via the circulation. Two genes in particular caught our attention. Hepcidin is a regulator of cellular iron use by virtue of its interaction with ferroportin (Ganz, 2011), and is primarily expressed in the adult liver. Interferon regulatory factor 7 (IRF7) is a transcription factor primarily expressed by macrophages that mediates inflammation by virtue of its activation of interferon β (IFNβ) (Ford and Thanos, 2010; Honda et al., 2005). Misexpression of either of these would be detrimental to effective erythropoiesis (Ganz and Nemeth, 2012; Kim and Nemeth, 2015; Nagata, 2007; Yoshida et al., 2005a). Our data show little expression in the wild-type or knockout cells or in the wild-type cells from the *Nan* strain; however, their levels are dramatically increased in the *Nan*/+ cells (Fig. 1C) [also observed in an independent experiment (Fig. S1A), thus providing us with data from a total of 12 biological replicates]. These are not the result of global or regional changes; in each case, expression of the adjacent genes remain unaffected irrespective of the genotype (Fig. 1C). Total KLF1 levels are only slightly lower in *Nan*/+ compared with wild type (Fig. 1D and Fig. S1B).

Although EKLF expression is restricted to erythroid progenitors and their progeny, we verified that the ectopic expression results from the erythroid compartment by depleting adherent cells and expanding E13.5 fetal liver cells in erythropoietin. qRT-PCR analysis of RNA shows that these erythroid-enriched cells express KLF1 at similar levels, that *Nan*/+ cells exhibit the dramatic decrease in dematin (*Epb4.9*) expression previously seen with total fetal liver material, and that there is dysregulation of hepcicin (*Hamp*) and *Irf7* (Fig. 1E). We conclude that *Hamp* and *Irf7* are mis-expressed in the erythroid cell by virtue of the presence of the *Nan* mutant variant of KLF1.

Erythroferrone is a new EKLF target that is part of the disrupted intrinsic expression pattern generated in the Nan-EKLF red cell

Hepcidin levels are suppressed by erythroferrone (*Fam132b*) (Kautz et al., 2014), a gene whose expression in the erythroid cell is stimulated by erythropoietin when iron mobilization is desired. We found that erythroferrone is regulated by KLF1 levels in the erythroid cell, as its expression is virtually absent in the knockout (Fig. 2A). Of relevance here, its levels are also dramatically deficient in *Nan*/+ cells (Fig. 2A,B; Fig. S1C); as with other similarly affected genes (Siatecka et al., 2010b), this is not due to haploinsufficient levels of wild-type EKLF (not shown). The two likely KLF1-binding sites in this gene are category II (Siatecka et al., 2010b), and thus are predicted to be negatively affected by the presence of the *Nan* variant.

The implication that erythroferrone is a KLF1 target was directly tested using the KLF1-null rescue system with which stably expressed cytoplasmic ER/KLF1 or ER/ Nan-KLF1 can be induced to translocate to the nucleus by treatment of the cells with tamoxifen (Coghill et al., 2001; Hodge et al., 2006). Although the treatment protocol is limited to 1 h, these rescued cells show robust chromatin association by ER/KLF1 to one of these sites (a category II site in intron 1) but not by ER/Nan-KLF1 (Fig. 2C). In addition, RNA begins to accumulate within this short time period only in the wild-type rescue (Fig. 2D).

We found additional evidence for disarray in iron utilization components within the *Nan*/+ erythroid cell (Gillinder et al., 2016). For example, when comparing *Nan*/+ cells with wild type we see a slight decrease in transferrin receptor (*Tfrc*) levels, and a slight increase in ferroportin (*Slc40a1*) in spite of the increase in hepcidin (Fig. 3A). This yields a lower level of iron in the red cell (Ganz and Nemeth, 2012; Tolosano, 2015). Coupled to this, heme oxygenase (*Hmox1*) levels increase while heme transporter HRG1 (*Slc48a1*) levels decrease in the *Nan*/+ cells compared with wild type (Fig. 3B), yielding less heme in the erythroid cell (Korolnek and Hamza, 2015; Narla and Mohandas, 2015).

In light of this, we examined expression patterns of other genes involved in iron utilization or in the inflammation pathway (reviewed by Ganz, 2011; Ginzburg and Rivella, 2011; Guida et al., 2015; Kim and Nemeth, 2015; Li et al., 2014; Nagata, 2007; Rivella, 2015; Yoshida et al., 2005b) to address indirect or Nan-KLF1-independent causes for the inefficient erythropoiesis (Gillinder et al., 2016). Expression is either unchanged [hemochromatosis (*Hfe*), IRP1 (*Aco1*), aconitase (*Aco2*), Flvcr1 (*Mfsd7b*); Fig. S2A] or remains insignificantly expressed [*Bmp6*, hemojuvelin (*Hfe2*), *Gdf11*, *Pigf*; not shown]. Genes involved in regulation of hepcidin, such as *Tmprss6*, are not altered (Fig. S2B) or not expressed (*Gdf15*, *Atoh8* and *Sox2*; not shown), although the level of *Stat3*, an upstream regulator of hepcidin, is increased in *Nan*/+ cells (Fig. S2B) in spite of undetectable *Il6* (not shown). Levels of erythroid regulators such as *Twsg1*, HIF2α (*Epas1*), or IRP2 (*Ireb2*) are not altered (Fig. S2C). With respect to genes involved in a predicted inflammatory response due to *Irf7* expression, we see no change in expression of *Irf3*, *Irf2* or *Tlr2* (Fig. S2D), and no expression of *Tlr6* (not shown). We conclude that the misexpression of hepcidin and IRF7, and the depletion of erythroferrone expression in the red blood cell are not the result of a generalized response to external stimuli but rather are caused by the intrinsic presence and inappropriate binding of Nan-KLF1.

Effects of Nan-EKLF expression early in development

Embryos that express only the Nan-EKLF variant (i.e. *Nan/Nan* or *Nan*/−) are lethal by E10-11 and display a striking dysmorphology that extends beyond the fetal erythroid cell (Heruth et al., 2010). To model these early stages, we established embryonic stem cells (ESCs) from *Nan/Nan* and +/+ littermates and differentiated them to embryoid bodies (EBs). EBs synchronously recapitulate

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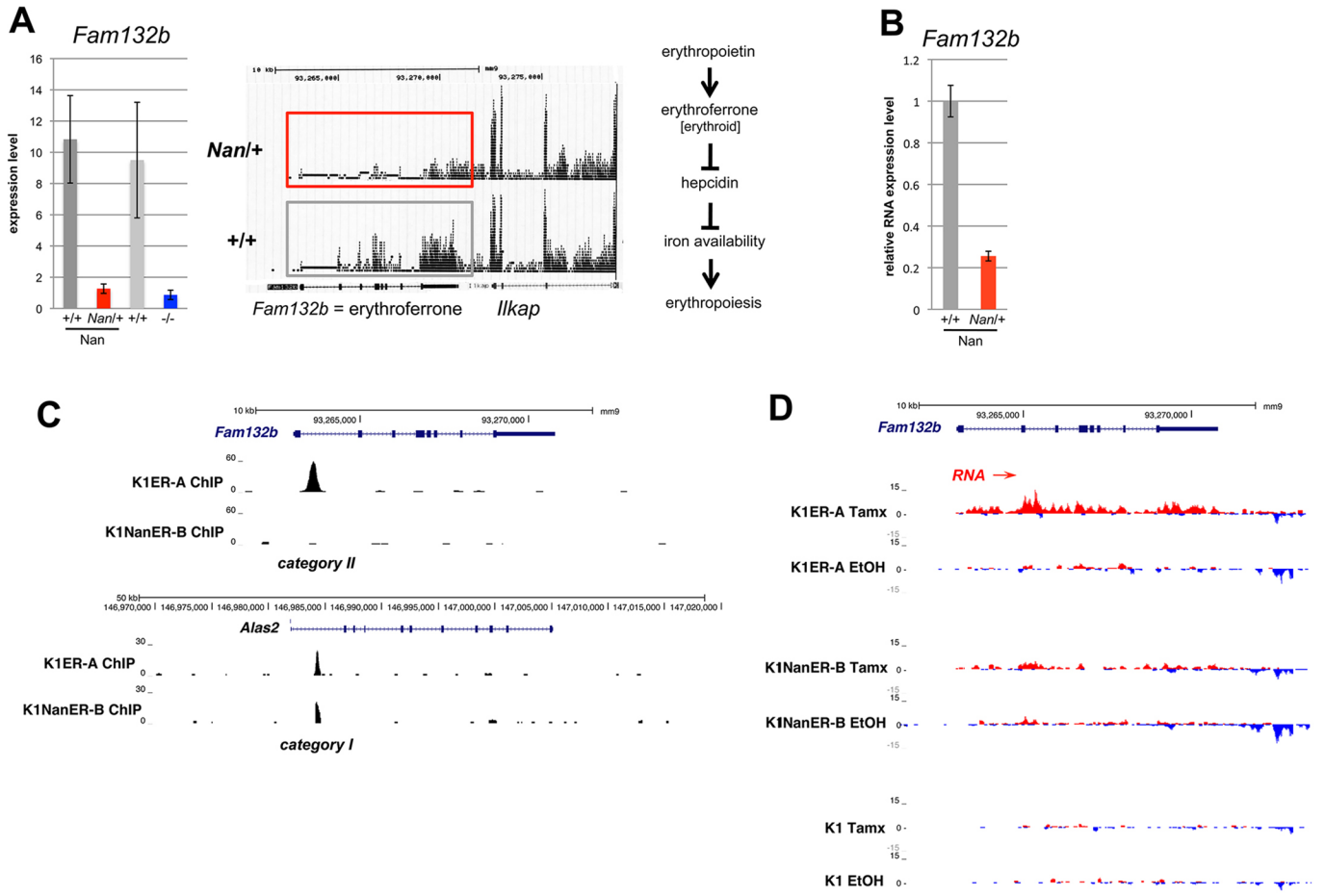

Fig. 2. Altered expression of erythroferrone (Fam132b) in Nan mice fetal liver samples or rescued EKLF-null erythroid cells. (A) RNA-seq expression data from biological triplicates of wild-type (+/+) (dark gray) and Nan/+ (red) littermates ('Nan'), or from wild-type (+/+) (light gray) and EKLF-null (–/–) (blue) littermates were analyzed for expression of *Fam132b* (erythroferrone). A representative browser expression profile from one sample each is shown [wild type (+/+) (dark gray) and Nan/+ (red)]; the adjacent gene is unaffected by Nan-EKLF expression status. A flow chart showing the relationship of erythroferrone to upstream and downstream effectors is shown on the right. (B) Total RNA from adherent cell-depleted erythroid cells isolated from wild-type (+/+) or Nan/+ littermates ('Nan') were monitored by q-RT-PCR for expression of *Fam132b* (erythroferrone). Results are the average of three biological replicates each performed in triplicate. (C) EKLF-null erythroid cells were used to monitor EKLF association with *Fam132b* chromatin (ChIP) after a 1 h tamoxifen-induced rescue with wild-type EKLF (K1ER-A) or Nan-EKLF (K1NanER-B) (Coghill et al., 2001); Alas2 was monitored as a positive control. Analysis is limited to 1 h post-treatment to avoid loss of cell number following KLF1 induction of CDK inhibitors and the ensuing cell cycle arrest (Pilon et al., 2008; Siatecka et al., 2010a; Tallack et al., 2009, 2007; Gnanapragasam et al., 2016). Results are representative of two experiments. (D) RNA expression was monitored across the *Fam132b* locus during a 1 h tamoxifen-induced rescue with the wild-type EKLF (K1ER-A), Nan-EKLF (K1NanER-B) or parental K1 line; treatment with ethanol (EtOH) is the negative control in each case. 4-Thiouridine tagging was used to identify nascent RNA (Gillinder et al., 2016). Results are representative of two experiments.

erythropoiesis (Choi et al., 2005; Kennedy and Keller, 2003); in our hands, the onset of *Klf1* and primitive erythropoiesis begins at day 4 and peaks by day 6, when definitive erythropoiesis is well underway to eventual dominance (Adelman et al., 2002; Bruce et al., 2007; Lohmann and Bieker, 2008). *Nan/Nan* EBs demonstrate morphological defects by day 6 of differentiation, as they are smaller in comparison with +/+ EBs (Fig. 4A). Although the onset of the CD44 early definitive erythroid cell surface marker (Chen et al., 2009; Liu et al., 2013) is normal in both sets by day 6 (Fig. 4B, left), *Nan/Nan* EBs are defective in generating late (primitive or definitive) erythroid cells, as judged by the absence of Ter119 expression in these cells at day 6 or day 7 (Fig. 4B, left), which attains normal levels (Lohmann and Bieker, 2008; Soni et al., 2014) in the wild-type EBs.

Although this may result from an intrinsic erythroid deficit, we also tested whether the *Nan* cells could exert an extrinsic effect on wild-type EB differentiation by mixing equivalent numbers of +/+ and *Nan/Nan* cells at d0 and then monitoring erythroid status during differentiation via cell surface marker analysis as before. Again, although CD44 levels are not affected, no evidence of Ter119 expression is evident even though the number of +/+ cells included in the culture was no different from before (Fig. 4B, right). We conclude that the dysmorphology of the non-erythroid tissues seen in the *Nan* embryo may well result from the ectopic expression pattern and resultant secretory proteins seen within the Nan-expressing cell.

Effects of Nan-EKLF expression in the adult

We next addressed whether the altered expression patterns of *Hamp* and *Irf7* seen in the fetal liver are also manifested in the *Nan*/+ adult, and could explain part of the anemia exhibited by the *Nan* mouse. The adult *Nan*/+ mouse exhibits low mean corpuscular volume and mean corpuscular hemoglobin, reticulocytosis and splenomegaly (Siatecka et al., 2010b), suggesting defective erythropoiesis possibly due to iron deficiency (Andrews, 2009). Consistent with this, circulating erythropoietin levels are higher in *Nan*/+ adults than

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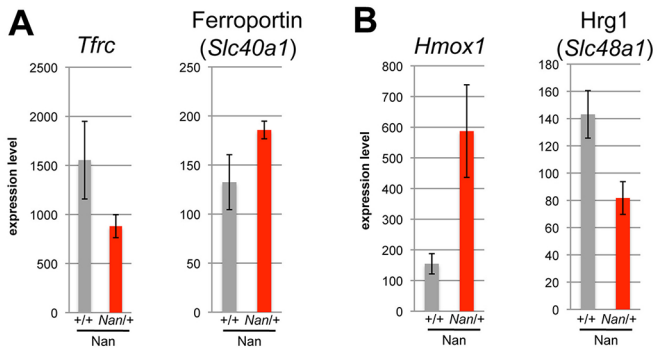

Fig. 3. Expression of iron- and heme-utilization targets in fetal liver samples. (A,B) RNA-seq expression data from biological triplicates of wild-type (+/+) and Nan/+ littermates ('Nan') were analyzed for expression of genes coding for iron (A) and heme (B) transport proteins as indicated.

in matched littermates (Fig. 5A). *Hamp* and *Irf7* expression levels are significantly higher in spleen and bone marrow RNA from the *Nan*/+ mouse than in the wild type (Fig. 5B) (although hepcidin expression is lower in bone marrow than in spleen). To probe the possibility of systemic changes, we monitored serum levels of hepcidin and found that its levels are significantly increased in the circulation in *Nan*/+ mice compared with wild type (Fig. 5A). Analysis of serum IFNβ levels show an even more dramatic change (Fig. 5A). Serum analyses in the adult reflect alterations in the iron use components we had observed in the *Nan*/+ fetal erythroid cell (Fig. 3), as there is a slight but significant increase in serum bilirubin and a decrease in serum iron (Fig. 5C). Together, these add up to an iron- and heme-deficient red cell environment that contributes to the overall anemia of the mouse.

Increased hepcidin production can be achieved by IL6-mediated stimulation of the liver, particularly as a result of an inflammatory response (Nemeth et al., 2004a). As a result, we checked for IL6 in the serum, but did not find any detectable levels in either the wild-type or *Nan*/+ adult (Fig. 5D). This removes inflammation as a causal effector and negates a possible liver contribution to the circulatory hepcidin levels we see in the Nan/+ adult.

Collectively, the developmental data suggest that the anemia in adult *Nan*/+ mice is exacerbated not only by the intrinsic defects in erythroid expression based on the category I versus category II distinction, but also by the neomorphic genetic output via the new recognition site. This leads to expression of secreted molecules that work directly against establishing a steady-state replenishment of erythroid cells to combat the anemia.

In vitro and *in vivo* testing of altered DNA binding by Nan-EKLF protein in the *Nan*/+ adult

The DNA-recognition properties of the Nan-KLF1 protein variant (Gillinder et al., 2016) suggest a possible mechanism for the effect; i.e. by binding to novel target site(s) within the *Hamp* and *Irf7* genes not normally recognized by the wild-type KLF1 protein. Inspection of the gene loci of *Hamp* and *Irf7* reveal a number of potential Nan-KLF1 target sites (5′CCMNGCCCN) that meet this criterion (Fig. 6A). A subset were tested *in vitro* by quantitative gel shift assays for binding to wild-type or Nan-KLF1 proteins. As controls, we obtained dissociation constants for known category I (p21 site 3) and category II (dematin and E2F2 site 2) DNA sites binding to wild-type or Nan-KLF1 protein (Fig. 6B). Using this approach, we monitored binding to the predicted new sites in the hepcidin (*Hamp*) and *Irf7* genes and found that binding affinities are substantially higher for Nan-KLF1 compared with wild type (Fig. 6C), supporting the predictions made from the global variant analysis.

As a final test, we checked for *in vivo* interaction of KLF1 or Nan-KLF1 to these new sites. As we do not have an antibody that can distinguish wild-type KLF1 from Nan-KLF1, we simply monitored whether the adult *Nan*/+ spleen shows a novel KLF1 ChIP compared with wild type at any of the potential sites of *Hamp* and *Irf7*. KLF1 is well expressed within the red pulp of the normal adult spleen (Miller and Bieker, 1993; Southwood et al., 1996). The analyses (Fig. 6D) show that the putative sites are indeed occupied

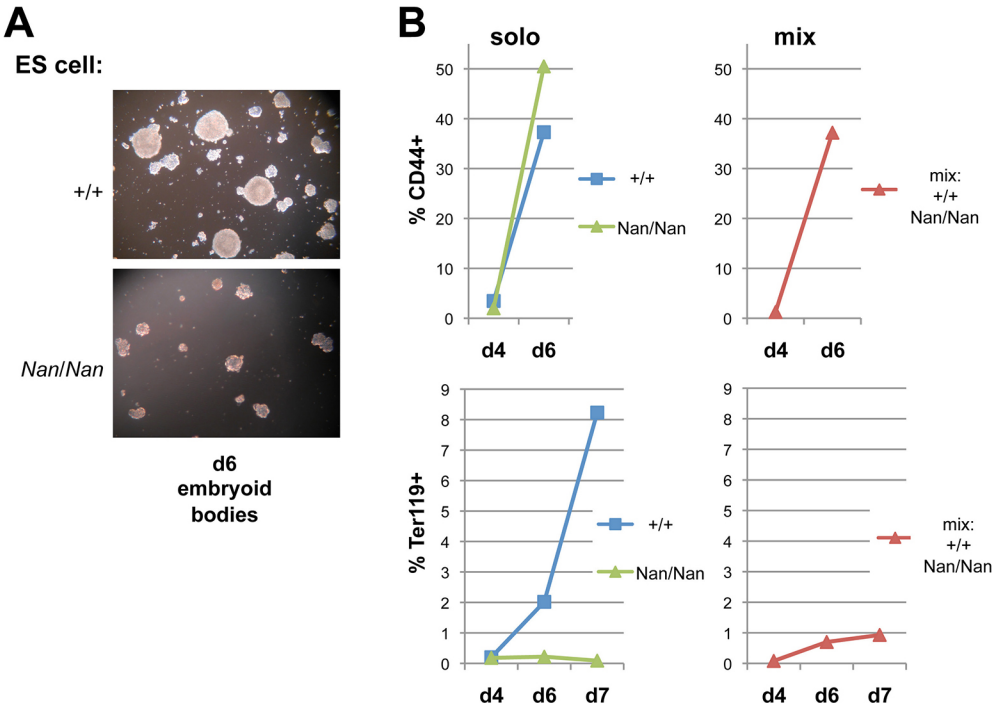

Fig. 4. Effects of Nan on embryoid body differentiation. Embryonic stem (ES) cells derived from wild-type (+/+) or Nan/Nan littermates were differentiated to embryoid bodies (EBs) and analyzed at day (d) 4, d6 or d7.
(A) Photomicrographs of d6 EBs concurrently generated from wild-type or Nan/Nan ES cells (same magnification).
(B) Cell-surface marker expression of CD44 (early erythroid) and Ter119 (late erythroid) was performed on d4, d6 or d7 EBs concurrently generated from wild-type or Nan/Nan ES cells. (Left) Results from individual cultures. (Right) Results after having mixed equivalent numbers of ES cells (wild type with Nan/Nan as indicated) at d0.

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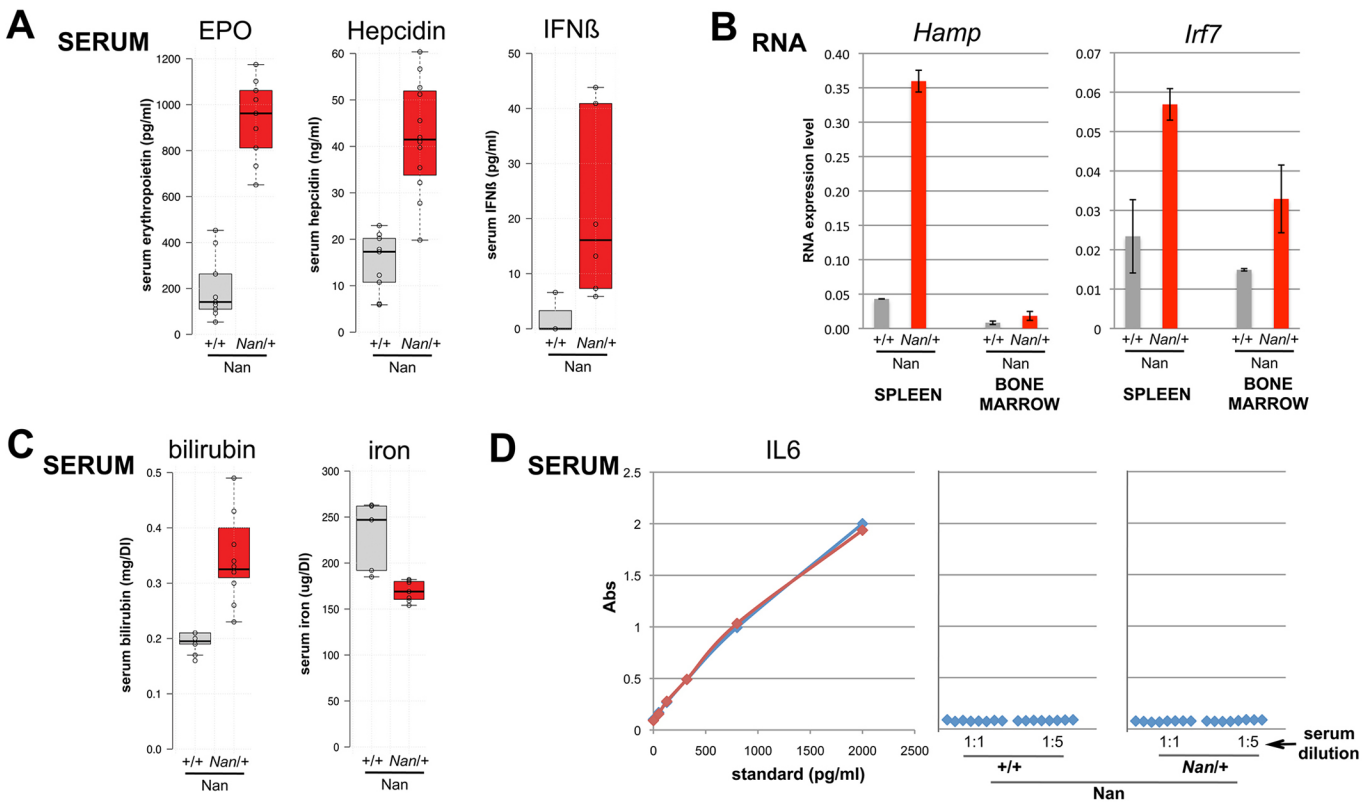

Fig. 5. Analysis of expression changes in adult Nan/+ mice. (A) Sera from wild-type (+/+) or Nan/+ adult littermates ('Nan') were monitored for expression of erythropoietin (EPO) (n=6, each analyzed at three dilutions, P<0.0001), hepcidin (n=11, each analyzed in duplicate, P<0.0001) or IFNβ (n=4, each analyzed in duplicate, P=0.049). Data are average±s.e.m. (B) Spleen and bone marrow RNA from wild-type (+/+) or Nan/+ adult littermates ('Nan') was monitored by qRT-PCR for expression of hepcidin (*Hamp*) or *Irf7*. Results are from two experiments each performed in triplicate and analyzed at the same time. Although Irf7 is regulated differently in the adult bone marrow compared with the fetal liver (Kingsley et al., 2013; Liang et al., 2015), its levels are nonetheless significantly increased in the adult Nan/+ bone marrow. (C) Sera from wild-type (+/+) or Nan/+ adult littermates ('Nan') were monitored for expression of bilirubin (n=10-12, P<0.0001) or iron (n=5-7, P=0.0025). Data are average±s.e.m. (D) Sera from wild-type (+/+) or Nan/+ adult littermates ('Nan'), were monitored for expression of IL6 (n=8, each analyzed at two dilutions as indicated) and compared with IL6 standards (duplicates are shown).

only in the chromatin derived from the *Nan*/+ mouse. Collectively, the *in vitro* and *in vivo* data demonstrate that the variant DNA recognition property of Nan-KLF1 is responsible for altered binding and subsequent ectopic protein expression in the *Nan*/+ erythroid cell.

DISCUSSION

Our studies demonstrate that an apparently minor conservative change in a single amino acid (E to D) of a crucial erythroid transcription factor can result in significant phenotypic changes. In Nan-KLF1, the residue in question is absolutely conserved across the whole KLF family in all species examined. This leads to a limitation on its ability to recognize the consensus wild-type DNA element (Siatecka et al., 2010b). However, a remarkable finding from the global analyses was that a novel, atypical site is newly recognized (Gillinder et al., 2016), leading to a significant number of expressed genes that are not normally present in the mammalian red cell. We have shown how this can lead to defects of gene expression within and outside the cell.

Within the wild-type erythroid compartment, neither hepcidin nor IRF7 is expressed. However, unanticipated phenotypic changes arise from two physiologically nonproductive cycles generated by the *Nan*/+ red cell (Fig. 7). In one case, misexpression of hepcidin and deficient expression of erythroferrone lead to a decrease in iron availability and lower red cell numbers. In the second case, misexpression of IRF7 induces IFNβ expression, which has a repressive effect on erythroid development. The organism responds to the defects by inducing erythropoietin to replenish erythropoiesis, leading to splenomegaly. However, this is futile, as the very cells being expanded are those that are secreting hepcidin and IFNβ, leading to an ill-designed feedback inhibition. This cycle is superimposed upon the intrinsic erythroid deficits, leading to a worsening of the red cell anemia. The increase in hepcidin is both direct (via binding of Nan-EKLF to its atypical site) and indirect (via low erythroferrone expression in the *Nan*/+ environment), as ablation of EKLF (and thus low erythroferrone) is not sufficient to substantially increase hepcidin levels. Remarkably, these deficits occur even in the presence of a normal wild-type copy of KLF1.

More globally, iron is indispensable for all tissues, being a crucial component of essential enzymes and multiprotein complexes that contain heme and iron-sulfur clusters (Zhang et al., 2014). As a result, heme-containing proteins play crucial roles in all cells (Sawicki et al., 2015); however, heme can also play lineage-specific roles in differentiation (Philip et al., 2015). Proper heme levels are also crucial for normal fetal development, as defects can lead to limb deformities in addition to abnormal erythropoiesis (Keel et al., 2008). Not surprisingly then, increased circulatory hepcidin can have systemic effects; for example, decreasing heme- and nonheme-iron absorption to the duodenum (Cao et al., 2014). In the present case, the levels of hepcidin in the *Nan*/+ mouse are high enough to exert a physiological effect (Nemeth et al., 2004b).

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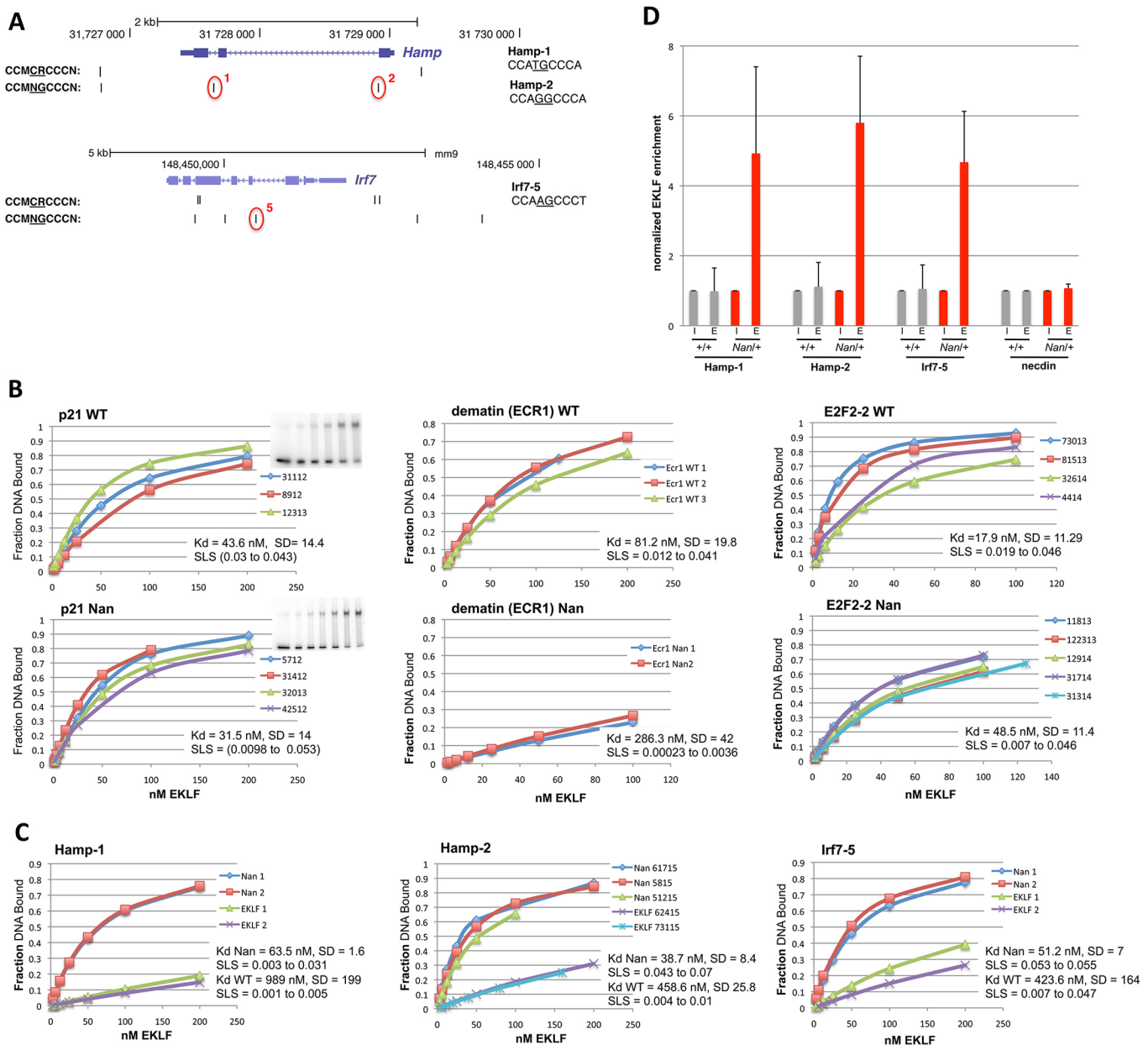

Fig. 6. Binding of Nan-EKLF to novel genomic regions. (A) Localization of wild-type EKLF binding consensus (5′CCMCRCCCN) or Nan-EKLF-binding consensus (5′CCMNGCCCN) across the hepcidin (*Hamp*) and *Irf7* genomic loci are shown. Circled regions (red) show the location and sequences used for further analysis: Hamp-1, Hamp-2 and Irf7-5 (right). (B) *In vitro* quantitative gel-shift analyses were performed to assess the binding affinities of wild-type and Nan-EKLF proteins to representative category I (at the p21 gene) or category II (ECR1 at the dematin gene and E2f2 site 2) elements (Siatecka et al., 2010b). Multiple DNA concentrations were tested and selected, as described in the Materials and Methods: 12.5 to 50 nM for p21 (P=0.32, not significant), 25 nM for ECR1 (P=0.0045), and 0.5 to 12.5 nM for E2f2 site 2 (P=0.0052), all by unpaired t-test. Excel Solver software was used to determine Kd values along with the standard deviation (s.d.); SLS is the sum of the squares of the errors and indicates the range of error over the curves. Also shown (as inserts on the p21 data) are typical gel data generated and analyzed for all the experiments. GST alone showed no binding to any of these probes (not shown). These provide us with a range of well-discriminated affinities, with nanomolar binding constants that are equivalent at p21 but are 2- (E2F2) to 3.5- (dematin) fold higher with Nan-KLF1 compared with wild type. (C) *In vitro* quantitative gel shift analyses were performed to assess the binding affinities of wild-type and Nan-EKLF proteins to the novel Hamp-1, Hamp-2 and Irf7-5 elements. All were analyzed at 5 nM DNA, except Hamp1 ‘EKLF 2’ and IRF7-5 ‘EKLF 2’, which were at 25 nM. (D) *In vivo* chromatin immunoprecipitation was performed at selected regions (as shown in A) with IgG (I) or 7B2 anti-EKLF (E) antibodies. Necdin was included as a negative control. Spleens from wild-type (+/+) or Nan/+ adult littermates (‘Nan’) were used for the analyses. Results are from two separate experiments, each performed in triplicate.

As a result, we suggest that Nan-EKLF expression contributes to the dysmorphology of *Nan* embryos seen during early stages of development and during ES cell differentiation, to the fetal liver erythroid misregulation, and to the chronic anemia of the adult (Fig. 7). In this context, it is of interest that a DAVID analysis of the most significantly upregulated gene categories in *Nan*/+ RNA shows that those in the ‘secreted’ and ‘signal’ groupings are the most numerous (Fig. S3).

Intriguingly, low iron availability via hepcidin expression coupled with expression of IFNβ resembles the anemia of chronic

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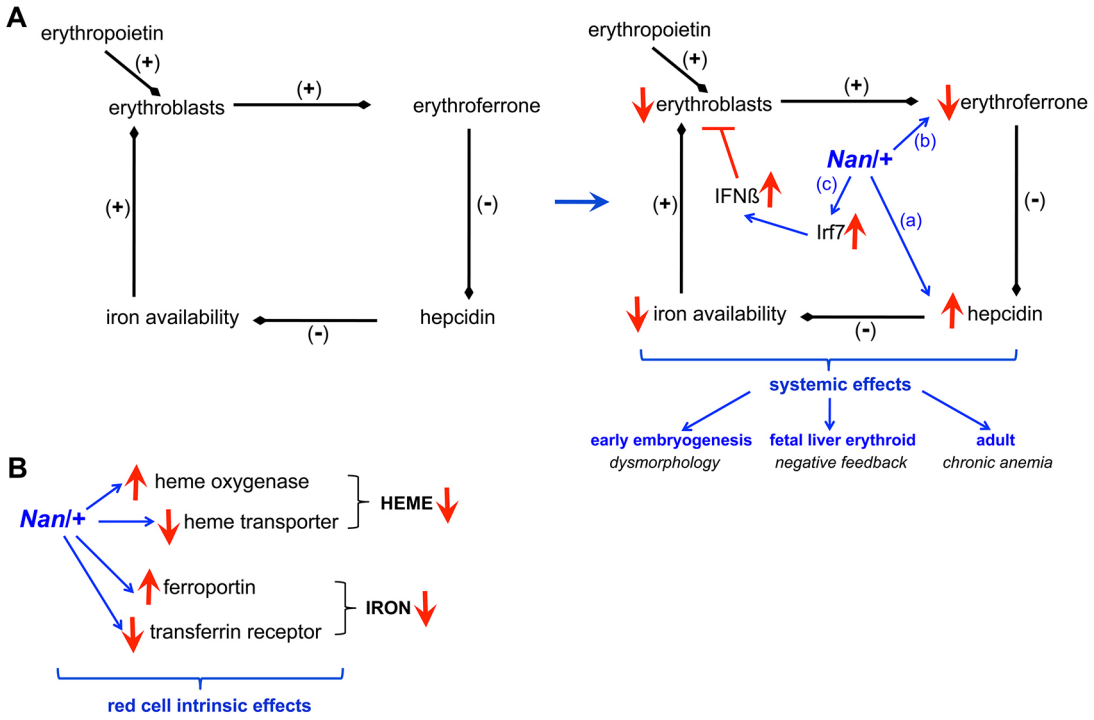

Fig. 7. Summary and model of systemic and intrinsic effects of the Nan-EKLF mutation. (A) (Left) During normal wild-type steady-state, adult erythropoiesis is kept in balance by the levels of hepcidin, which negatively regulates iron availability when erythroblast levels are replete, but is negatively regulated by erythroid erythroferrone when iron is needed for erythroid expansion, such as after stimulation by erythropoietin. (Right) In the case of Nan/+ adults, the direct increase of hepcidin by Nan-EKLF (a) and the low level of expression of erythroferrone (b) decrease iron availability, negatively affecting erythroblast expansion. Coupled to this, misexpression of Irf7 (c) leads to secretion of IFNβ, superimposing another negative cycle on erythroid expansion. The net result is a stimulation of erythroblast proliferation and the accompanying splenomegaly that is non-productive and does not address the anemia, which remains chronic. These effects in the adult form part of an array of developmental deficits that occur in spite of the erythroid-restricted expression of Nan-EKLF, including dymorphology in the embryo and embryoid body, and defective erythropoiesis in the fetal red cell. (B) Coupled to the systemic effects summarized in A, intrinsic expression changes (based on fetal liver expression, although also likely in the adult) in the Nan/+ red cell contribute to defective erythropoiesis by altering genes that play roles in heme and iron uptake.

inflammation (Goodnough et al., 2010), where iron restriction sensitizes cells to the inhibitory effects of inflammatory cytokines (Richardson et al., 2013). However, we do not detect any IL6, the key inducer of hepcidin and mediator of inflammation (Hom et al., 2015; Wang and Babitt, 2016).

To our knowledge, finding genetic effects of a tissue-restricted factor that occur outside of its normal expression realm is novel, and thus may be an unrecognized contribution to human disease. In the present case, the most direct connection is with CDA type IV (Iolascon et al., 2012), which is caused by a mutation in human KLF1 at the same amino acid (Arnaud et al., 2010; Jaffray et al., 2013; Singleton et al., 2011). The change is non-conservative (E to K), so although not all characteristics of the *Nan* mouse may apply, individuals with CDA exhibit altered globin switching and membrane deficits that are highly reminiscent of the present model (discussed by Siatecka et al., 2010b). Of greater interest are the non-erythroid aspects of a subset of these individuals, such as short stature, high serum iron and bilirubin levels, and altered gonads. These suggest that there may very well be secreted factors that are aberrantly/ectopically expressed in these human cells as a direct result of the single amino acid change seen in the human KLF1 CDA mutant.

It may appear surprising that Nan-KLF1 is able to recognize its atypical site within genes that are presumably not easily accessible outside their normal cell of expression. However, recent studies with KLF4, which is highly related to KLF1 (Bieker, 2001), suggest that zinc fingers 2 and 3 play a necessary and dominant role in targeting its recognition site within nucleosomes (Soufi et al., 2015). In the present case, the amino acid change is located within zinc finger 2 and thus could enable Nan-KLF1 to play a role as a pioneer factor at ectopic sites in spite of their lower accessibility in the red cell.

Our present studies, as with the earlier analyses of the Nan-KLF1 protein gene regulation (Siatecka et al., 2010b), re-emphasize that the effects of KLF1 mutation are not strictly due to a dominant-negative outcome. This is clearly different from the cell-intrinsic defects seen with the haploinsufficient or most of the other mutant KLF1 proteins that have been identified in humans (Borg et al., 2011; Helias et al., 2013; Perkins et al., 2016; Singleton et al., 2012; Tallack and Perkins, 2013; Waye and Eng, 2015). What is seen in the present case is a new variant with novel properties that dramatically changes *Nan*/+ red cell identity.

MATERIALS AND METHODS

General protocols

Establishment of the *Nan* mouse strain has been previously described (Heruth et al., 2010; Lyon, 1983; Siatecka et al., 2010b). All protocols have been reviewed and approved by the IACUC at Mount Sinai. RNA was isolated from E13.5 fetal livers or adult spleens using Trizol. Total RNA was purified with a Qiagen RNeasy kit. qRT-PCR was performed using published procedures (Siatecka et al., 2010b). Global RNA-seq was performed by the Mount Sinai Facility or by the JAX facility and analyzed as described (Gillinder et al., 2016). The DAVID analysis tool v6.7 (https://david-d.ncifcrf.gov/) was used as described (Huang et al., 2009).

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Cell isolation and manipulation

K1-ER or K1-Nan-ER cells (originally described by Coghill et al., 2001, recently authenticated and reanalyzed by Gillinder et al., 2016) were grown and treated with doxycycline as described (Coghill et al., 2001; Hodge et al., 2006). Analysis is limited to 1 h post-treatment to avoid loss of cell number following KLF1 induction of CDK inhibitors and the ensuing cell cycle arrest (Gnanapragasam et al., 2016; Pilon et al., 2008; Siatecka et al., 2010a; Tallack et al., 2009, 2007). As a result, only immediate-early effects can be detected. Nascent RNA expression was monitored after a concurrent 1 h treatment of cells with 4-thiouridine, as described previously (Gillinder et al., 2016).

Mouse ESCs derived from wild-type and *Nan/Nan* blastocysts (littermates) were established as described previously with minor modifications (Meissner et al., 2009). These were maintained and differentiated to embryoid bodies as per established protocols (Choi et al., 2005; Kennedy and Keller, 2003) described previously (Manwani et al., 2007). Ter119-PE and CD44-FITC antibodies were from eBiosciences.

Erythroblasts were derived from wild-type and *Nan*/+ E12.5 fetal livers and depleted of adherent cells as previously described (England et al., 2011; Gnanapragasam et al., 2016). Briefly, cells were harvested, and plated on gelatin dishes for 2 days. This was followed by a 1-day culture in erythroid growth media prior to harvest for RNA analysis.

ChIP analysis of EKLF binding was performed using a modification of previous procedures (Lohmann and Bieker, 2008; Siatecka et al., 2015). Briefly, cells from fetal livers or spleens (7-10×10^6 per immunoprecipitation) were crosslinked with 1.0% formaldehyde. Chromatin was sonicated, immunoprecipitated with antibodies against mouse IgG (557273; BD Pharmingen) or EKLF (7B2), and purified on magnetic protein G Dynabeads (10004D; Life Technologies). Eluted and precipitated genomic DNA was amplified and quantified by PCR.

Serum testing

Serum from adult mice was analyzed using ELISA kits for erythropoietin (R&D Systems), IL6 (Thermo Fisher), hepcidin (Intrinsic Lifesciences) or IFNβ (PBL Assay Science), following the manufacturers' instructions. Bilirubin and iron were measured on a Beckman Coulter AU680 Chemistry Analyzer; only samples with low hemolysis [i.e. hemoglobin value lower than 100 (Beckman-Coulter)] were used for these analyses.

Quantitative protein-DNA binding analysis

Quantitative gel shifts were performed using GST-tagged EKLF zinc fingers (Bieker and Southwood, 1995), purified as previously described (Soni et al., 2014) and incubated with ^{32}P-labeled DNA oligonucleotide probes in 25 mM HEPES (pH 7.5), 16 mM KCl, 50 mM NaCl, 2 µM $ZnCl_2$, 0.6 mM β-mercaptoethanol and 8% glycerol for 30 min on ice at various concentrations (see figures and legends). A 10-fold molar excess of polydeoxyinosinic-deoxycytidylic acid (dIdC, Sigma-Aldrich) relative to the DNA probe was used to reduce non-specific binding. Protein concentrations were determined by saturation binding to the p21 gene EKLF site, to which both EKLF ZF and Nan ZF bind similarly. A fixed amount of DNA was incubated with increasing amounts of protein and DNA, and protein concentrations were adjusted to maximize the curve fits. Samples were electrophoresed on 8% acrylamide gels in 0.5× TBE at 150 volts for 1.5 h at room temperature. Quantitation was performed on a Molecular Dynamics Phosphorimager. Kd values were determined by curve fitting with Excel Solver using the following equation and minimizing the squared error by varying K_{dis}:

$$\text{Fraction of DNA bound} = \frac{D_{tot} + P_{tot} + K_{dis} - \sqrt{[(D_{tot} + P_{tot} + K_{dis})^2 - 4 \cdot D_{tot} \cdot P_{tot}]}}{2 \cdot D_{tot}},$$

where D_{tot} is the DNA concentration, P_{tot} is the protein concentration, and K_{dis} is the dissociation constant. Results for E2f2 site 2 and wild-type EKLF in Fig. 6B appear more variable than others because different amounts of poly(dIdC) were used (20- to 400-fold molar excess) to counteract non-specific binding that occurred at high protein to DNA ratios. The four best fits (SLS≤0.045) using 0.5 to 2.5 nM DNA and poly(dIdC) were averaged. For high-affinity binding, DNA amounts were chosen such that 70 to 100% of the probe was bound at highest protein concentration and there was detectable binding in the low protein samples: once established, these same conditions were used for lower affinity interactions.

To generate probes, one strand of DNA oligonucleotide was end-labeled with polynucleotide kinase and γ-P^{32} ATP, and then annealed to the unlabeled complimentary strand. For some experiments, only labeled DNA was used and concentrations were determined with Quant-it PicoGreen from Invitrogen. For other experiments, the labeled DNA was used a tracer with known quantities of unlabeled DNA. Similar results were obtained with these methods.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.P., L.X., C.D.T., M.D., K.G., M.S. and D.N. performed experiments. L.L.P. and A.C.P. provided data and analysis. J.J.B. directed experiments, provided data and analysis, and wrote the manuscript with input from all authors.

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Data availability

The RNA-seq data referred to in this study (Gillinder et al., 2016) are available from Gene Expression Omnibus (GEO) under accession GSE71396.

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.145656.supplemental

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