

## The mouse *Klf1 Nan* variant impairs nuclear condensation and erythroid maturation

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## **Author contributions**

I.C., T.v.D., H.J.G.v.d.W, R.S., M.v.L., F.G. and S.P. designed the experiments. I.C., N.G, R.S., S.H., A.M. and T.v.D. performed the experiments. H.J.G.v.d.W analyzed the 4C-seq and RNA-seq data and wrote the bioinformatics section. Z.O. and W.F.J.v.IJ. performed next generation sequencing experiments. The paper was written by I.C., H.J.G.v.d.W , M.v.L., F.G., T.v.D. and S.P. The authors have no conflicts of interest to declare.

## ABSTRACT

1 Krüppel-like factor 1 (KLF1) is an essential transcription factor for erythroid development, as  
2 demonstrated by *Klf1* knockout mice which die around E14 due to severe anemia. In humans, >65  
3 KLF1 variants, causing different erythroid phenotypes, have been described. The *Klf1 Nan* variant, a  
4 single amino acid substitution (p.E339D) in the DNA binding domain, causes hemolytic anemia and is  
5 dominant over wildtype KLF1. Here we describe the effects of the *Nan* variant during fetal  
6 development. We show that *Nan* embryos have defects in erythroid maturation. RNA-sequencing of  
7 the *Nan* fetal liver cells revealed that Exportin 7 (*Xpo7*) was among the ~780 deregulated genes. This  
8 nuclear exportin is implicated in terminal erythroid differentiation; in particular it is involved in nuclear  
9 condensation. Indeed, KLF1 *Nan* fetal liver cells had larger nuclei and reduced chromatin  
10 condensation. Knockdown of XPO7 in wildtype erythroid cells caused a similar phenotype. We  
11 conclude that reduced expression of XPO7 is partially responsible for the erythroid defects observed  
12 in *Nan* erythroid cells.

13

## 14 INTRODUCTION

15 Erythropoiesis is the process of red blood cell production; defects in this process lead to anemia and  
16 thus insufficient oxygen delivery to tissues and subsequent organ failure. Therefore, the formation of  
17 red blood cells has to be tightly controlled during embryonic development and homeostasis in the  
18 adult.

19 KLF1 (previously known as EKLF) is a well-characterized, erythroid-specific transcription factor and  
20 one of the critical regulators of red blood cell maturation. KLF1 acts mainly as an activator and its  
21 target genes are involved in multiple processes of erythroid differentiation, including cell cycle  
22 regulation (1, 2), hemoglobin metabolism (3), and expression of membrane skeleton proteins (4, 5).  
23 The importance of KLF1 is illustrated by *Klf1* knockout embryos which die around E14 due to the lack  
24 of functional erythrocytes (6, 7). In contrast, heterozygous *Klf1*<sup>+/-</sup> mice survive into adulthood,  
25 showing that haploinsufficiency for KLF1 does not have a severe phenotype (8). KLF1 has a  
26 N-terminal transactivation domain and a C-terminal DNA binding domain, composed of three zinc  
27 fingers. They mediate specific DNA binding to 5'-CACCC-3' motifs (9). Variants in human *KLF1* are  
28 found across the entire gene. The majority are missense variants in the three zinc fingers, which  
29 presumably alter the DNA binding/sequence recognition properties of KLF1. Mutations in KLF1 are  
30 associated with different phenotypes in humans (10), such as In(Lu) blood group (11), hereditary  
31 persistence of fetal hemoglobin (HPFH) (12), zinc protoporphyria (13), increased HbA2 (14), and  
32 congenital dyserythropoietic anemia (CDA) type IV (15).

33 The Neonatal anemia (*Nan*) mouse is an ethylnitrosourea (ENU)-induced semi-dominant hemolytic  
34 anemia model first described in 1983 by Mary Lyon (16), who positioned the variant on chromosome 8  
35 (17). In 2010, *Klf1* was identified as the gene responsible for this phenotype, due to a single point  
36 mutation in the second zinc finger (p.E339D) (18, 19). While *Klf1 Nan* homozygous mice die around  
37 E10, KLF1 *Nan* heterozygous mice survive into adulthood displaying life-long hemolytic anemia. This  
38 indicates that the *Nan* variant affects the function of wildtype KLF1 protein, as this phenotype does not  
39 occur in *Klf1* haplo-insufficient mice (6-8, 18, 19). Indeed, the DNA binding properties of *Nan* KLF1  
40 may be altered due to steric clash between the carboxyl group of p.339D and the methyl group of  
41 thymidine, resulting in the deregulation of a subset of target genes (19), although alternative models  
42 have been proposed (18).

43 Until now, research has focused on the effects of the *Nan* variant in adult mice (18-20). Given that  
44 KLF1 expression begins around E7.5 (21), it is of interest to investigate the impact of aberrant KLF1  
45 activity during development. Here we investigated erythropoiesis during different stages of fetal  
46 development and observed impaired red blood cell maturation at E12.5, as assessed by flow  
47 cytometry analysis of the CD71 and Ter119 markers. Expression profiling of E12.5 fetal liver cells  
48 revealed 782 deregulated genes in *Nan* mutant samples including a host of known KLF1 targets such

49 as Dematin and E2F2 (1, 4, 22). Intriguingly, the nuclear exportin XPO7, which has recently been  
50 implicated in nuclear condensation and enucleation during erythroid maturation (23), was one of the  
51 deregulated genes. XPO7 expression was significantly downregulated in the presence of the *Nan*  
52 variant erythroid progenitors, contributing to increased nuclear size. This partially explains the  
53 erythroid defects observed in *Nan* erythroid cells and provides a novel link between KLF1 and nuclear  
54 condensation.

55

## 56 MATERIAL AND METHODS

### 57 Mice

58 All animal studies were approved by the Erasmus MC Animal Ethics Committee. The mouse strains  
59 used were *Klf1 Nan* mutant (16) and *Klf1* knockout (6). Genotyping was performed by PCR using DNA  
60 isolated from toe biopsies. For *Nan* genotyping, the PCR product was digested with DpnII. Embryos  
61 were collected at E12.5, E13.5, E14.5 and E18.5; tail DNA was used for genotyping. Primer  
62 sequences are detailed in Supplementary Materials and Methods.

63

### 64 Blood analysis

65 Peripheral blood was collected from the mandibular vein of adult mice, and standard blood parameters  
66 were measured with an automated hematologic analyzer (Scil Vet ABC, Viernheim, Germany).

67

### 68 Cell culture and transduction

69 I/11 erythroid progenitors and primary mouse fetal liver cells were cultured as described (24). To  
70 induce differentiation of I/11 cells we used StemPRO-34 SFM (10639-011, life technologies)  
71 supplemented with 500 µg/mL iron-saturated transferrin (Scipac) and Epo (Janssen-Cilag, 10 U/mL).  
72 Lentiviral shRNAs targeting XPO7 were obtained from the Sigma MISSION shRNA library. The clones  
73 used are detailed in Supplementary Materials and Methods.

74

### 75 RNA isolation and RT-qPCR analyses

76 RNA was extracted using TRI reagent (Sigma-Aldrich). To synthesize cDNA, 2 µg of RNA were used  
77 together with oligo dT (Invitrogen), RNase OUT (Invitrogen), and SuperScript reverse transcriptase II  
78 (Invitrogen) in a total volume of 20 µL for 1 hour at 42°C. 0.2 µL of cDNA was used for amplification by  
79 RT-qPCR. Other experimental details and primer sequences are detailed in Supplementary Materials  
80 and Methods.

81

### 82 Protein extraction and western blotting

83 Total protein extracts from mouse fetal liver cells were prepared according to (25). To visualize protein  
84 expression, cell lysates of  $\sim 3 \times 10^6$  cells were loaded on 10% SDS-polyacrylamide gels for  
85 electrophoresis. The gels were transferred to nitrocellulose blotting membrane 0.45 µm (10600002,  
86 GE Healthcare) and probed with specific antibodies. Membranes were stained for Tubulin (T5168,  
87 Sigma-Aldrich) as loading control, and for XPO7 (sc390025, Santa Cruz).

88

### 89 Flow cytometry, cell sorting, enucleation- and cell morphology analysis

90 These procedures are described in detail in Supplementary Materials and Methods.

91

92 **RNA-sequencing and analysis**

93 RNA-seq was performed according to manufacturer's instructions (Illumina; San Diego, CA, USA), as  
94 described(26). The sequenced reads were mapped against the mouse genome build mm10 using  
95 TopHat 2.0.6 (27) with the transcriptome gene annotation of Ensembl v73 (28). Further details of the  
96 bioinformatics analyses are described in Supplementary Materials and Methods.

97

98 **Chromosome Conformation Capture Combined with high-throughput Sequencing (4C-seq) and**  
99 **data analysis**

100 4C-seq experiments were carried out as described (29, 30). Briefly, 4C-seq template was prepared  
101 from E13.5 fetal liver or fetal brain cells. In total, between 1 and 8 million cells were used for analysis.  
102 Further experimental details and of the bioinformatics analyses are described in Supplementary  
103 Materials and Methods.

104

105 **Statistical tests**

106 Statistical analysis of blood parameters was performed by using analysis of variance with Bonferroni  
107 correction; flow cytometry data and gene expression results were analyzed by using Mann-Whitney  
108 tests. Excel 2010 (Microsoft, Redmond, WA) was used to draw the graphs. Values plus or minus  
109 standard deviation are displayed in all figures.

110

## 111 RESULTS

### 112 Characterization of *Nan* fetal liver cells

113 The effect of the KLF1 *Nan* variant has been studied in adult mice (18-20), but data on its effect during  
114 gestation is limited. Hence, to study this variant during embryonic development, we used a *Nan* mouse  
115 model carrying one mutant allele (*Nan*+, from now on called *Nan*). At E12.5, E14.5, and E18.5, *Nan*  
116 embryos were paler than wildtype littermates, indicating anemia, but otherwise looked phenotypically  
117 normal. Flow cytometry analysis of E12.5, E14.5, and E18.5 fetal liver cells used the Kit, CD71,  
118 Ter119 and CD44 markers to trace red blood cell differentiation. A severe downregulation in  
119 expression of the Ter119 marker was observed at all three stages (Figure 1A,B). The CD71/Ter119  
120 double-positive population was significantly decreased in the *Nan* samples, while the CD71  
121 single-positive population showed an increase. No significant differences were observed for Kit and  
122 CD44 in the *Nan* variant (Supplementary Figure 1A,B). In addition, similar results were obtained when  
123 assaying embryonic blood, with Ter119 being highly downregulated (Figure 1C,D). These results  
124 indicate that *Nan* embryos display delayed erythroid maturation compared to wildtype controls. This is  
125 in line with the observation that a higher percentage of cells is positive for CD71 in adult blood  
126 (Supplementary Figure 2A,B), indicative of higher percentage of circulating reticulocytes (19).  
127 Consistent with this notion, analysis of standard blood parameters revealed a significant increase in  
128 red cell distribution width (RDW) in the *Nan* mice (Supplementary Figure 2C). Furthermore, we  
129 observed minor, yet significant, decreases in RBC (red blood cell), HGB (total hemoglobin), HCT  
130 (hematocrit), MCH (Mean Corpuscular Hemoglobin), MCHC (Mean Corpuscular Hemoglobin  
131 Concentration) values. Interestingly, when comparing *Nan* E14.5 fetal liver cytopins to wildtype  
132 controls, we observed a marked increase in the average size of the erythroid cells and their nuclei  
133 (Figure 1E). Taken together, these data show that erythroid maturation is impaired in *Nan* animals.

134

### 135 Identification of deregulated genes in E12.5 *Nan* fetal livers

136 In order to identify genes that are affected by the *Nan* variant, a genome-wide RNA-seq was  
137 performed on samples derived from E12.5 *Nan* and wildtype fetal livers ( $N=6$  each), as at this stage  
138 the fetal liver is mainly composed of erythroid cells. 782 genes appeared to be deregulated in the *Nan*  
139 mutants (false discovery rate [FDR]  $<0.01$ , absolute fold change equal or greater than 1.5), of which  
140 437 were upregulated and 345 downregulated (Figure 2A,B and Supplementary Table 1). Strikingly,  
141 even though KLF1 has been mainly described as a transcriptional activator, the majority of the  
142 deregulated genes displayed increased activation in the *Nan* erythroid cells. We postulate that this  
143 might be due to secondary effects of KLF1 on other transcriptional regulators and/or aberrant activity  
144 of KLF1 *Nan*. To validate the data, we checked the expression of *Epb4.9* and *E2f2*, genes known to  
145 be down-regulated in *Nan* erythroid cells (19) (Figure 2D, left panel and Supplementary Figure 3).



146 Indeed, a significant decreased expression of the transcripts of these two genes was detected in *Nan*  
147 fetal livers. Moreover a significant 2-fold down-regulation of BCL11A, a known target of KLF1 (12, 31),  
148 was observed indicating that the KLF1 *Nan* variant affects its expression (Figure 2D, left panel and  
149 Supplementary Figure 3). Given the role of BCL11A and KLF1 in globin switching, the expression  
150 levels of the  $\beta$ -like globin genes were checked; the embryonic *Hbb-bh1* gene was upregulated and the  
151 KLF1 target gene *Hbb-b1* was downregulated, consistent with previous reports (19). In addition, the  
152 embryonic *Hba-x* gene was upregulated in E12.5 *Nan* fetal livers (Figure 2D, right panel and  
153 Supplementary Figure 3). Collectively, these data are in accordance with the notion that intact KLF1  
154 fulfils a crucial role in developmental regulation of globin gene expression (8) and deregulation of  
155 embryonic globin expression in adult *Nan* mice (19).

### 156 157 **The nuclear exportin XPO7 is downregulated in *Nan* erythroid cells**

158 The expression of the *Xpo7* gene, encoding a nuclear exportin, was prominently downregulated in  
159 *Nan* E12.5 fetal livers (~4-fold decrease; Figure 2A). This raised our interest since XPO7 was recently  
160 implicated in terminal erythroid differentiation, as a protein involved in enucleation (23). To corroborate  
161 the RNA-seq data, XPO7 expression in *Nan* E14.5 fetal livers was reduced by approximately 90% in  
162 the *Nan* samples as determined by RT-qPCR (Figure 3A). Transcripts using the canonical first exon  
163 were barely detectable in all samples (not shown). Importantly, XPO7 protein levels were also reduced  
164 in *Nan* fetal liver cells (Figure 3B). To investigate whether XPO7 expression is dependent on KLF1,  
165 XPO7 mRNA and protein levels were measured in *Klf1 null* erythroid cells. In E13.5 *Klf1 null* fetal  
166 livers (6) expression of XPO7 mRNA and protein is significantly reduced (Figure 3C,D). Remarkably,  
167 downregulation of XPO7 was also observed in *Klf1 wt/ko* fetal livers, although to a lesser extent than  
168 observed in *Klf1 null* fetal livers (Figure 3C,3D). Thus, similar to BCL11A (12, 31), activation of XPO7  
169 by KLF1 is dose-dependent. In agreement with the notion that KLF1 is a direct activator of the *Xpo7*  
170 gene, KLF1 binds to the canonical promoter and first intron of the *Xpo7* gene in mouse (32) and  
171 human (33) erythroid cells (Supplementary Figure 4).

172

173

174 **The chromatin conformation of the *Xpo7* locus is not affected in *Nan* erythroid cells**

175 Since KLF1 is required to form an active chromatin hub in the  $\beta$ -globin locus (34), 4C-seq experiments  
176 were performed on the *Xpo7* locus in E13.5 wildtype fetal livers and fetal brains and *Nan* mouse fetal  
177 livers (Figure 4A). The canonical promoter of *Xpo7* was used as viewpoint to investigate potential  
178 changes in chromatin conformation (Figure 4B). Interestingly, a loop was identified between the  
179 canonical promoter of *Xpo7* (situated at the beginning of exon 1a) and the exon that produces the  
180 erythroid-specific form of *Xpo7* (exon 1b), indicating that these two regions are in spatial proximity in  
181 erythroid cells (Figure 4B), whereas this loop has lower contact frequencies in fetal brain. However,  
182 few local changes in the chromatin conformation were found between wildtype and *Nan* samples  
183 (Figure 4B). The experiment was repeated using the erythroid-specific promoter as view point. This  
184 confirmed the results obtained with the canonical promoter as viewpoint. (Figure 4C). We suggest that  
185 this loop might recruit transcription factors binding to the area of the canonical promoter to the vicinity  
186 of the erythroid promoter, thereby facilitating expression of the erythroid-specific *Xpo7* transcript.

187  
188 ***Nan* mouse fetal liver cells present defects in nuclear condensation**

189 Since *Xpo7* has been implicated in enucleation of erythroid cells *in vitro* (23) enucleation in *Nan* fetal  
190 livers was analyzed. This was quantified in E14.5 fetal livers by flow cytometry using the erythroid  
191 marker Ter119 and Hoechst-33342 as a nuclear stain. Similar percentages of enucleated cells were  
192 observed between *Nan* and control fetal liver samples (Figures 5A,B). Similar results were obtained  
193 with E12.5 and E18.5 fetal liver cells (data not shown). To check whether the flow cytometry analysis  
194 could indeed discriminate nucleated from enucleated cells, we sorted the Hoechst-positive and  
195 Hoechst-negative populations and prepared cytopins. This showed all the Hoechst-negative cells  
196 identified by flow cytometry to indeed be enucleated (Figure 5C). In addition, assessing enucleation  
197 levels of mouse fetal liver cells cultured in proliferative medium and in differentiation medium from  
198 embryos at E12.5 and E14.5 by flow cytometry, similar ratios of nucleated *versus* enucleated cells  
199 were found in control and *Nan* samples (Supplementary Figure 5A,B). Nevertheless, a striking  
200 increase in the percentage of large cells in the fetal livers of the *Nan* embryos was observed.  
201 Quantification of cell size using flow cytometry revealed a significant increase in average cell size at  
202 E12.5, E14.5 and E18.5 in the *Nan* samples (Figure 5D). In line with this finding the nuclear area of  
203 the *Nan* fetal liver cells was significantly increased when compared to control fetal liver cells in  
204 cytopin slides stained with the nuclear dye Hoechst 33342 (Figure 5E). These data are consistent  
205 with the notion that XPO7 is involved in nuclear condensation, a process that precedes enucleation.  
206 However, despite the impaired nuclear condensation, the cells still are still able to undergo enucleation  
207 as we observed similar ratios of nucleated *versus* enucleated cells in control and *Nan* fetal liver cells.

208

209 **XPO7 knock down in I/11 cells mimics the phenotype of *Nan* cells**

210 The role of XPO7 in erythroid differentiation was further analyzed by knocking down XPO7 in the  
211 factor-dependent immortalized mouse erythroid cell line I/11 (35). Using three different shRNAs, an  
212 efficiency of ~70% knockdown was reached as shown by Western blot (Figure 6A). Before  
213 differentiation a minor difference in expression of the surface markers CD71 and Ter119 and no  
214 difference in cell size between the control and the knockdown cells was observed (data not shown). In  
215 contrast, upon transfer to differentiation medium, the maturation of XPO7 knockdown cells was  
216 impaired, as shown by CD71 and Ter119 flow cytometry analysis (Figure 6B), and the average cell  
217 size was increased (Figure 6C). In addition, using an ImageStream flow cytometer showed the mean  
218 and median size of the nuclear area to be increased upon XPO7 knockdown when the cells were  
219 cultured under differentiation conditions. This is consistent with the notion that XPO7 is required for  
220 nuclear condensation during terminal erythroid differentiation. Collectively, these findings indicate that  
221 XPO7 is partially responsible for the phenotype of *Nan* mice, establish that the *Xpo7* gene is a novel  
222 erythroid target gene of KLF1, and that nuclear condensation is a process previously unrecognized to  
223 be regulated by KLF1.

224

## 225 DISCUSSION

226 Erythropoiesis is a complex process that involves many players whose coordinated activity ensures  
227 the production of functional red blood cells. One of these players is KLF1, a transcription factor with  
228 multiple roles during terminal erythroid differentiation. Firstly, it is essential for globin regulation, in  
229 particular for direct activation of  $\beta$ -globin (6, 7). In addition, it acts as a master regulator of genes  
230 activated during differentiation of red blood cells, such as membrane proteins, heme synthesis  
231 enzymes and cell cycle regulators (2, 4, 22). Hence, it comes as no surprise that *Klf1* knockout  
232 embryos die due to severe anemia, and that the phenotype is not rescued by exogenous expression  
233 of a  $\beta$ -like globin gene (36). Accordingly, *KLF1* variants can lead to diverse phenotypes in humans  
234 (10). One example is a missense variant in the second zinc finger of human KLF1 (p.E325K) that  
235 causes CDA type IV (15). This variant is believed to affect binding of KLF1 to its target genes thereby  
236 exerting a dominant-negative effect on wildtype KLF1 protein. Similar effects have been described for  
237 the *Nan* mouse model. These mice have a missense variant, p.E339D, in a position homologous to  
238 that of the human CDA type IV variant (18, 19). Studies on the effect of the *Nan* variant in adult mice  
239 have revealed that these animals display life-long anemia (18-20).

240 In this paper we present our findings on the effects of the *Nan* variant on definitive fetal erythropoiesis  
241 and show that erythroid maturation is impaired in *Nan* fetal livers at E12.5, E14.5 and E18.5. We  
242 identified 782 differentially expressed genes in *Nan* versus control E12.5 fetal livers. In agreement with  
243 a previous report on erythropoiesis in adult *Nan* mice (19), the expression of globin genes is altered in  
244 *Nan* fetal livers. In particular, the upregulation of embryonic  $\beta$ h1 globin can be explained by the  
245 significantly lower expression of BCL11A in *Nan* embryos, which normally suppresses  $\beta$ h1 expression  
246 (37). *Xpo7*, encoding a nuclear exportin, was one of the most significantly downregulated genes. It  
247 caught our attention since a recent paper described that *Xpo7* is required for nuclear condensation  
248 and enucleation during terminal erythroid differentiation *in vitro* (23). In addition, the observation that  
249 XPO7 expression was also reduced in *Klf1* knockout fetal livers indicated that the *Xpo7* gene might be  
250 a direct target of KLF1. Supporting this notion, data mining of ChIP-seq results revealed that KLF1  
251 binds to the *Xpo7* locus in mouse (32) and human (33) erythroid cells. Collectively, these data  
252 suggested that, similar to the  $\beta$ -globin locus (34), KLF1 might have a role in the spatial organization of  
253 the *Xpo7* locus. 4C-seq analysis of the *Xpo7* locus demonstrated that it adopts a different  
254 conformation in fetal liver cells compared to fetal brain cells. The presence of the *Nan* variant doesn't  
255 appear to mediate any major changes in the chromatin conformation of the *Xpo7* locus. We note that  
256 the promoter of the *Xpo7* gene contains so-called 'category II' KLF1 binding sites(19) which are  
257 recognized by wildtype KLF1 only. The presence of such 'category II' sites is a hallmark of  
258 downregulated genes in *Nan* erythroid cells. This suggests that in *Nan* cells wildtype KLF1 is still able  
259 to bind to the *Xpo7* promoter and organize the erythroid-specific 3D conformation of the locus, but is

260 unable to activate transcription efficiently. An interesting observation is the presence of a loop  
261 between the promoter of the canonical *Xpo7* promoter (in front of exon 1a) and the erythroid-specific  
262 promoter (in front of exon 1b), which is absent in the fetal brain control. This loop is likely the  
263 consequence of recruitment of the two promoters to the same transcription factory (38). Previous work  
264 has shown that XPO7 knockdown in cultured mouse fetal liver cells impairs chromatin condensation  
265 and enucleation during terminal erythroid differentiation (23). Although in *Nan* mice enucleation still  
266 occurs, the reduced XPO7 expression due to the *Nan* variant impairs chromatin condensation during  
267 terminal erythroid differentiation. We propose that this contributes to the maturation defects of *Nan*  
268 erythrocytes in fetal and adult definitive erythropoiesis. Indeed, knockdown of XPO7 in immortalized  
269 mouse erythroblasts cells leads to impaired maturation of the cells, evident by dysregulation of the  
270 flow cytometry markers CD71 and Ter119 and the presence of larger cells with larger nuclei in the  
271 cultures. Our data are in reasonable agreement with the recent publication on the role of XPO7 in  
272 erythroid maturation (23). It is important to keep in mind that we cannot compare the levels of XPO7  
273 protein between our system and that of Hattangadi et al. (23). An emerging question is how *Nan* cells  
274 manage to enucleate in the presence of reduced levels of XPO7. One possibility is that the level of  
275 XPO7 present *in vivo* in *Nan* mice might suffice for correct enucleation of the erythroblasts but still  
276 affects nuclear condensation. Alternatively, downregulation of XPO7 might just slow down nuclear  
277 condensation, but the cells eventually manage to shed their nucleus when condensation is completed.  
278 Lastly, a protein with a role similar to that of XPO7 may substitute for it, thus enabling enucleation. We  
279 favour a scenario in which chromatin condensation is crucial for enucleation (39-41), with XPO7 as an  
280 important effector. It is not clear whether enucleation can happen before nuclear condensation is  
281 completed. Our study suggests that impaired nuclear condensation contributes to the erythroid  
282 maturation defects observed in the *Nan* mice.

283 Understanding the role of KLF1 during erythroid maturation and the enucleation process has clinical  
284 significance for the production of red blood cells *in vitro* for transfusion purposes. In recent years,  
285 many efforts have been made to produce erythrocytes *in vitro* starting from hematopoietic stem cells,  
286 embryonic stem cells or induced pluripotent stem cells (42-44). Efficient enucleation is one of several  
287 challenges that have to be overcome in order to produce sufficient numbers of fully functional  
288 erythrocytes *in vitro*. More in depth knowledge of this process might guide the development of  
289 improved strategies to achieve this goal.

290

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395

396 **Figure legends**

397 **Figure 1. Flow cytometry analysis of erythroid cells isolated from *Nan* embryos.** (A) Examples of  
398 flow cytometry profiles of CD71 and Ter119 staining of E12.5, E 14.5 and E18.5 wildtype and *Nan*  
399 mouse fetal livers. (B) Quantification of CD71+, CD71+ Ter119+ and Ter119+ populations. n indicates  
400 the number of embryos. \* indicates *p value* <0.01. (C) Examples of flow cytometry profiles of CD71  
401 and Ter119 staining of E12.5, E 14.5 and E18.5 wildtype and *Nan* mouse fetal blood. (D)  
402 Quantification of CD71+, CD71+ Ter119+ and Ter119+ populations. n indicates the number of  
403 embryos. \* indicates *p value* <0.01. (E) Cytospins of E14.5 wildtype and *Nan* mouse fetal liver cells  
404 stained with May Grünwald-Giemsa and O-dianisidine.

405

406 **Figure 2. RNA-seq analysis of wildtype and *Nan* fetal liver cells.** (A) Hierarchical clustered heat  
407 map with scaled Z-score color key of normalized counts of 782 differentially expressed genes in 6 WT  
408 (+/+) and 6 *Nan* (*Nan*/+) E12.5 fetal liver samples. Samples with the same genotype are indicated by  
409 black (WT) and cyan (*Nan*) horizontal bars; gene clusters are indicated by green (upregulated in *Nan*)  
410 and purple (downregulated in *Nan*) vertical bars. False discovery rate [FDR] <0.01, fold-change equal  
411 or greater than 1.5. (B) Schematic representation of the number of downregulated and upregulated  
412 genes in the *Nan* E12.5 fetal livers. (C) Log2 values of fold-change for selected genes. \* indicates  
413 FDR <0.01.

414

415 **Figure 3. XPO7 expression in wildtype, *Nan* and *Klf1* knockout fetal liver cells.** (A) XPO7 mRNA  
416 relative values in wildtype and *Nan* E14.5 fetal livers. \* indicates *p value* <0.01. n indicates the number  
417 of embryos. (B) Western blot analysis of XPO7 protein in wildtype and *Nan* E14.5 fetal livers and  
418 quantification.  $\beta$ -tubulin was used as loading control. \* indicates *p value* <0.01. n indicates the number  
419 of embryos. (C) KLF1 and XPO7 mRNA relative expression values in wildtype, KLF1 heterozygotes  
420 and KLF1 knockout E13.5 fetal livers. \* indicates *p value* <0.01. n indicates the number of embryos.  
421 (D) Western blot analysis of XPO7 protein in wildtype, KLF1 heterozygotes and KLF1 knockout E13.5  
422 fetal livers and quantification.  $\beta$ -tubulin was used as loading control. \* indicates *p value* <0.01. n  
423 indicates the number of embryos.

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425



426 **Figure 4. 4C-seq analysis of the *Xpo7* locus.** (A) Schematic representation of chromosome number  
427 14. The green box indicates the zone where the *Xpo7* gene resides. The RefSeq mm10 *Xpo7* gene is  
428 indicated by rectangles (exons) and arrows (introns) that point to the direction of transcription . The  
429 location is indicated in Mega basepairs (Mb). The erythroid-specific first exon is indicated by a red box.  
430 (B-C) 4C-seq representation of the chromosome contact frequencies detected using the canonical  
431 promoter of *Xpo7* (B) and the region of the erythroid specific *Xpo7* exon (C) as viewpoints. The mean  
432 of a running windows of 21 restriction fragment-ends of the median value of the biological replicates  
433 with a maximum of 3000 are indicated by colored lines. Loci with a statistically significant (FDR <0.05)  
434 higher contact frequencies and reads per million >250 in wildtype fetal liver compared to the fetal brain  
435 are indicated by light grey boxes. Loci with a statistically significant (FDR <0.05) higher contact  
436 frequencies and reads per million >250 in fetal brain compared to the wildtype fetal liver brain are  
437 indicated by dark grey boxes. The red dotted line indicates the view point and the red arrow the  
438 position of the loop. Purple, *KLF1* *+/+* fetal liver; Orange, *KLF1* *Nan*/*+* fetal liver; Grey, *KLF1* *+/+* fetal  
439 brain.

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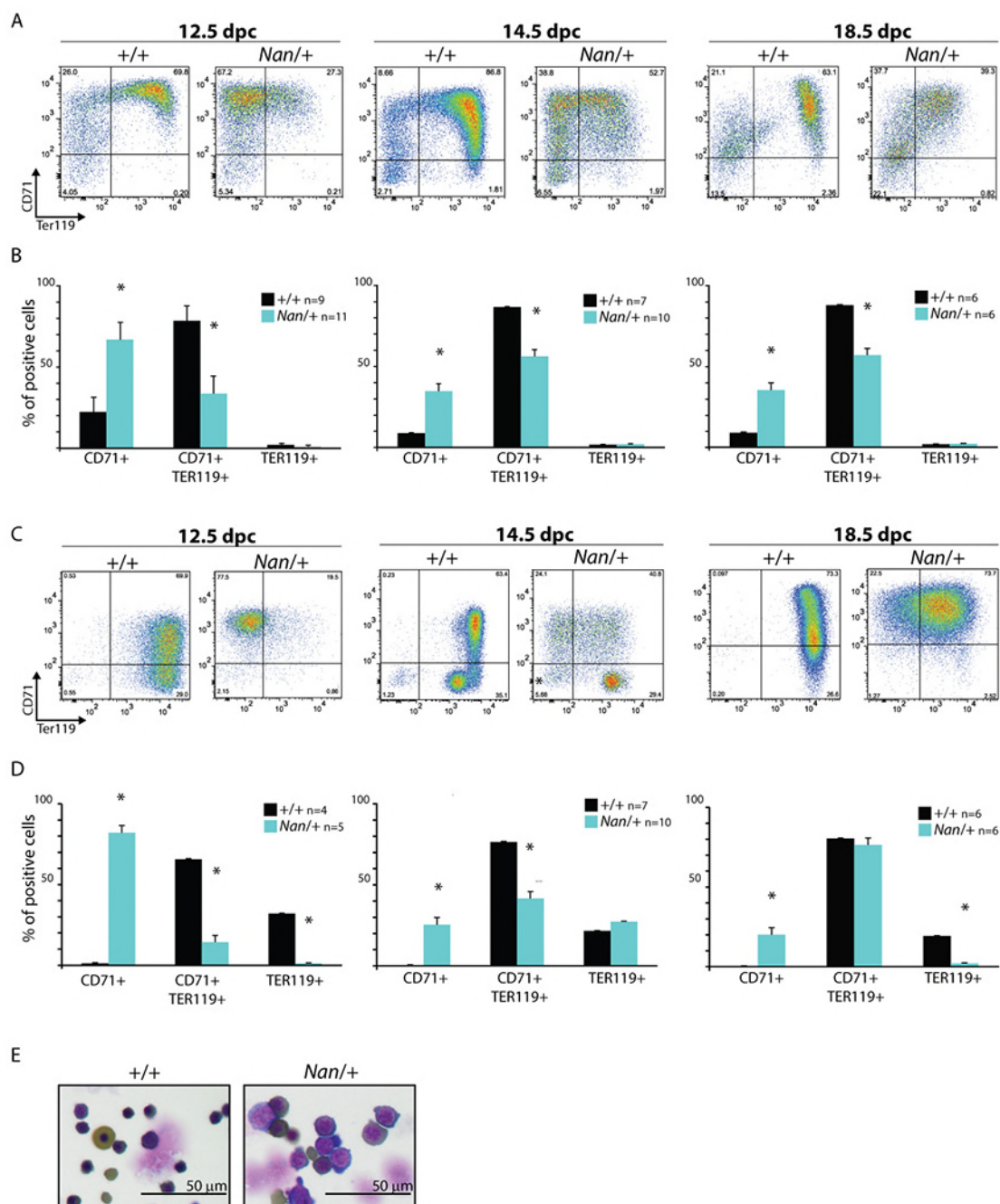
441 **Figure 5. Analysis of enucleation and cell size of *Nan* fetal liver cells.** (A) Gating strategies of  
442 Hoechst- and Ter119-stained E14.5 fetal liver cells. Red, Hoechst+ population; Green, Hoechst-  
443 population. (B) Quantification of the number of nucleated (Hoechst+) and enucleated (Hoechst-) cells.  
444 n indicates the number of embryos. (C) Cytospins stained with May Grünwald-Giemsa and  
445 O-dianisidine of Hoechst- wildtype and *Nan* sorted populations. (D) Representative FSC-A value flow  
446 cytometry plots of E12.5, E14.5 and E18.5 wildtype and *Nan* fetal liver cells and quantification. \*  
447 indicates *p* value <0.01. n indicates the number of embryos. (E) Relative nuclear area size  
448 quantification of E12.5, E14.5 and E18.5 wildtype and *Nan* fetal liver cells. \* indicates a *p* value <0.01.  
449 n indicates the number of embryos.

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451

452 **Figure 6. XPO7 knockdown in I/11 immortalized mouse erythroid progenitor cells.** (A) Western  
453 blot analysis indicating the efficiency of XPO7 knockdown using 3 different shRNAs.  $\beta$ -tubulin was  
454 used as loading control. (B) Example of flow cytometry profiles of CD71 and Ter119 staining of I/11  
455 cells transduced with either control, sh#1, sh#2 or sh#3 lentiviruses in differentiation conditions. The  
456 percentage of cells in the CD71/Ter119 double-positive quadrant is  $50.2 \pm 1.92$  for control cells and  
457  $43.5 \pm 3.0$  for XPO7 knockdown cells ( $p=0.039$ , four independent experiments). (C) Representative  
458 FSC-A value flow cytometry plots of I/11 cells transduced with either control, sh#1, sh#2 or sh#3  
459 lentiviruses in differentiation conditions. (D) ImageStream area quantification (arbitrary units) of I/11  
460 cells transduced with either control, sh#1, sh#2 or sh#3 lentiviruses in differentiation conditions. The  
461 total number of cells counted, the mean, the median and the standard deviation are shown below the  
462 histograms. On top a representative cell from the control sample is depicted.  
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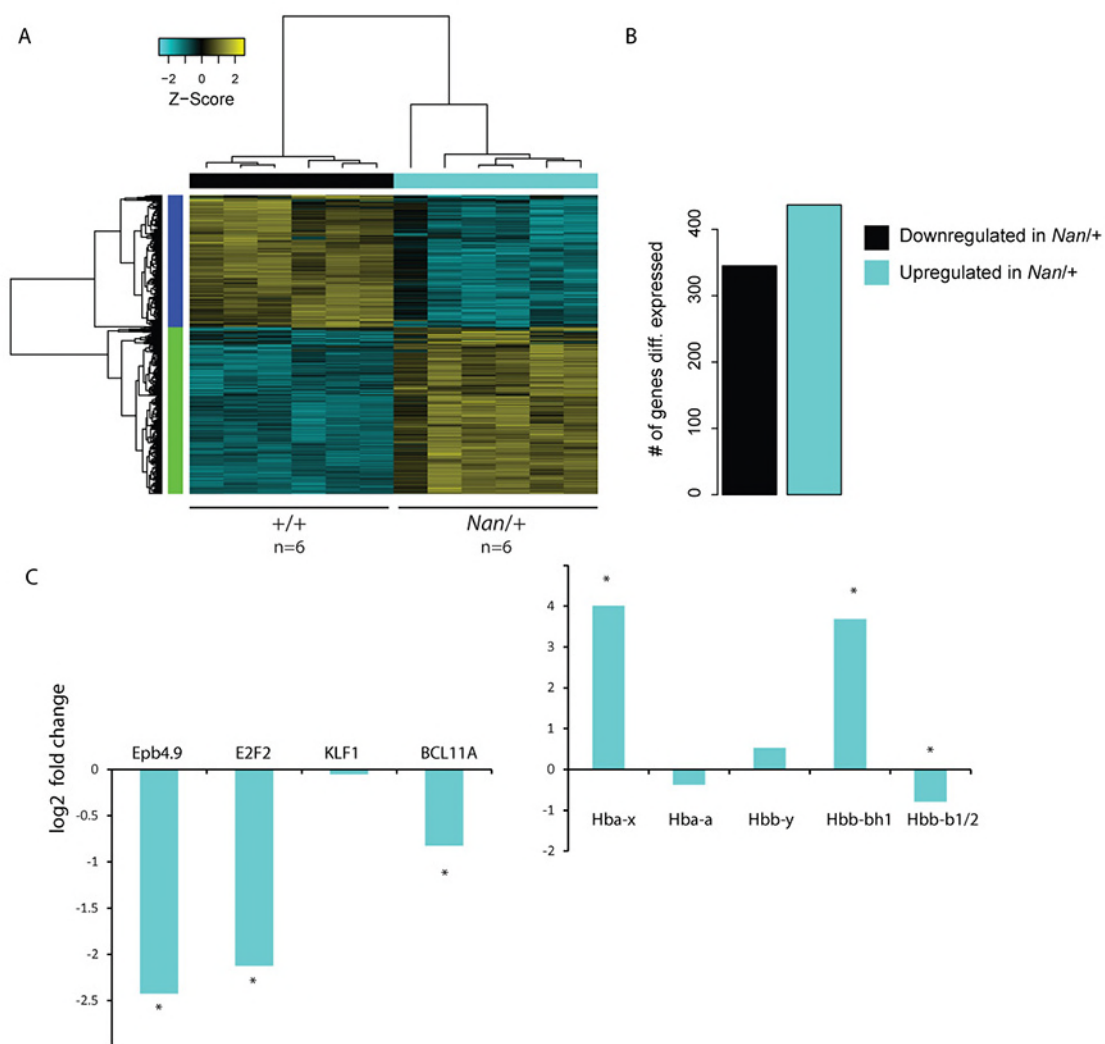
465 **Figure 1. Cantú et al.**



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468 **Figure 2. Cantú et al.**

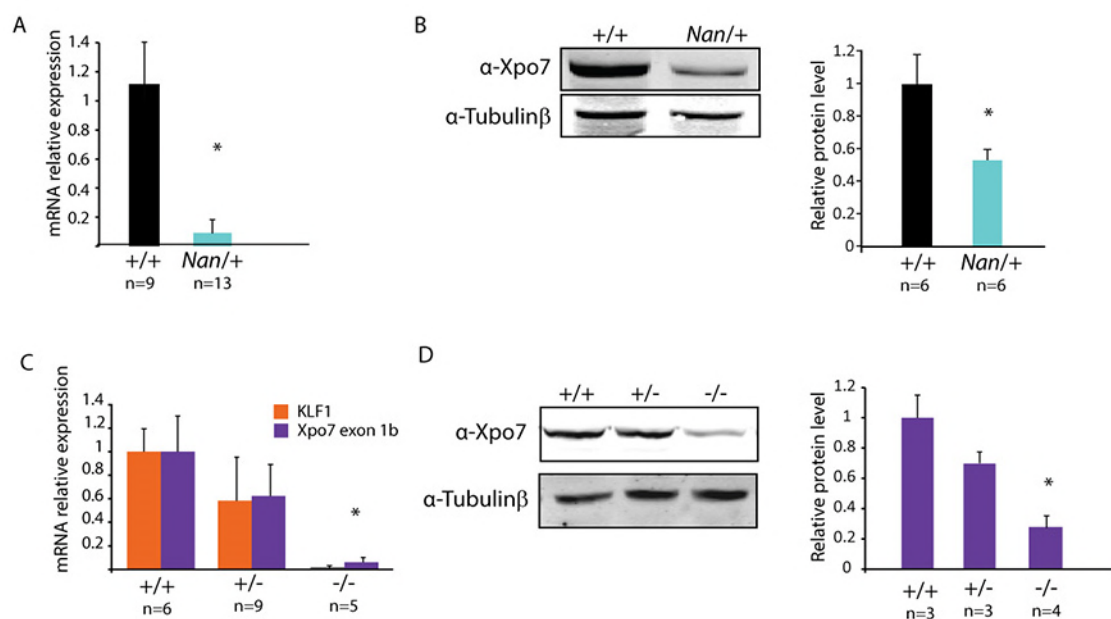


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472 **Figure 3. Cantú et al.**

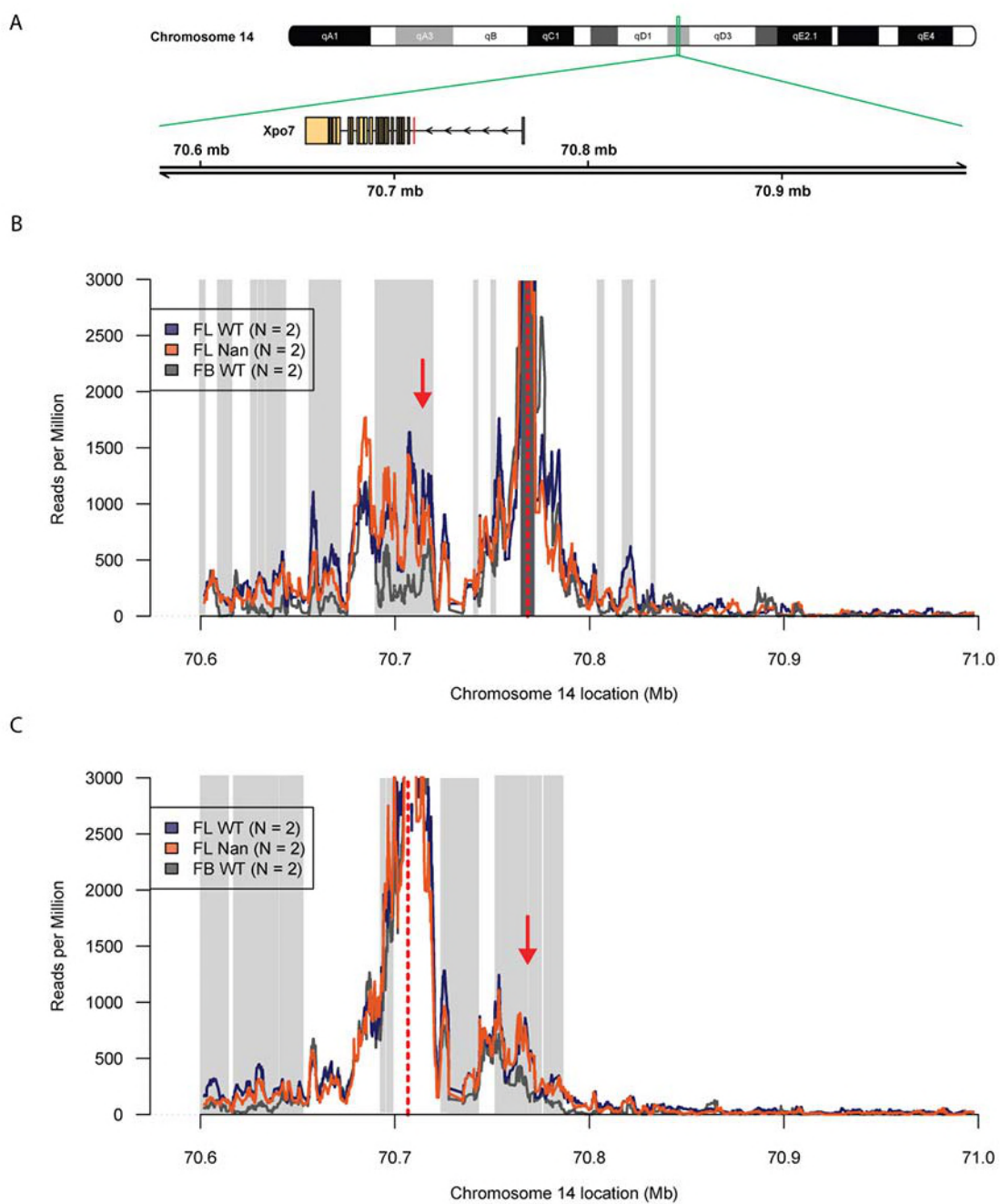


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476 **Figure 4.** *Cantú et al.*

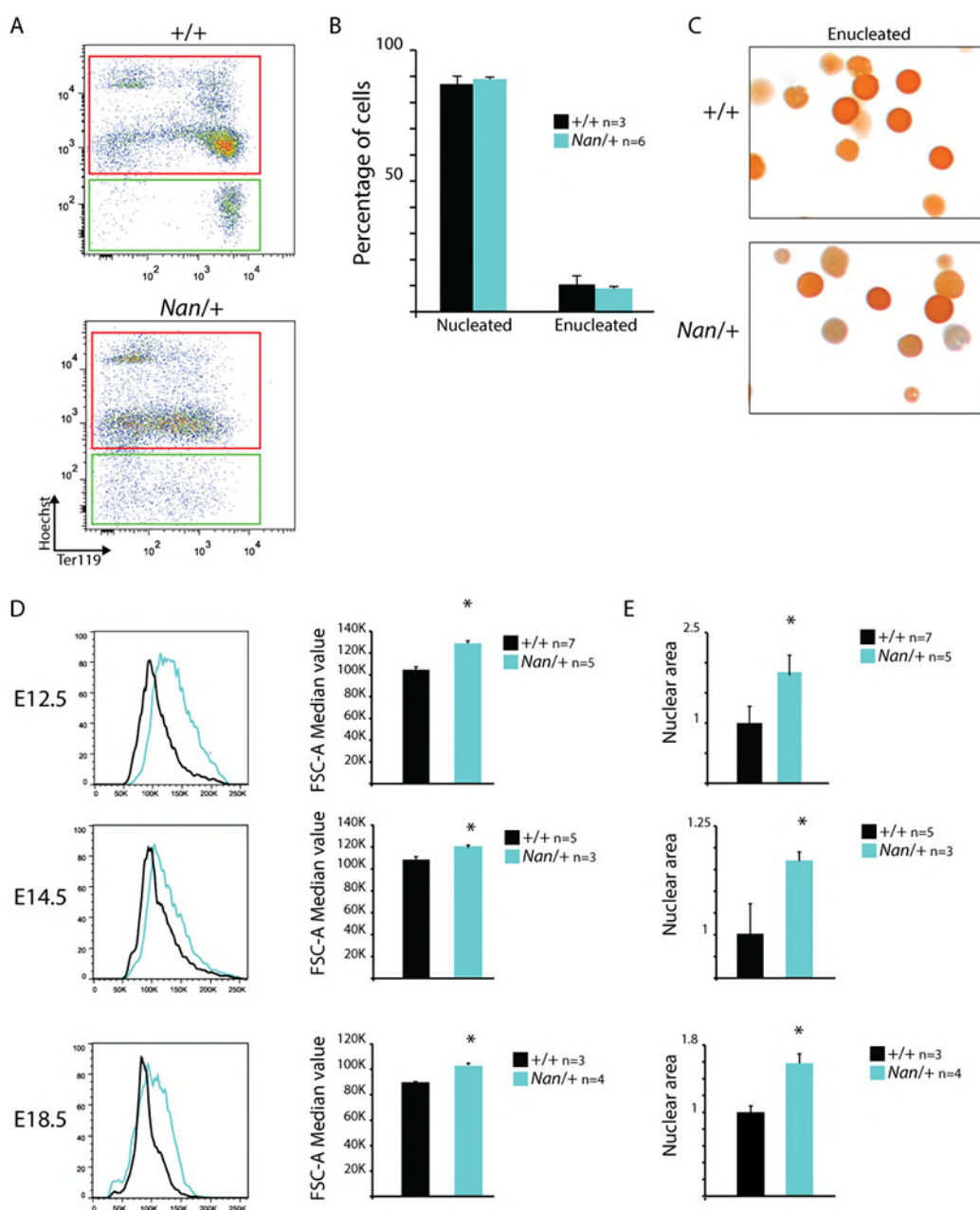


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480 **Figure 5. Cantú et al.**

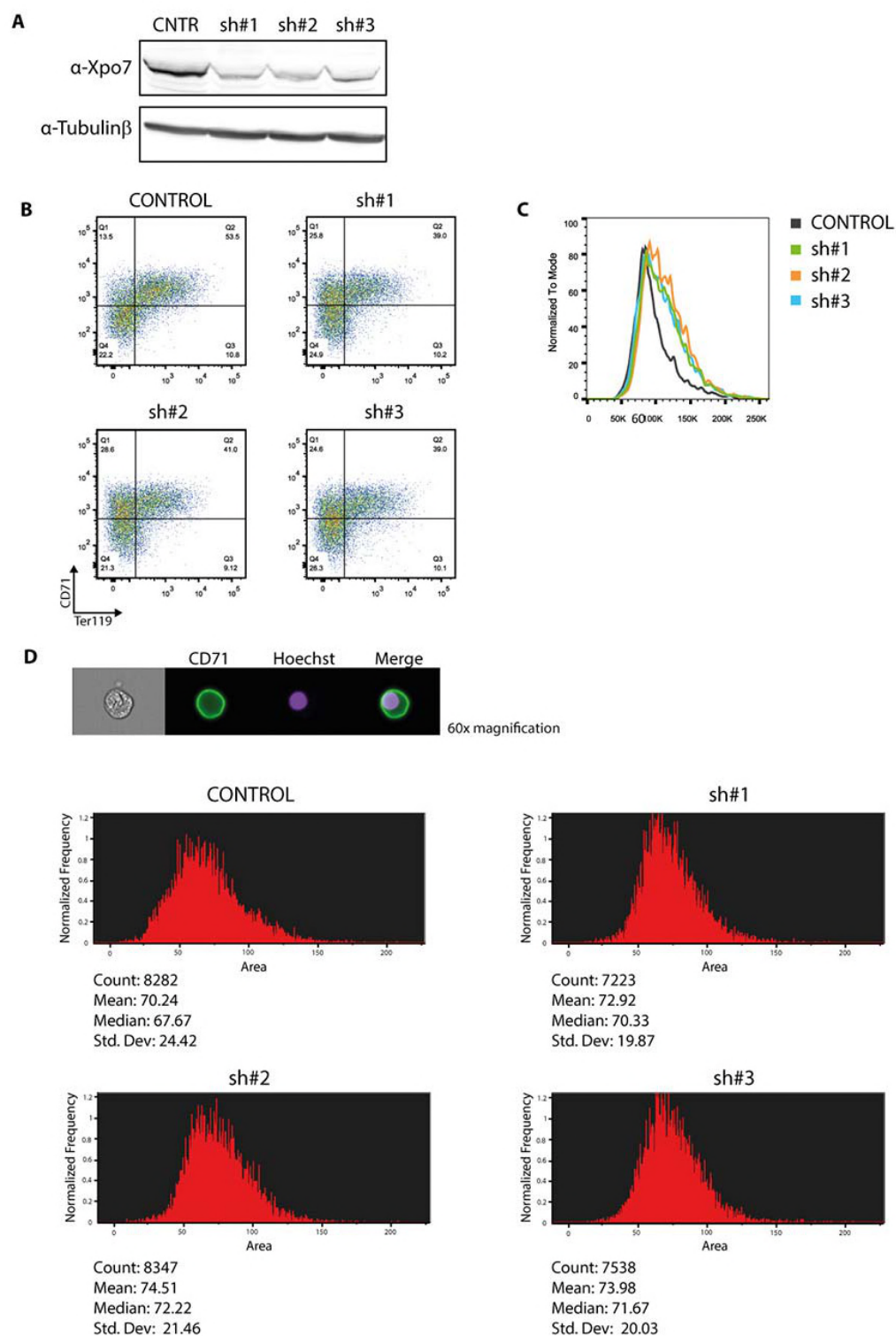


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484 **Figure 6. Cantú et al.**



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