NuRD mediates activating and repressive functions of GATA-1 and FOG-1 during blood development

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GATA transcription factors interact with FOG proteins to regulate tissue development by activating and repressing transcription. FOG-1 (ZFPM1), a co-factor for the haematopoietic factor GATA-1, binds to the NuRD co-repressor complex through a conserved N-terminal motif. Surprisingly, we detected NuRD components at both repressed and active GATA-1/FOG-1 target genes *in vivo*. In addition, while NuRD is required for transcriptional repression in certain contexts, we show a direct requirement of NuRD also for FOG-1-dependent transcriptional activation. Mice in which the FOG-1/NuRD interaction is disrupted display defects similar to germline mutations in the *Gata1* and *Fog1* genes, including anaemia and macrothrombocytopaenia. Gene expression analysis in primary mutant erythroid cells and megakaryocytes (MKs) revealed an essential function for NuRD during both the repression and activation of select GATA-1/FOG-1 target genes. These results show that NuRD is a critical co-factor for FOG-1 and underscore the versatile use of NuRD by lineage-specific transcription factors to activate and repress gene transcription in the appropriate cellular and genetic context.

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Introduction

Lineage-specific gene expression patterns are established by the combinatorial action of transcription factors and their

co-factors. Maturation of a given cell lineage requires the activation of gene expression and the simultaneous repression of genes that mark earlier stages of development or alternative cell fates. GATA-1 is a haematopoietic zinc-finger transcription factor that activates all known erythroid and megakaryocyte (MK)-specific genes (Crispino, 2005; Ferreira *et al*, 2005). In addition to activating gene expression, GATA-1 can function as transcriptional repressor (Welch *et al*, 2004). Genes directly repressed by GATA-1 include *Gata2* (Grass *et al*, 2003), *Kit* (Munugalavadla *et al*, 2005; Jing *et al*, 2008) and *Myc* (Rylski *et al*, 2003).

The regulation of most, but not all, GATA-1-dependent genes requires binding to FOG-1 (ZFPM1), a multi-type zinc-finger protein whose expression in the haematopoietic system is restricted largely to erythroid cells and MKs (Tsang *et al*, 1997). FOG-1 contains nine zinc fingers, four of which are capable of binding GATA-1. The GATA-1/FOG-1 interaction is direct and requires the N-terminal zinc finger of GATA-1, and at least one of the four GATA-1-binding zinc fingers of FOG-1 (Tsang *et al*, 1997; Fox *et al*, 1999). Point mutations in GATA-1 that disrupt FOG-1 binding impair erythroid and MK development in engineered mice and human patients, establishing the importance of this interaction *in vivo* (Crispino *et al*, 1999; Nichols *et al*, 2000).

Chromatin immunoprecipitation (ChIP) experiments revealed that FOG-1 colocalizes with GATA-1 at genes in which GATA-1 functions as an activator and repressor (Wang *et al*, 2002; Letting *et al*, 2004; Pal *et al*, 2004; Jing *et al*, 2008). How FOG-1 cooperates with GATA-1 during activation and repression of transcription is unclear. Biochemical approaches to address this question revealed interactions of FOG-1 with several protein complexes that modulate GATA-1/FOG-1 activity, including TACC3, CtBP2 and NuRD (Fox *et al*, 1999; Garriga-Canut and Orkin, 2004; Hong *et al*, 2005; Rodriguez *et al*, 2005). TACC3 is capable of competing with GATA-1 for FOG-1 binding and might sequester FOG-1 in the cytoplasm thus inhibiting erythroid maturation (Garriga-Canut and Orkin, 2004). CtBP2 might link FOG-1 to other co-repressor molecules, but it remains uncertain to what extent this interaction contributes to FOG-1 function *in vivo* (Katz *et al*, 2002). Affinity purification of proteins associated with the N-terminal repression domain of FOG-1 identified a high-affinity interaction with NuRD (Hong *et al*, 2005). The composition of the NuRD complex can vary (Bowen *et al*, 2004), but in the context of FOG-1 contains the ATPase Mi-2 β , MTA-1, MTA-2, p66, RbAp46 (RBBP7), RbAp48 (RBBP4), MBD3 and the histone deacetylases HDAC1 and HDAC2 (Hong *et al*, 2005). NuRD bears features of a classical co-repressor complex because of the presence of histone deacetylases and its association with diverse transcriptional repressors (Bowen *et al*, 2004; Denslow and Wade, 2007; Manavathi and Kumar, 2007).

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The interaction between FOG-1 and NuRD is mediated by a small, conserved motif contained within the N-terminal 12 amino acids of FOG-1 that is also present in FOG-2 (Hong *et al*, 2005; Roche *et al*, 2008). Other molecules known to repress transcription, including Bcl11A, Bcl11B, SALL1-4, EBFAZ and Evi3, bear an identical motif at their N-termini (for reference see Hong *et al*, 2005). For FOG and SALL molecules, this motif has been shown to be necessary and sufficient for NuRD binding, and point mutations that disrupt this interaction impair transcriptional repression in transfection-based assays (Lin *et al*, 2004; Hong *et al*, 2005; Lauberth and Rauchman, 2006; Roche *et al*, 2008). Truncated forms of SALL1 found in patients with Townes–Brock syndrome, a dominantly inherited developmental disorder characterized by multiple birth defects, maintain the minimal repression motif suggesting that the disease is caused by interference with the normal SALL1/NuRD interaction (Kiefer *et al*, 2003). Moreover, the zinc-finger transcriptional co-repressor ZFX1B bears a similar motif near its N-terminus that also mediates NuRD binding (Verstappen *et al*, 2008). Mutations or deletions affecting this region are associated with rare forms of Mowat–Wilson syndrome, a disorder associated with mental retardation (Zweier *et al*, 2006; Verstappen *et al*, 2008). These examples illustrate the importance of NuRD recruitment by select transcription factors to ensure normal development. So far, no germ line mutations within the NuRD-binding motif of FOG-1 or FOG-2 have been described in human patients.

The association of FOG-1 with NuRD immediately suggested a mechanism by which GATA-1/FOG-1 repress transcription. Indeed, NuRD is detectable *in vivo* at genes that are repressed by GATA-1 in a FOG-1-dependent manner (Hong *et al*, 2005; Rodriguez *et al*, 2005). However, further exploration suggested broader functions for NuRD during haematopoietic gene transcription. Here, we report that NuRD is present not only at repressed, but also active FOG-1-regulated genes in erythroid cells and MKs. Surprisingly, transfection assays using GATA-1/FOG-1-dependent reporter genes showed that NuRD is directly required for transcriptional activation. To assess the *in vivo* function of the GATA-1/FOG-1/NuRD axis during haematopoietic development, we generated mice with a triple point mutation in FOG-1 that abrogates NuRD binding.

Homozygous mutant animals revealed defects in the erythroid and MK lineages, consistent with a function for NuRD in most of FOG-1 biology. Alterations in gene expression in mutant animals included the failure to activate and repress select GATA-1/FOG-1-regulated genes. The dual function of NuRD during transcriptional activation and repression suggests that the classification of NuRD as co-repressor might not do justice to its versatile function in gene expression.

Results and discussion

NuRD broadly occupies active and repressed GATA-1/FOG-1 target genes

Earlier ChIP experiments that were performed in cells expressing a conditional form of GATA-1 suggested that the Mi-2 β subunit of NuRD is recruited to select sites at the *Gata2* and *Kit* genes upon their repression by GATA-1, consistent with NuRD serving as a GATA-1/FOG-1 co-repressor (Hong *et al*,

2005; Rodriguez *et al*, 2005). Here, we sought to extend these findings by (1) examining a total of three subunits of NuRD that would more reliably reflect the presence of the NuRD complex, (2) analysing multiple regulatory regions of the *Gata2* and *Kit* genes through which GATA-1 represses their expression, (3) studying additional genes that are repressed by GATA-1 and (4) investigating genes that are directly activated by GATA-1.

ChIP experiments were carried out in the erythroid cell lines G1E and G1E-ER4. G1E-ER4 cells were derived from the GATA-1-deficient cell line G1E that lacks an intact GATA-1 gene and is developmentally arrested at the proerythroblast stage (Weiss *et al*, 1997). G1E-ER4 cells contain a stably integrated form of GATA-1 fused to the ligand-binding domain of the oestrogen receptor (GATA-1-ER). Treatment with oestradiol leads to activation of GATA-1 followed by erythroid maturation along with the activation and repression of GATA-1 target genes (Weiss *et al*, 1997). This system faithfully recapitulates erythroid maturation and has been invaluable for numerous studies of erythroid biology and transcriptional regulation.

We began by examining FOG-1 and NuRD occupancy across ~ 100 kilobases (kb) of the *Kit* gene that is expressed in immature erythroid cells driven in part by transcription factor GATA-2. During terminal erythroid maturation, GATA-1 activation leads to loss of GATA-2 binding and repression of *Kit* in a FOG-1-dependent manner (Jing *et al*, 2008). We and others earlier identified sites of high GATA-1 occupancy at multiple positions including -114.5 , $+4.7$, $+58.1$ and $+72.8$ kb with respect to the transcription start site (TSS) (Munugalavada *et al*, 2005; Jing *et al*, 2008). Thus, the study of the *Kit* locus under dynamic conditions is well suited to examine possible spatial and temporal correlations between FOG-1 and NuRD occupancy. In parental G1E cells lacking GATA-1, FOG-1 was detected at $+72.8$ kb and at low levels at additional sites of the locus (Figure 1A). GATA-1 and FOG-1 were near background levels at control regions -224.9 kb and the silent CD4 gene. Low levels of FOG-1 might reflect the presence of GATA-2 that also binds FOG-1 (Figure 1A) (Jing *et al*, 2008). In G1E-ER4 cells treated with oestradiol, FOG-1 levels remained high at $+72.8$ kb and increased at sites of high GATA-1 occupancy (Figure 1A). When we examined the distribution along the *Kit* locus of two NuRD components, MTA-2 and RbAp46, three findings were especially noteworthy. First, the levels of MTA-2 and RbAp46 closely correlated with each other and tended to be high at sites with high FOG-1 occupancy (Figure 1A). Second, both proteins showed occupancy significantly above background throughout the *Kit* locus including sites with little or no GATA-1/FOG-1 binding (Figure 1A). This might result from spreading along the chromatin fibre and is consistent with the ability of NuRD to associate with chromatin in a non-targeted manner (Li *et al*, 2002). Third, surprisingly, both MTA-2 and RbAp46 were detected at the active *Kit* locus before its repression by GATA-1, but not at control regions (Figure 1A). To examine whether NuRD occupies other genes repressed by GATA-1, we chose the *Gata2* gene that contains several known GATA-1-binding sites (-77 , -3.9 , -2.8 and -1.8 kb with respect to the TSS) (Grass *et al*, 2003, 2006; Martowicz *et al*, 2005), and the *Myc* and *Lyl1* genes (Rylski *et al*, 2003; Johnson *et al*, 2007). Notably, at all three genes, MTA-2 and RbAp46 occupancy was high before repression and changed very little on

GATA-1-induced silencing (Figure 1B and C), mirroring our observations at the *Kit* locus.

These results indicated that the presence *per se* of NuRD is not prohibitory to active transcription, and prompted us to explore whether NuRD also occupies genes that are directly activated by GATA-1. Indeed, at three sites of the β -globin

locus in which GATA-1 occupancy is high, including DNase 1 hypersensitive sites (HS) 2 and 3 of the locus control region and the *Hbb-b1* promoter (Horak *et al*, 2002; Johnson *et al*, 2002; Letting *et al*, 2003; Im *et al*, 2005), significant levels of MTA-2 and RbAp46 were observed before activation of GATA-1 when compared with the controls at *Kit*-224.9 and

Figure 1 FOG-1 and NuRD proteins occupy active and repressed GATA-1 target genes. ChIP at the repressed GATA-1 target genes *Kit* (A), *Gata2* (B), *Myc* and *Lyl1* (C) using antibodies anti-GATA-1, GATA-2, FOG-1, MTA2, RbAp46 and the corresponding control IgG, in G1E and G1E-ER4 cells after treatment with oestradiol for 24 h. (D) FOG-1 and NuRD ChIP at sites in which GATA-1 functions as an activator. HS2, DNase hypersensitive sites 2. The regions at 224.9 kb upstream of the *Kit* promoter and in the first intron of *CD4* serve as negative control. The results are averages of 4–10 independent experiments. Error bars represent s.e.m. Statistical significance was determined by comparing the occupancy at each site and negative control regions; * $P < 0.05$.

Figure 1 Continued.

CD4 (Figure 1D). Surprisingly, NuRD levels remained high or even increased on activation of *Hbb-b1* transcription by GATA-1 (Figure 1D). High levels of NuRD at active genes seems to be a general phenomenon, as similar results were found at the GATA-1-activated target genes *Klf1*, *Band3* (*Slc4a1*) and *Eraf* (Figure 1D).

We next examined the occupancy of Mi-2 β , one of the defining subunits of NuRD. Using two independently derived antibodies, A301-081A and A301-082A, Mi-2 β was found at

both GATA-1-activated and repressed genes in a manner very similar to that of RbAp46 and MTA-2 (Supplementary Figure S1). However, we noticed a discrepancy between these results and those earlier obtained with different Mi-2 β antibodies that had detected strongly inducible increases in Mi-2 β occupancy at positions at the *Gata2* and *Kit* genes (Hong *et al*, 2005; Rodriguez *et al*, 2005). To resolve this issue, we introduced HA-tagged Mi-2 β into G1E-ER4 cells through retrovirus. ChIP analysis with HA antibodies showed

that the spatial distribution of HA-Mi-2 β across the *Kit* locus closely resembled that of RbAp46 and MTA-2 (Supplementary Figure S2A). We also observed a modest, but reproducible, two-fold increase of HA-Mi-2 β near the +4.7 kb region of *Kit* and -2.8 kb of *Gata2* in addition to similar increases at several other sites (Supplementary Figure S2A, B and C). In addition, at HS2, HS3, *Hbb-b1*, *Klf1*, *Eraf* and *Band3* (*Slc4a1*), HA-Mi-2 β was present in the absence of GATA-1 and slightly increased further following GATA-1 activation (Supplementary Figure S2D).

Two observations deserve comments: first, HA-Mi-2 β displayed a more general trend towards increased occupancy on GATA-1 activation than RbAp46 and MTA-2. This might be a result of over-expression or of variation in the populations of the cells analysed. Second, at some sites, such as the *Hbb-b1* promoter, changes in FOG-1 occupancy were more pronounced than those of NuRD proteins. This might be due to changes associated with transcriptional activation that either influence NuRD recruitment and/or epitope recognition, or to posttranslational modifications that regulate the FOG-1/NuRD interaction similar to what has been observed with SALL1 (Lauberth *et al*, 2007). Nevertheless, together, the above results indicate that at least three components of the NuRD complex are present at GATA-1/FOG-1-activated and repressed genes, and that occupancy of NuRD can be further augmented on gene activation. Therefore, a simple recruitment mechanism does not account for the distinction between activated versus repressed GATA-1/FOG-1 target genes.

We next examined whether NuRD occupies GATA-1/FOG-1-activated genes in cultured primary foetal liver-derived MKs. We found that MTA-2 and RbAp46 were enriched at all active genes examined, including α *IIb* (*Itga2b*), *Mpl*, *Gp9*, *Gp1b α* and *Pf4* when compared with a control region or the silent erythroid GATA-1/FOG-1 target gene *Hbb-b1* (Supplementary Figure S3). This indicates that NuRD recruitment to active GATA-1/FOG-1-regulated genes is not limited to erythroid cells, but is a general phenomenon.

NuRD as transcriptional co-activator

The presence of the Mi-2 β component of NuRD has been observed at the actively transcribed CD4 locus, and loss of Mi-2 β in gene-targeted mice resulted in reduced CD4 expression, suggesting that NuRD functions as an activator or anti-silencer at this gene (Williams *et al*, 2004; Naito *et al*, 2007). However, it remained unresolved whether Mi-2 β acted in the context of the entire NuRD complex. Moreover, inferences as to a direct function of Mi-2 β in transcription activation are complicated by the presence of increased levels of the related protein Mi-2 α in mutant mice, and the possibility that changes in gene expression are an indirect consequence of Mi-2 β loss (Williams *et al*, 2004). Similarly, Mi-2 α was shown to activate transcription by MYB, but it was suggested that Mi-2 α might function in the absence of other NuRD components in this context (Saether *et al*, 2007).

To examine a direct requirement for NuRD during transcriptional activation, we made use of an assay that measures transcriptional activation by FOG-1. Specifically, we and others have shown that FOG-1 augments the transcriptional activity of GATA-1 in reporter assays incorporating the MK-specific promoter of the α *IIb* gene that bears adjacent GATA and ETS elements (Gaines *et al*, 2000; Wang *et al*, 2002; Pang *et al*, 2006). Consistent with earlier results,

FOG-1 increased the activity of GATA-1 when co-expressed with an α *IIb*-luciferase reporter gene in 3T3 fibroblasts (Figure 2A). Deletion of 45 amino acids comprising the NuRD-binding domain (Δ 45) of FOG-1 strongly impaired activation by FOG-1 (Figure 2A). Moreover, a triple point mutation (R3G,R4G,K5A) that abolishes NuRD binding (Supplementary Figure S4) had the same effect as the deletion (Figure 2A). One concern was that the point mutations might compromise binding to unknown molecules that function as co-activators. Although numerous attempts failed to detect any non-NuRD polypeptides that interact with this domain of FOG-1, we measured FOG-1 activity under conditions in which key NuRD proteins were depleted. We chose the MTA proteins, as they bind to the repression domain at the N-terminus of FOG-1 (Hong *et al*, 2005). Twenty-four hours before transfection with the reporter construct, 3T3 cells were exposed to a mixture of siRNAs directed against MTA-1, MTA-2 and MTA-3 as described earlier (Roche *et al*, 2008) or with two different control siRNAs. Knockdown of >90% of control was achieved for MTA-1 and MTA-2 as determined by western blotting (Figure 2B). MTA-3 is not detectable by western blotting in 3T3 cells (Roche *et al*, 2008). Generally, mock transfection or transfection with two distinct control siRNAs lead to a slight reduction in overall reporter activity when compared with untreated cells (Figure 2C). However, loss of MTA proteins specifically and completely abrogated activation by FOG-1 (Figure 2C). Importantly, the FOG-1-independent activation by GATA-1 was not significantly affected. A second set of siRNAs against MTA-1, MTA-2 and MTA-3 produced a less pronounced, but consistent effect (Supplementary Figure S5). To further rule out non-specific effects of MTA knockdowns on high-level transcription, we examined the transcriptional activator GAL4-VP16 in the context of a reporter gene bearing 5 GAL4-binding sites. Knockdown of MTA proteins did not significantly impair GAL4-VP16-mediated activation (Figure 2D), further supporting a specific function for NuRD during FOG-1-dependent transcriptional activation.

Together, these results show a direct requirement of NuRD for transcriptional activation. Moreover, as the N-terminus of FOG-1 binds stoichiometrically and with high affinity to the entire NuRD complex *in vitro* and *in vivo* (Hong *et al*, 2005), this suggests that the complex as a whole and not just a subset of NuRD proteins is associated with active transcription. The consistent presence of NuRD at both GATA-1/FOG-1-activated and repressed genes *in vivo* together with the requirement for the FOG-1/NuRD interaction for both transcriptional activation (Figure 2) and repression (Hong *et al*, 2005) in transfection assays suggests that NuRD can function distinctly depending on gene context. Whether this context determines the specific activity of NuRD or affects the recruitment or loss of additional components are questions that need to be addressed in future studies.

Methylation of lysine 4 of histone H3 (H3K4) leads to loss of NuRD binding to the N-terminal tail of histone H3 *in vitro* (Nishioka *et al*, 2002; Zegerman *et al*, 2002) providing a possible mechanism by which NuRD is displaced from active chromatin. However, our ChIP studies indicate that NuRD is not displaced from active genes. It is possible that instead of displacing NuRD, H3K4 methylation interferes with the way NuRD engages chromatin to deacetylate histones. Another possibility is that modification of FOG-1 alters the way it

Figure 2 NuRD binding is required for transcriptional activation by FOG-1. Transient transfection of 3T3 cells with constructs expressing GATA-1, FOG-1 and a luciferase reporter gene driven by the α 1b promoter. FOG-1 mutants bearing triple point mutations (tri-mut) or a deletion of the NuRD-binding domain (Δ 45) fail to substantially activate GATA-1-dependent transcription (**A**). Bars denote averages of five independent experiments. Error bars represent s.e.m. * $P < 0.05$; NS: not significant. (**B**) Western blots of cells co-transfected with siRNAs against MTA-1 and MTA-2. NS, non-specific bands. (**C**) Transient transfections as in (**A**), but 24 h before transfection with GATA-1, FOG-1 and the reporter gene, cells were additionally transfected with MTA-1, MTA-2 and MTA-3 siRNAs, no siRNAs (mock), or two different control siRNAs (ctr1 and ctr2). Note that the exposure to an additional round of transfection leads to non-specific reduction in GATA-1/FOG-1 activity. The results are averages of 3–12 independent experiments. Error bars represent s.e.m. * $P < 0.05$; NS: not significant. (**D**) 3T3 cells were treated with control siRNA (siRNA ctr2) or siRNA against MTA-1 and MTA-2 (siRNA/MTA) as in (**C**); 24 h later, GAL4-VP16 or GAL4 alone were co-transfected with a reporter gene driven by the thymidine kinase promoter and containing 5 GAL4-binding sites; $n = 3$ (error bars denote s.e.m.; NS: not significant).

interacts with NuRD at active versus repressed genes. The transcriptional repressor SALL1 binds to NuRD through an N-terminal motif that is identical to that of FOG-1 (Lauberth and Rauchman, 2006). Phosphorylation of serine 2 leads to disruption of this interaction *in vitro* (Lauberth *et al.*, 2007). Whether FOG-1 is phosphorylated or modified in any other way to regulate NuRD binding remains unknown, but deserves further investigation. Finally, it has become clear that the presence of HDACs *per se* does not necessarily specify transcriptional repression. In addition to histones, HDACs deacetylate nuclear factors and can influence transcription positively and negatively (Smith, 2008).

Disruption of the FOG-1/NuRD interaction in mice

We showed earlier that the K5A substitution in FOG-1 impaired NuRD binding and transcriptional repression, but did not completely abrogate it (Hong *et al.*, 2005). Therefore, we also altered amino-acids R3 and R4 that contribute to the NuRD interaction. To verify that the triple mutation disrupts NuRD binding, we generated GST fusion proteins containing amino-acids 1–45 of FOG-1 and single (K5A) and triple (R3G, R4G, K5A) amino-acid substitutions. When exposed to *in vitro* translated MTA-1, MTA-2, RbAp48 or RbAp46,

wild-type GST-FOG-1–45 associated efficiently with all of these proteins (Supplementary Figure S4A). The K5A substitution greatly diminished these associations, but residual binding was observed. However, the triple substitution completely abolished association with NuRD components (Supplementary Figure S4A). Correspondingly, when the N-terminus of FOG-1 was fused to the DNA-binding domain of GAL4, the single K5A mutation led to a significant loss of transcriptional repression towards a GAL4-regulated reporter gene in transfected cells. However, the triple mutation essentially abolished all of transcriptional repression (Supplementary Figure S5B). Finally, the triple mutation also abrogated the FOG-1/NuRD interaction in the context of full-length FOG-1 without diminishing FOG-1 protein levels (not shown).

To determine the function of the FOG-1/NuRD axis on GATA-1-regulated haematopoietic lineages *in vivo* and to evaluate the dual function of NuRD in gene expression under conditions in which FOG-1 is expressed at physiological levels, we engineered mice in which the FOG-1/NuRD interaction is disrupted. We used recombineering (Copeland *et al.*, 2001) to generate a targeting vector to 'knock-in' (ki) the triple mutation into the *Fog-1* gene (*Zfpml1*; for details see

Supplementary Figures S6A and S5B–D). Mice heterozygous for the *ki* mutations were bred to homozygosity and studied as outlined below. Generally, the triple point mutation did not significantly change FOG-1 protein levels in erythroid cells or MKs (Supplementary Figure S6E).

Defective erythropoiesis in homozygous mutant animals

Among 293 live born offspring from heterozygous crosses, 97 (33.1%) were wild type (+/+), 149 (50.9%) +/ki and only 47 (16%) ki/ki, consistent with embryonic or perinatal mortality of ki/ki mice (Supplementary Table S1). As the frequency of homozygous embryos was close to normal at E14.5, the predominant loss of ki/ki animals likely occurred between E14.5 and birth (Supplementary Table S1). The ki/ki animals that survived past their birth seemed normal with regard to appearance, weight and activity, and were fertile. The exact time points and cause(s) of death need to be investigated further, but might result from a combination of haematopoietic and cardiac defects. Nevertheless, these results show that the FOG-1/NuRD interaction is important for normal murine development.

When 6–7-week-old mice were examined, marked spleno-megaly was evident in all ki/ki animals with spleens weighing more than three times that of +/+ or +/ki littermates (Figure 3A and B), consistent with extramedullary haematopoiesis. Other organs, including heart and liver, were grossly normal in morphology and weight (Figure 3B). A modest increase in heart weight in ki/ki animals might be secondary to their anaemia (see below). Peripheral blood analysis of ki/ki mice revealed signs of a mild compensated anaemia as reflected in increased reticulocyte counts, red cell distribution width and haemoglobin distribution width, but no microcytosis (normal mean corpuscular volume). Other erythroid indices were within the normal range, including red blood cell count, haemoglobin concentration and haematocrit (Figure 3C). Smears of peripheral blood were generally unremarkable with regard to erythroid cells (Figure 3D). We also observed a two-fold increase in neutrophil granulocytes and monocytes in ki/ki animals, whereas the remaining white cell parameters were normal (Supplementary Table S2). The effects of the FOG-1 mutation on the myeloid lineages will be explored in future studies.

Histological sections of ki/ki bone marrow appeared normal with regard to overall cellularity (Supplementary

Figure 3 (A) Spleens from 6-week-old mice with indicated genotype. (B) Organ weights plotted as per cent of total body weight ($n=4$). (C) Red blood cell indices of wild-type (+/+, $n=15$), heterozygous (+/ki, $n=11$) and homozygous mutant (ki/ki, $n=10$) animals. Error bars represent s.e.m. P -values were determined by two-tailed t -tests. * $P<0.001$. (D) Giemsa–Wright-stained peripheral blood smears from +/+ and ki/ki mice. Note scarcity of normal sized platelets and the presence of enlarged platelets.

Figure 4 Erythroid defects in *ki/ki* erythroid cells from bone marrows (A, C, E, G) and spleens (B, D, F, H). (A, B) Representative flow cytometric analysis using CD71 and Ter119 antibodies. R1, R2, R3 and R4 represent proerythroblasts, basophilic, polychromatic and orthochromatic erythroblasts, respectively (Socolovsky *et al*, 2001) and were plotted as per cent of total (C, D) or per cent of Ter119+ cells (E, F). Error bars represent s.e.m. *n* = 7 (C, D) *n* = 9 (D, F). **P* < 0.05. (G, H) Colony assays measuring BFU-E and CFU-E from bone marrows and spleens. The results were plotted as fold changed comparing +/+ with *ki/ki*-derived samples. *N*-values are indicated for each graph. **P* < 0.05.

Figure S6). We next examined the maturation of erythroid cells in the bone marrow by determining the Ter119/CD71 profile (Socolovsky *et al*, 2001) from 6-week-old mice. The *ki/ki* mice displayed a significant reduction in the total number of Ter119+ cells indicative of impaired erythropoiesis (Figure 4A). Moreover, the levels of Ter119 per cell were reduced ~two-fold. However, the maturation from basophilic erythroblasts to orthochromatic erythroblasts was not significantly impaired (Figure 4A, C and E). When cultured under the appropriate conditions, the *ki/ki* bone marrow contained fewer erythroid progenitors as reflected in an ~50% reduc-

tion in BFU-E and CFU-E colonies, whereas myeloid progenitors were normal in number (Figure 4G).

The *ki/ki* spleens displayed a marked increase in the number of haematopoietic cells, including that the erythroid (Ter119+) compartment (Figure 4D) commensurate with the increase in spleen size, and a relative decrease of the white pulp (Supplementary Figure S6). The *ki/+* spleens were normal (not shown). The severity of the phenotype varied significantly between *ki/ki* animals. Among the Ter119+ cells, the proerythroblast (R1), basophilic (R2) and polychromatic (R3) compartments were markedly expanded in

ki/ki spleens when compared with wild type (Figure 4D and F). The ki/ki erythroid cells largely maintained their ability to progress normally throughout later stages of maturation, although the progression from basophilic to orthochromatic erythroblasts appeared to be somewhat impaired (Figure 4B and D). The number of BFU-E, CFU-E and myeloid progenitors in ki/ki spleens was dramatically increased consistent with splenic compensation (Figure 4H).

The analysis of ki/ki animals described above was performed between 6 and 8 weeks of age and their phenotypes were consistent. Interestingly, in a subset of animals aged over 1 year, the anaemia and splenomegaly worsened progressively. In these cases, the anaemia was accompanied by peripheral reticulocytosis (Supplementary Figure S8A, B) and a pronounced expansion of the precursor populations (R1 + R2) relative to the mature cells in the bone marrow and spleens (Supplementary Figure S8C) and increased cell death (Supplementary Figure S8D) consistent with ineffective erythropoiesis. Although the causes underlying this age-related deterioration are unclear, the variation in penetrance among siblings is likely because of differences in their genetic background.

To investigate whether erythroid defects occur at earlier stages of development, we examined erythropoiesis in foetal livers. Notably, the total number of cells contained in ki/ki E12.5 foetal livers was reduced by 50% when compared with wild-type counterparts (Figure 5A). Moreover, ki/ki foetal liver erythroid cells displayed a delay in maturation as determined by Ter119/CD71 expression (Figure 5B). To examine the cause for the reduction in erythroid cell numbers, we measured cell cycle progression by propidium iodide staining and flow cytometry in erythroid populations at the proerythroblast and basophilic stages of maturation (Supplementary Figure S9A). A reduction in the fraction of cells in S/G2/M-phases was detected in both populations, indicating a significant delay in cell cycle progression in ki/ki erythroblasts (Supplementary Figure S9A). The fractions of apoptotic and dead cells were determined by staining the cells with annexinV and by measuring uptake of 7-amino-actinomycin D (7-AAD). The percentages of both apoptotic cells (AnnexinV⁺ 7-AAD⁻ cells) and dead cells (7-AAD⁺ cells) were significantly increased in ki/ki foetal liver (Supplementary Figure S9B, C). The combination of the delay in cell cycle progression and decreased cell viability explains the reduced cell numbers found at E12.5. Together with the delay in erythroid maturation, this accounts for the peripheral anaemia in mutant embryos at this stage of development (not shown).

At E14.5, erythroid maturation, cell cycle progression and cell viability improved approximating those of normal littermates (Figure 5A and data not shown). Thus, the most severe erythroid defects occur transiently and early in development, but can surface again in aged specimens.

Defective megakaryopoiesis in homozygous mutant animals

The most prominent change found on peripheral blood smears was a reduction in platelet numbers with the occasional presence of dramatically enlarged platelets in ki/ki animals (Figure 4D), consistent with macrothrombocytopenia. Flow cytometry showed that the number of blood platelets was reduced to ~11% of that found in +/+ or ki/+

animals (Figure 6A). Ultrastructural analysis revealed heterogeneity among ki/ki platelets (Figure 6B). Strikingly, the majority of platelets exhibited a paucity of α -granules and increased numbers of lysosomal-like vacuoles (Figure 6B and data not shown) resembling those found in mice and human patients with macrothrombocytopenia owing to mutations in GATA-1 that impair binding to FOG-1 (Nichols *et al*, 2000; Freson *et al*, 2001; Chang *et al*, 2002). Despite defects in platelet numbers and appearance, the ki/ki animals had no overt bleeding diathesis indicating the presence of residual platelet function.

In histological sections, the number of MKs in the ki/ki bone marrows appeared to be in the normal range with cells displaying an apparent decrease in cytoplasmic to nuclear ratio when compared with +/+ MKs (Supplementary Figure S6). As another measure of the maturation of MKs in the spleen and bone marrow, we determined their ploidy using propidium iodide staining. Surprisingly, we found virtually normal degrees of ploidy in ki/ki MKs, suggesting that the FOG-1/NuRD interaction is dispensable for endoreduplication (Supplementary Figure S10). When cultured *in vitro* in the presence of thrombopoietin, ki/ki bone marrows were unable to produce large acetyl-cholinesterase positive MK colonies (Figure 6C). However, the number of MK occurring in two-cell colonies or as single cells was increased. Thus, *in vitro* conditions uncovered a requirement for the FOG-1/NuRD interaction in the proliferative capacity of maturing MKs. This suggests that *in vivo* a compensatory mechanism might exist, perhaps triggered by the decreased platelet count, that leads to normal numbers of MKs.

Together, these results show an essential function of the FOG-1–NuRD interaction for normal MK development. Mice that are null for FOG-1 displayed a more dramatic phenotype with complete failure to produce any MKs (Tsang *et al*, 1998), indicating that FOG-1 performs functions in addition to interacting with NuRD.

Deregulation of gene expression in mutant erythroid cells and MKs

To examine the underlying causes for the defective erythroid development in ki/ki mice, we measured the expression of key erythroid GATA-1/FOG-1 target genes in stage-matched erythroid cells. Total mRNA was purified from splenic TER119⁺, CD71⁺ erythroid precursors from wild-type, +/ki and ki/ki littermates. Transcript levels were analysed by real-time RT-PCR and normalized to GAPDH. First, we examined the GATA-1/FOG-1-activated genes β -globin (*Hbb-b1*), *Eraf* (AHSP), *Band3* (*Slc4a1*), *Klf1*, *Tal1*, *Fog-1* (*Zfpml1*) and *Gata1*. We found their expression to be essentially unchanged (Figure 7), although *Hbb-b1* levels showed a modest trend towards reduced expression in the ki/ki erythroid cells. This suggests that the FOG-1/NuRD interaction is dispensable for the activity of most activated GATA-1/FOG-1 target genes, consistent with the relatively normal maturation of splenic ki/ki erythroid cells. When Ter119⁺, CD71⁺ cells from bone marrow were examined, levels of *Hbb-b1* and *Hba-a1* were significantly reduced, whereas the remaining genes were expressed normally (Supplementary Figure S10). Of note, as *Hbb-b1* and *Hba-a1* are GATA-1/FOG-1 target genes, this implies a direct function of NuRD for their activation.

We next turned our attention to genes that are directly repressed by GATA-1 in a FOG-1-dependent (*Kit*, *myc*, *Gata2*)

Figure 5 Hypocellularity (A) and erythroid maturation defects in *ki/ki* erythroid cells from E12.5 foetal livers as revealed by flow cytometric analysis using CD71 and Ter119 antibodies (B). R1, R2, R3 and R4 represent immature erythroblasts, proerythroblasts, basophilic and polychromatic/orthochromatic erythroblasts, respectively (Zhang *et al*, 2003) and were plotted as per cent of total cells. (C) R1, R2 and R3, populations from (B), were analysed by flow cytometry for surface expression of Kit. Note residual expression of kit in R2 to R4 populations, which might reflect a delay in kit silencing. Error bars represent s.e.m. $n = 4$ E12.5 litters and $n = 3$ E14.5 litters. * $P < 0.05$. ** $P < 0.005$.

and -independent (*Lyl1*) manner. Strikingly, splenic *ki/ki* erythroid cells displayed a ~75-fold increase in *Gata2* expression, whereas the other genes were expressed at normal or near-normal levels (Figure 7). Likewise, in bone marrow erythroblasts (Supplementary Figure S11) and in E12.5 and E14.5 foetal liver erythroblasts (not shown), *Gata2* expression was substantially elevated with normal or

close to normal levels of *Kit* and *Lyl*. *Gata2* is normally silenced as erythroid cells mature and accumulate GATA-1 (Weiss *et al*, 1994). Our results indicate that NuRD is required for the normal silencing of *Gata2*, but not of all repressed GATA-1 targets.

This gene-selective requirement of NuRD cannot be explained by differences in NuRD recruitment, as the

Figure 6 (A) Platelet counts of wild-type (+/+, $n = 15$), heterozygous (+/ki, $n = 11$) and homozygous mutant (ki/ki, $n = 10$) animals. Error bars represent s.e.m. * $P < 0.001$; ** $P < 0.05$. (B) Electron micrographs of +/+ and ki/ki platelets. (C) Reduced MK potential in ki/ki mice as revealed by colony assays from bone marrows. ki/ki bone marrows produced markedly fewer CFU-Mk colonies in but increased numbers of single MKs. Right panel: acetyl-cholinesterase (AChE) stain of representative colonies.

occupancy of NuRD proteins was comparable at NuRD-dependent and -independent genes (Figure 1). NuRD-independent genes might have compensatory mechanisms in place that can substitute for NuRD during transcriptional repression. It is also possible that the subunit composition of NuRD varies between genes in a way that influences the activity of the complex. Finally, as the mere presence of NuRD does not reflect the activity of a gene, it is possible that gene-specific transcription factors modulate the catalytic activity of NuRD. The stimulation of histone acetyltransferase activity by nuclear factors has been observed earlier (e.g. Chen *et al*, 2001). In this manner, FOG-1 or neighbouring molecules might alter the specific activities of the HDACs or the ATPase of Mi-2 β . For example, SCL and its associated proteins have been recently shown to be present at activating, but not repressive GATA elements (Tripic *et al*, 2009). Moreover, in the context of MK-specific genes, select Ets proteins such as Fli1 specify transcriptional activation by FOG-1 (Wang *et al*, 2002). It is conceivable that co-operating transcription factors such as SCL and Fli1 modulate NuRD in a way to promote positive transcriptional outcomes. Perhaps, similar to our results, although the SWI/SNF complex can bind to DNA and chromatin without sequence specificity, a

transcription activation domain contributes to targeting and stimulation of the catalytic activity of this complex to trigger nucleosome remodelling (Gutierrez *et al*, 2007).

Using a gene complementation assay, we earlier observed a delay in *Kit* silencing in erythroid cells expressing mutant FOG-1 (Hong *et al*, 2005). To examine whether this is the case in the ki/ki mice, *Kit* expression was measured by flow cytometry at maturation stage-matched erythroid cells from foetal livers. Interestingly, we observed a delay in silencing of *Kit* expression in ki/ki E12.5 foetal liver erythroblasts with substantially elevated fractions of cells in the R3 and R4 populations expressing *Kit* when compared with +/+ controls (Figure 5C). Elevated *Kit* expression was also observed in the R3 population by RT-PCR (not shown). Whether the delay in *Kit* repression is a direct consequence of failed FOG-1 function or an indirect consequence of the maturation delay remains uncertain. It is also possible that elevated GATA-2 levels in ki/ki erythroblasts contribute to sustained *Kit* expression (Jing *et al*, 2008). Forced *Kit* expression has been shown to antagonize GATA-1-induced cell cycle arrest in G1E cells (Munugalavada *et al*, 2005). Therefore, additional mechanisms need to be invoked to explain the slowing of the cell cycle progression in ki/ki foetal liver erythroid cells.

GATA-1 and GATA-2 contribute to the expression of most if not all MK-specific genes. The function of FOG-1 during megakaryopoiesis is entirely dependent on GATA factors (Chang *et al*, 2002). To examine a requirement for FOG-1/NuRD during MK gene expression, we cultured primary MKs from E13.5 foetal livers under serum-free conditions in the presence of thrombopoietin for 5 days and prepared total RNA. Real-time RT-PCR was used to measure the expression of the MK-specific genes α IIb, *Mpl*, *Gp9*, *GP1b α* , *Pf4* and *Gp6*. All of the genes analysed were reduced in ki/ki mice when compared with +/ki or +/+ counterparts. However, the extent of expression was affected differently. For example, *Mpl* expression was only reduced by ~40% in ki/ki MKs, whereas *Gp6* expression was reduced by almost six-fold (Figure 7C). Thus, there are differences in NuRD requirement among distinct genes. In contrast to mature erythroid cells, Gata2 expression is maintained throughout megakaryopoiesis. Yet, ki/ki MKs displayed increased levels of *Gata2*, suggesting that GATA-1 limits the expression of this gene through FOG-1 and NuRD. Culture of MKs under serum-free conditions for 5 days does not completely eliminate the presence of other haematopoietic cells. However, the contribution by such cells was comparable between cultures from +/+, +/ki and ki/ki mice (not shown), and hence does not explain the differences in gene expression. In addition, contribution by non-MKs does not account for the different degrees of reduced gene expression.

These results show that NuRD is required *in vivo* for the activation of MK-specific gene expression by FOG-1 through GATA-1 and likely also GATA-2. These findings are consistent with our experiments showing that NuRD is directly required for transcriptional activation in reporter assays using a promoter of a MK-expressed gene (Figure 2) and the presence of NuRD proteins at active megakaryocytic genes (Supplementary Figure S3). Thus, the classification of NuRD as co-repressor does not account for all of its functions during gene expression.

In concert, the work presented here reveals that NuRD has a function in several key functions of FOG-1 in the erythroid

Figure 7 Expression of GATA-1-activated (A) and repressed genes (B) in stage-matched (R3) +/+, +/- and ki/ki erythroid cells as determined by real-time RT-PCR, normalized to GAPDH and plotted as fold change from +/+ samples. (C) mRNA levels of indicated genes in MKs cultured under serum-free conditions from E13.5 MKs determined by qRT-PCR. The results are averages of seven independent experiments. Error bars represent s.e.m. * $P < 0.05$.

and MK lineages. In addition to its function in promoting the development of these two lineages, FOG-1 is also involved in restricting the formation of the GATA-1-dependent mast cell and eosinophil lineages in which FOG-1 is normally down-regulated. In fact, forced expression of FOG-1 in either cell type can block their differentiation (Querfurth *et al*, 2000; Cantor *et al*, 2008). Our preliminary work suggests that suppression of mast cell differentiation by FOG-1 requires NuRD as several mast cell-specific genes are deregulated not only in haematopoietic precursor cells, but also in mature erythroid cells and MKs from ki/ki mice (Gregory *et al*, in preparation). Moreover, colony assays revealed that MK-erythroid precursors have the potential to form mast cells (Gregory *et al*, in preparation). As up-regulation of *Gata2* is the most salient difference in gene expression between +/+ and ki/ki haematopoietic cells, it will be interesting to determine the relative contributions of GATA-2 over-expres-

sion versus direct effects of the FOG-1 mutation on haematopoietic differentiation.

In the context of GATA-4, FOG-1 contributes to normal heart morphogenesis (Katz *et al*, 2003). Our ki/ki mice display defects in this functions as well (unpublished observations). This suggests that NuRD is required for normal FOG-1 function in most if not all FOG-1-dependent tissues. Yet, in all cell types investigated, the phenotypes caused by disrupted NuRD binding are less severe than those associated with complete loss of FOG-1. Given the interaction of FOG-1 with diverse protein partners, many of whom still remain to be discovered, this hardly comes as a surprise. On the other hand, it was unexpected to observe defects in erythroid development in ki/ki mice as expression of a mutant form of FOG-1 lacking the N-terminal 254 amino acids was capable of rescuing erythroid maturation of a FOG-1^{-/-} cell line (Cantor *et al*, 2002). However, the erythroid phenotype in

mutant animals is relatively mild and might be missed in a cell line-based system. Moreover, forced expression of proteins can obscure subtle defects in their functions.

A key advantage of our approach to selectively disrupt a specific molecular interaction between a nuclear factor and its co-regulator is that it allows examining this interaction *in vivo* under conditions of normal protein expression. An alternative approach is to knock out a component of NuRD itself. Indeed, conditional deletion of Mi-2 β produced broad defects in haematopoiesis affecting stem cell homeostasis, and erythroid and myeloid compartments (Yoshida *et al*, 2008). However, interpretation of these findings in the context of erythroid gene regulation is difficult as Mi-2 β -depleted cells can express increased amounts of Mi-2 α (Williams *et al*, 2004). Other unknowns include the intactness of the NuRD complex in the absence of a key subunit (Kaji *et al*, 2006), and the effects, direct or indirect, that result from interactions of Mi-2 β with diverse nuclear factors (Denslow and Wade, 2007; Manavathi and Kumar, 2007; Bowen *et al*, 2004).

The generation of the FOG-1 mutation in mice not only established an essential function of NuRD in the transcriptional circuitry surrounding GATA-1, but yielded an unexpected requirement of NuRD for activation of transcription. The mutant animals will be useful for future studies examining the GATA-1/FOG-1/NuRD axis during various aspects in development involving FOG-1. For example, recent reports implicated GATA-1, FOG-1 and NuRD in the developmental silencing of foetal haemoglobin expression in human beings (Chen *et al*, 2008; Harju-Baker *et al*, 2008). Breeding the human β -globin gene cluster (Gaensler *et al*, 1993) into the FOG-1 mutant mice will allow for testing this model *in vivo*.

Materials and methods

Plasmids

GST-FOG-1(45), GAL4-FOG-1(45) and GAL4-VP16 were generated by PCR and subcloning into pGEX-6P1 (Amersham Biosciences) and pcDNA3-Gal4 (gift from M Poncz), respectively. pcDNA3-GAL4 contains amino-acids 1-147 of GAL4. Related constructs with point mutations were generated with Site-Directed Mutagenesis (Stratagene). pGEM7Zf(+)-rMTA1 and pcDNA3CF-mMTA2 were gifts from W-M Yang (Yao and Yang, 2003). CMV-RbAp48 was a gift from N Barlev. pcDNA3-RbAp46 was generated by RT-PCR from murine RNA and inserted between the BamH1 and EcoR1 sites of pcDNA3 (Invitrogen). Reporter vector G5-TK-luciferase was gift from W-M Yang (Yao and Yang, 2003).

G1E cell culture, transfections, antibodies and ChIP

G1E cells were maintained as described (Weiss *et al*, 1997). Where indicated, cells were treated with 1 μ M oestradiol (21 h). siRNAs were transiently transfected into 3T3 cells with Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). siRNAs directed against MTA-1 (M-047751-00; L-047751-01), MTA-2 (M-046370-00; L-046370-00) and MTA-3 (M-058830-00; L-058830-01) were from Dharmacon, whereas control siRNAs were purchased from Ambion (AM4613). After 24 h, reporter plasmids were transiently transfected into 3T3 cells with Lipofectamine reagent according to the manufacturer's instructions (Invitrogen). Antibodies against MTA-1 (sc-9446), MTA-2 (sc-9447), FOG-1 (sc-9361) and GATA-1 (sc-265) RbAp46 (sc-8272) were from Santa Cruz, CA. Mi-2 β antibodies A301-081A and A301-082A were from Bethyl Laboratories, TX. ChIP assays were performed as described earlier (Letting *et al*, 2003) using real-time PCR with SYBR Green on an ABI Prism 7900 system (PE Applied Biosystems). ChIP primer sequences are listed below. The results were normalized to unprecipitated chromatin (input).

Haematological analysis

Periorbital blood samples were collected in EDTA-containing capillary tubes (catalogue no. 07 6011; RAM Scientific Inc.). A measure of 300 μ l blood was analysed on an Advia 2120 haematology analyzer using the mouse archetype software (Siemens Health Diagnostics Inc., Deerfield, IL). In addition, blood smears were stained with Wright Giemsa Stain.

Isolation and culture and isolation of primary MKs

Foetal livers were obtained from E13.5 embryos and cultured in serum-free medium containing 1% Nutridoma-SP (Roche Applied Science), 2 mM L-glutamine, 100 U/ml penicillin and streptomycin, 50 μ M β -mercaptoethanol and 50 ng/ml thrombopoietin (R&D Systems) for 5 days (Tong *et al*, 2007). Megakaryocytes of various maturation stages were harvested in CATCH buffer (Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS) containing 3.5% bovine serum albumin (BSA), 1 mM adenosine, 2 mM theophylline and 0.38% sodium citrate) by centrifugation at 70 g for 10 min. Cells were stained with May-Grünwald-Giemsa (Sigma Aldrich) and acetyl-choline esterase.

Bone marrow-derived megakaryocytes were isolated from the femurs and tibias and cultured either in the serum-free medium as above or in DMEM medium containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and streptomycin, and 50 ng/ml of Tpo for 5 days. Cells were collected by centrifugation at 37 g (400 rpm) for 10 min, resuspended in 5 ml PBS and spun at 9 g (200 r.p.m.) for 30 min at room temperature over a BSA step gradient containing 1.5% BSA and 3% BSA in PBS (Pang *et al*, 2006).

Flow cytometry

Erythroid cells were analysed using PE-conjugated anti-mouse TER-119, FITC-conjugated anti-mouse CD71 antibodies from BD Biosciences, MD, and APC-conjugated anti-mouse Kit, 7-AAD from eBioscience, CA. Total spleen, bone marrow and foetal liver-derived cells were incubated with anti-CD16/32 antibody (BD Biosciences) and 1% FBS to block non-specific binding to the Fc receptor and stained with the specific antibodies. To detect apoptotic cells, foetal liver cell suspensions were stained with APC-conjugated anti-mouse TER-119, PE-conjugated anti-mouse CD71 antibodies from BD Biosciences and 7-AAD from eBioscience, CA, and subsequently labelled using AnnexinV-FITC Apoptosis Detection Kit (Abcam, UK). For cell cycle analysis, foetal liver cells were labelled with APC-conjugated anti-mouse TER-119 and FITC-conjugated anti-mouse CD71 antibodies (BD Biosciences), permeabilized using BD Cytfix/Cytoperm Fixation/Permeabilization kit (BD Biosciences) and resuspended in PBS 1% serum containing 50 μ g/ml propidium iodide (BD Biosciences). Data were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analysed using FlowJo v8.7.1 (TreeStar, OR).

Colony assays

Bone marrow-derived cells were plated at a density of 1×10^5 cells/ml in methylcellulose medium (M3234; Stem Cell Technologies, BC, Canada) supplemented with 3 U/ml rhEpo (Amgen, CA), 10 ng/ml rmIL6 (Peprotech Inc., NJ), 10 ng/ml rmIL-3 (Invitrogen, CA) and 50 ng/ml rmSCF (Invitrogen). The BFU-E and myeloid colonies were scored by light microscopy 10–14 days after plating. For CFU-E assays, cells were cultured at a density of 2.5×10^4 cells/ml in methylcellulose medium containing 2 U/ml rhEpo. Colonies were scored after 3 days of culture. For the CFU-Mk assay, BM cells were cultured at a density of 1×10^5 cells/ml in MegaCult-C medium in the presence of 20 ng/ml rmIL6, 10 ng/ml rmIL-3 and 50 ng/ml rhTPO, as per manufacturers' instructions (Stem Cell Technologies). CFU-Mk colonies were fixed on day 8 and stained for acetylcholinesterase activity and counterstained with Harris' haematoxylin (Sigma, MO) before scoring of colonies.

Electron microscopy

Platelets were isolated by differential centrifugation of whole blood (Eslin *et al*, 2004); 1 ml of mouse blood was drawn from the inferior vena cava by using a 23G needle/1 ml syringe with 200 μ l of acid-citrate-dextrose for anti-coagulation. The total volume was adjusted to 3 ml using modified Tyrode's calcium-free buffer (Eslin *et al*, 2004). Platelet-rich plasma (PRP) was obtained by centrifugation at 900 rpm (200 g) for 4.5 min at room temperature. Prostaglandin E1 (PGE1) was added to the supernatant PRP fraction to a final

concentration of 1 $\mu\text{mol/l}$. The platelets were pelleted at 2000 r.p.m. (800 g) for 10 min at room temperature and gently resuspended in 100 μl of 1% paraformaldehyde (EM grade) fixing solution. The volume was adjusted to 1 ml for the electron microscopy study. The final concentration of platelets ranged between $3 \times$ and $5 \times 10^8/\text{ml}$ for wild-type mice. EM was performed at the University of Pennsylvania Biomedical Imaging Facility.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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