EMBO reports

scientific report

Gfi1 and Gfi1b act equivalently in haematopoiesis, but have distinct, non-overlapping functions in inner ear development

Katharina Fiolka[1], Ronna Hertzano[2], Lothar Vassen[1], Hui Zeng[1], Orit Hermesh[2], Karen B. Avraham[2], Ulrich Dührsen[3] & Tarik Möröy[1+]

[1]Institut für Zellbiologie (Tumorforschung), IFZ, Universitätsklinikum Essen, Essen, Germany, [2]Department of Human Genetics and Molecular Medicine, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel, and [3]Klinik für Hämatologie, Universitätsklinikum Essen, Essen, Germany

Gfi1 is a transcriptional repressor essential for haematopoiesis and inner ear development. It shares with its paralogue Gfi1b an amino-terminal SNAG repressor domain and six carboxy-terminal zinc-finger motifs, but differs from Gfi1b in sequences separating these domains. Here, we describe two knock-in mouse models, in which the N-terminal SNAG repressor domain was mutated or in which the Gfi1 coding region was replaced by Gfi1b. Mouse mutants without an intact SNAG domain show the full phenotype of Gfi1 null mice. However, Gfi1:Gfi1b knock-in mice show almost normal pre-T-cell and neutrophil development, but lack properly formed inner ear hair cells. Hence, our findings show that an intact SNAG domain is essential for all functions of Gfi1 and that Gfi1b can replace Gfi1 functionally in haematopoiesis but, surprisingly, not in inner ear hair cell development, demonstrating that Gfi1 and Gfi1b have equivalent and domain-dependent, cell type-specific functions.

Keywords: Gfi1b; Gfi1; granulocytes; inner ear hair cells; neutropenia

EMBO reports advance online publication 6 January 2006; doi:10.1038/sj.embor.7400618

INTRODUCTION

The gene *'growth factor independence 1'* (*Gfi1*) encodes a 55 kDa nuclear protein with six carboxy-terminal zinc-finger domains and a novel amino-terminal domain of 20 amino acids that is termed SNAG domain, because it is also found in the proteins Snail and Slug (Fig 1A; Grimes *et al*, 1996; Zweidler-McKay *et al*, 1996). A mutation in the SNAG domain that replaces a proline at amino acid position 2 by alanine (P2A) completely abrogates the activity of Gfi1 as a transcriptional repressor (Grimes *et al*, 1996; Zweidler-McKay *et al*, 1996), suggesting that the SNAG domain constitutes a crucial element for the function of Gfi1. Homology searches and *in silico* analyses showed that only one closely *Gfi1*-related gene exists, *Gfi1b*, that also encodes a protein with an N-terminal SNAG domain and six C-terminal zinc-fingers (Fig 1A; Rödel *et al*, 1998; Tong *et al*, 1998). However, the intermediary domain is much smaller in Gfi1b than in Gfi1 and is not conserved between both proteins (Fig 1A). Gfi1 and Gfi1b are differentially expressed during lympho- and haematopoiesis (for a review, see Möröy, 2005). In the myeloid lineage, granulocytes show high levels of Gfi1 expression, whereas Gfi1b is completely absent; conversely, Gfi1b is highest in erythroid precursors and megakaryocytes, whereas Gfi1 can barely be detected (Saleque *et al*, 2002; L.V. & T.M. unpublished).

Outside the haematopoietic system, Gfi1 is expressed in precursors of sensory neurons, the retina, specific cells of the lung and in the central nervous system (Wallis *et al*, 2003; Hertzano *et al*, 2004). Loss of Gfi1 in mice affects pre-T-cell differentiation (Yücel *et al*, 2003), the development of neutrophil granulocytes (Karsunky *et al*, 2002; Hock *et al*, 2003) and inner ear hair cells (Wallis *et al*, 2003; Hertzano *et al*, 2004). In contrast, the deletion of *Gfi1b* disrupts the development of erythroid cells and megakaryocytes, resulting in lethality at mid-gestation (Saleque *et al*, 2002).

Both Gfi1 and Gfi1b are highly similar proteins, with almost identical sequences in their SNAG and zinc-finger domains. However, the specific role of the intermediary domain of both proteins is largely unknown. To solve this enigma, and to test the specific domain requirement for Gfi1 and Gfi1b, we used gene targeting to knock-in a *Gfi1b* complementary DNA or a P2A *Gfi1* mutant, which mutates the SNAG domain in the *Gfi1* locus.

[1]Institut für Zellbiologie (Tumorforschung), IFZ, Universitätsklinikum Essen, Virchowstraße 173, 45122 Essen, Germany
[2]Department of Human Genetics and Molecular Medicine, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel
[3]Klinik für Hämatologie, Universitätsklinikum Essen, Hufelandstraße 55, 45122 Essen, Germany
[+]Corresponding author. Tel: +49 201 723 3380; Fax: +49 201 723 5904; E-mail: moeroey@uni-essen.de

Received 30 August 2005; revised 29 November 2005; accepted 30 November 2005; published online 6 January 2006

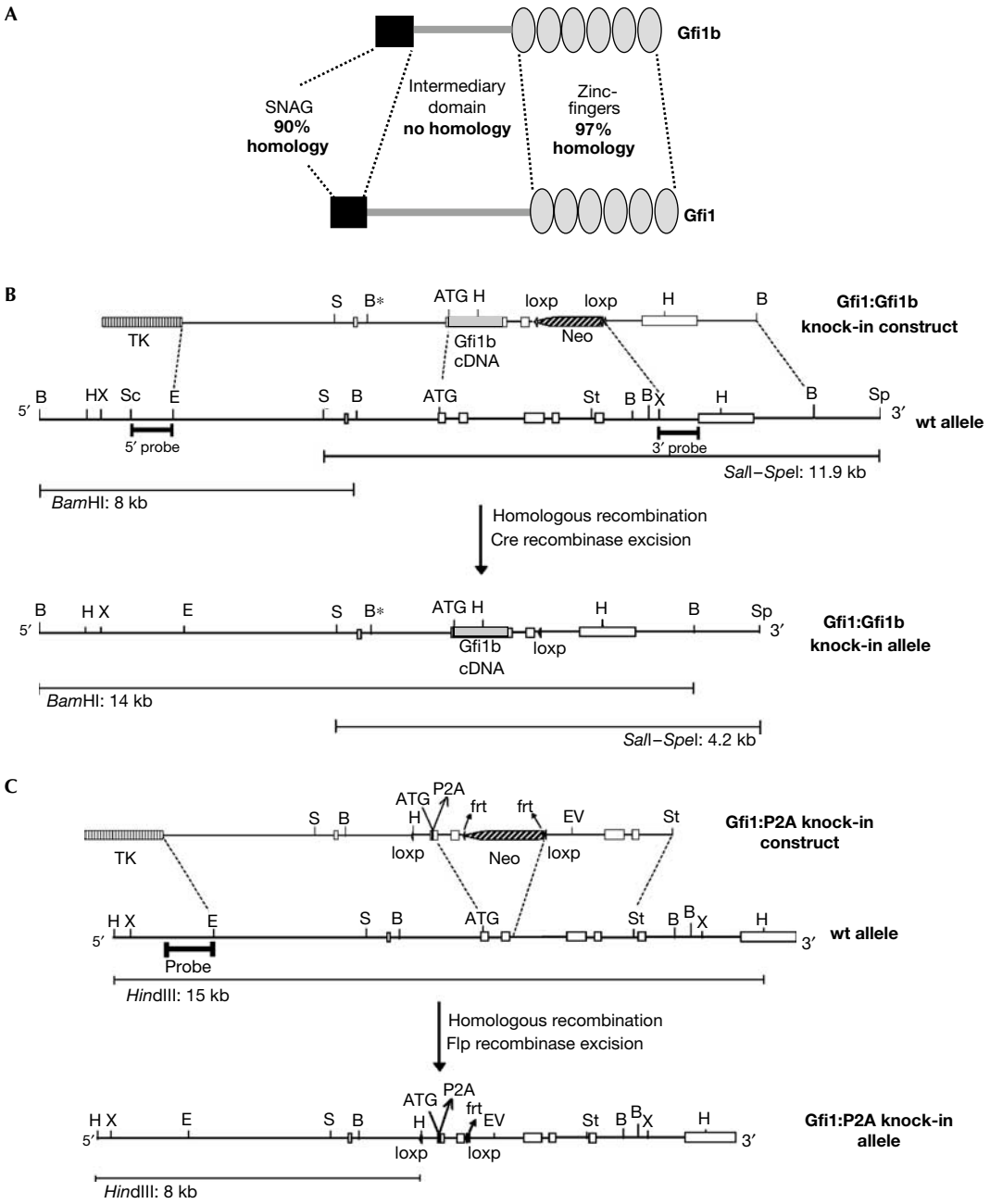


Fig 1 | Generation of Gfi1:Gfi1b and Gfi1:P2A knock-in mice. (**A**) Schematic structure of Gfi1 and Gfi1b proteins. (**B**) Schematic representation of the targeting vector (top), the murine *Gfi1* locus (middle) and the targeted allele (bottom). Exons are shown as open boxes and the *Gfi1b* coding region as a grey box. The removal of the neomycin resistance gene was achieved by mating to CMV-Cre transgenic mice. The probes used for genotyping by Southern blotting are indicated. B, *Bam*HI; B*, *Bam*HI deleted; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Sc, *Sac*I; Sp, *Spe*I; St, *Sty*I; TK, thymidine kinase; wt, wild type; X, *Xba*I. (**C**) Schematic representation of the knock-in strategy for introducing the P2A mutation into the *Gfi1* locus. Top: targeting vector; middle: *Gfi1* locus; bottom: targeted allele. Exons are depicted as open boxes. EV, *Eco*RV. Loss of the neomycin resistance gene was carried out by mating with Flp recombinase-expressing mice. Localization of the 5′ probe for Southern blotting is shown.

RESULTS AND DISCUSSION

Generation of Gfi1:Gfi1b and Gfi1:P2A knock-in mice

To test the extent of functional overlap between Gfi1 and Gfi1b, we generated a mouse mutant, in which the *Gfi1* coding region is replaced by *Gfi1b*. For this purpose, a gene-targeting vector was created in which exons 2–5 of *Gfi1* were deleted and the murine *Gfi1b* cDNA was inserted directly 3′ to the *Gfi1* translation initiation codon (Fig 1B). In this construct, 5′ regulatory sequences of the *Gfi1* locus as well as exons 6 and 7 with the poly-adenylation signal and the 3′ untranslated region were left intact.

Several correctly targeted embryonic stem (ES) cell clones were identified by Southern blotting, and injection of these cells into mouse blastocysts was carried out (supplementary Fig 1A online). The functionality of the newly introduced *Gfi1:Gfi1b* knock-in allele was confirmed by expression analysis of Gfi1 and Gfi1b in thymocytes (supplementary Fig 1B,C online).

In addition, we generated a second knock-in mouse mutant by introducing the crippling P2A mutation into the *Gfi1* locus to assess the significance of the N-terminal SNAG domain that mediates the repressor function in Gfi1 and Gfi1b. The gene-targeting construct replaced a region spanning exons 2–5 by the same sequence carrying a C to G substitution at position 4 of the *Gfi1* coding sequence, with the consequence that alanine instead of proline is encoded (Fig 1C). ES cells in which a homologous recombination event occurred were identified by Southern blotting (supplementary Fig 2A online) and were injected into mouse blastocysts to generate $Gfi1^{P2A/P2A}$ mutants. To exclude that remaining flox and frt sites affected the proper expression of the *Gfi1:Gfi1P2A* knock-in allele, we prepared thymocytes from these animals, compared the length of wild-type and knock-in transcript by reverse transcription–PCR and sequenced it (data not shown). Both transcripts had the same length and the knock-in transcript had no other mutations except the P2A exchange, indicating that the transcription of the *Gfi1:Gfi1P2A* allele was not disturbed (data not shown). Although it cannot be entirely ruled out that the remnant loxp and frt sites interfere with gene regulation, it is unlikely that they have an important role in our *Gfi1:Gfi1P2A* mutants, because we detect messenger RNA of proper length and sequence.

Expression of the Gfi1 P2A mutant protein was detectable in thymocytes of $Gfi1^{+/P2A}$ mice at about the levels of Gfi1 found in wild-type ($Gfi1^{+/+}$) thymus (supplementary Fig 2B online). However, in thymocytes from homozygous $Gfi1^{P2A/P2A}$ mice, the expression level of the Gfi1 mutants was significantly higher than wild-type levels (supplementary Fig 2B online). This upregulation of expression is most probably because of a loss of Gfi1 autoregulation in $Gfi1^{P2A/P2A}$ mutants. It has been shown *in vitro* that Gfi1 is able to repress its own promoter and that this activity depends on the presence of the SNAG domain (Doan *et al*, 2004; Yücel *et al*, 2004). Hence, mutation of the SNAG domain should lead to an upregulation of the *Gfi1* promoter in the absence of other intact Gfi1 proteins. Therefore, the elevated Gfi1 expression levels in thymocytes of $Gfi1^{P2A/P2A}$ mutants illustrate well the loss of repression activity of the P2A knock-in allele, and allow to draw the first conclusion that, not only in transfected cells but also *in vivo*, an intact SNAG domain is required for the function of Gfi1 as a transcriptional repressor.

Gfi1 domain requirement in haematopoiesis

Both mutants were used to further test the significance of the SNAG and intermediary domains in Gfi1 and to determine whether Gfi1b can replace Gfi1. As the development of pre-T-cells and granulocytes is severely affected in mice lacking Gfi1, we scrutinized both cell types in thymus and bone marrow. Comparison of thymocyte numbers of $Gfi1^{+/+}$, $Gfi1^{-/-}$ and $Gfi1^{1b/1b}$ thymocytes indicated that Gfi1b was able to almost fully replace Gfi1, whereas the $Gfi1^{P2A/P2A}$ mutant showed a significant loss of thymic cellularity, similar to the *Gfi1* null mutants (Fig 2A). The reduced number of thymocytes in *Gfi1*-deficient mice had previously been shown to be caused by defects in early, uncommitted c-Kit-positive T-cell progenitors (Yücel *et al*, 2003). Whereas wild-type mice showed about 67% c-Kit$^+$ cells among the CD4$^-$/CD8$^-$ pre-T-cell subset, $Gfi1^{-/-}$ animals showed a significantly reduced frequency (25%) of this subpopulation, as expected (Fig 2B). Replacement of *Gfi1* by *Gfi1b* almost completely restored the frequency of c-Kit$^+$ double-negative cells to wild-type levels, whereas the $Gfi1^{P2A/P2A}$ mutant remained at low knockout levels (Fig 2B). In contrast to previous studies (Doan *et al*, 2003) in which a unique function of Gfi1 and Gfi1b was proposed, the results presented here indicate that Gfi1b, which normally is absent or at least not readily detected in thymus (supplementary Fig 1C online), could replace Gfi1 almost completely in the development of the T-cell lineage. Nevertheless, it cannot be entirely ruled out that some aspects of T-cell differentiation are differentially regulated by the two proteins. However, it has to be concluded that an intact SNAG domain is absolutely necessary for the function of Gfi1 as a regulator of T-cell development, because the $Gfi1^{P2A/P2A}$ mutant showed the same defect as the complete knockout. As Gfi1b was able to replace Gfi1 almost fully in pre-T-cell development, it is likely that the intermediary domain is dispensable for the function of Gfi1 in the development of pre-T-cells.

The other main defects documented in *Gfi1*-deficient mice were a severe neutropenia and a pronounced increase in cells with monocytoid features (Karsunky *et al*, 2002; Hock *et al*, 2003). In bone marrow, $Gfi1^{1b/1b}$ knock-in mice had slightly reduced frequencies of granulocytes and showed a mild accumulation of cells with the immunophenotype of immature myeloid cells or monocytes (Mac-1^{lo}, Gr-1^{lo}) as compared with wild-type mice (Fig 2C). Although this phenotype resembled more the wild-type configuration than the drastic effects seen in the *Gfi1* knockout mouse, a complete rescue in terms of cell frequencies was not achieved by *Gfi1b*. Nevertheless, the typical signs that accompany the loss of neutrophils, as seen in *Gfi1* null mice, such as shortened lifespan, granuloma formation or an inherent predisposition to infections (Karsunky *et al*, 2002; Hock *et al*, 2003), were not observed in $Gfi1^{1b/1b}$ knock-in mice. In peripheral blood, $Gfi1^{1b/1b}$ knock-in and wild-type animals showed a similar distribution of granulocytes (Mac-1^{hi}/Gr-1^{hi}) or monocytes (Mac-1$^+$; Fig 2C, lower row). Neutropenia or a noticeable expansion of monocytoid cells, which was clearly present in the *Gfi1*-deficient mice, could not be detected in $Gfi1^{1b/1b}$ knock-in mice (Fig 2C), suggesting a full rescue of neutrophil development by Gfi1b. In sharp contrast, $Gfi1^{P2A/P2A}$ knock-in mice showed all phenotypes of the *Gfi1* null mouse, such as severe neutropenia and pronounced increase in monocytoid cells in all tissues examined (Fig 2C).

Blood smears and cytospins from bone marrow indicated that the $Gfi1^{P2A/P2A}$ mutant showed the *Gfi1* null phenotype, in that almost no mature neutrophilic granulocytes (cells with clearly segmented nuclei) were found and a large population of atypical monocytoid cells that is not detected in the wild-type controls (Fig 3A–C; supplementary Table 1 online). These cells resembled monocytes in that they had greyish-blue, often vacuolated cytoplasm and an indented, bean-shaped or lobed nucleus, but they were also reminiscent of metamyelocytes, frequently exhibiting a nuclear hole (Fig 3C). In both *Gfi*1 null and $Gfi1^{P2A/P2A}$ mice, a small percentage of almost mature appearing neutrophilic

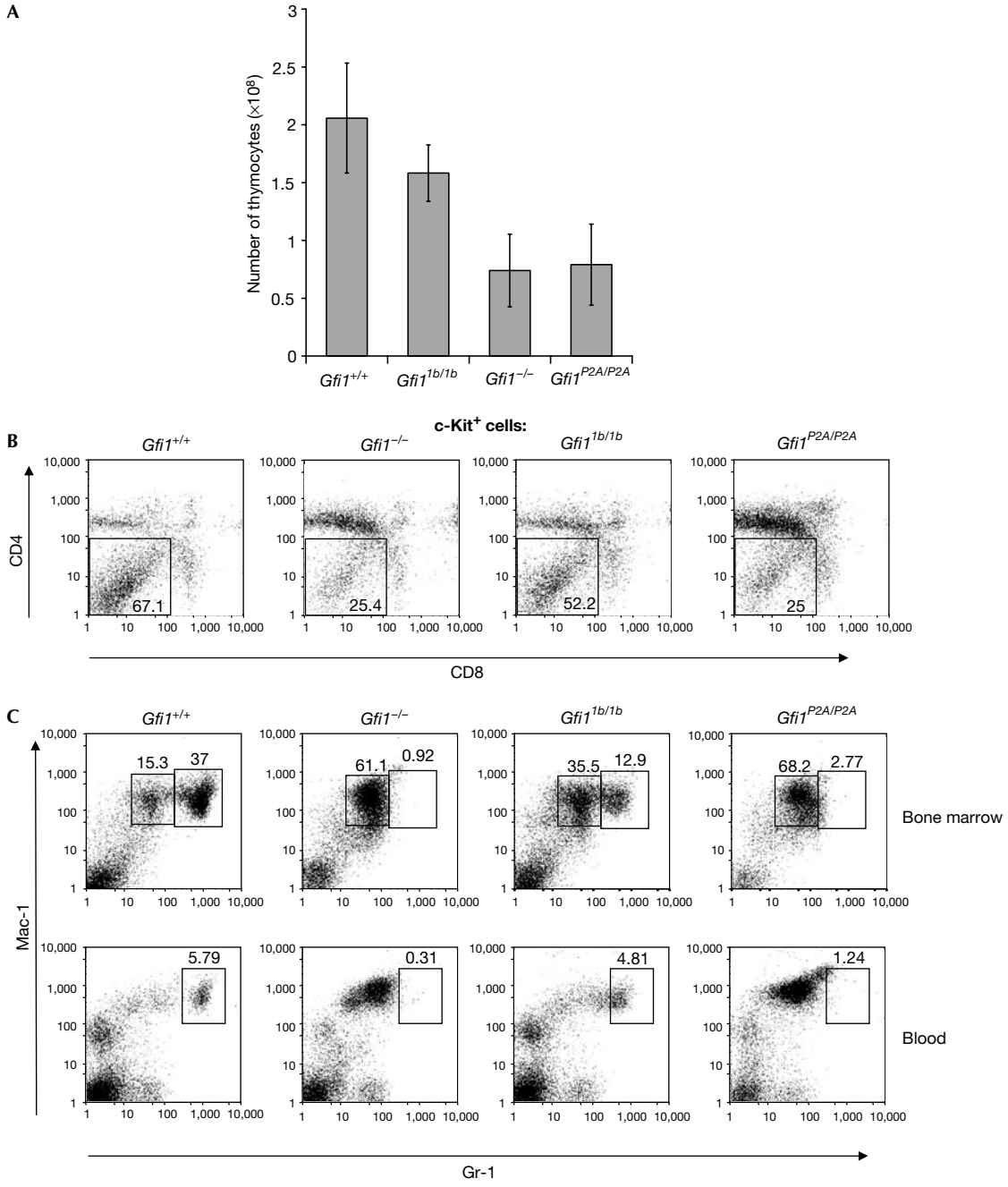


Fig 2 | Normal thymocyte and granulocyte development requires Gfi1 with an intact SNAG domain. (**A**) Total thymocyte numbers of 4- to 7-week-old animals ($n = 3$–8). (**B**) Fluorescence-activated cell sorting (FACS) analysis of CD4 and CD8 expression on electronically gated c-Kit$^+$ thymocytes ($n = 3$). (**C**) FACS analysis of bone marrow and blood cells stained with labelled anti-Mac-1 and anti-Gr-1 antibodies ($n = 4$).

granulocytes with subtle nuclear abnormalities (abortive segmentation, plump segments; Fig 3B,C) was detectable. Concurrent with this and the previously described *Gfi1* null phenotype, the proportion of lymphocytes was reduced in both blood and bone marrow of *Gfi1*$^{P2A/P2A}$ mutant to the same degree as in the full *Gfi1* knockout (supplementary Table 1 online). This suggests that an intact SNAG domain is an absolutely essential domain in Gfi1 for neutrophil development.

In contrast, the *Gfi1*$^{1b/1b}$ knock-in mutants were almost indistinguishable from the wild-type control, both in the differential cell count of the peripheral blood smear (supplementary Table 1 online) and in the appearance of the bone marrow cytospins, which showed regular maturation within the granulocytic lineage up to the stage of a mature neutrophil with a segmented nucleus (Fig 3A,B; supplementary Table 1 online). However, the proportion of mature granulocytes in the peripheral

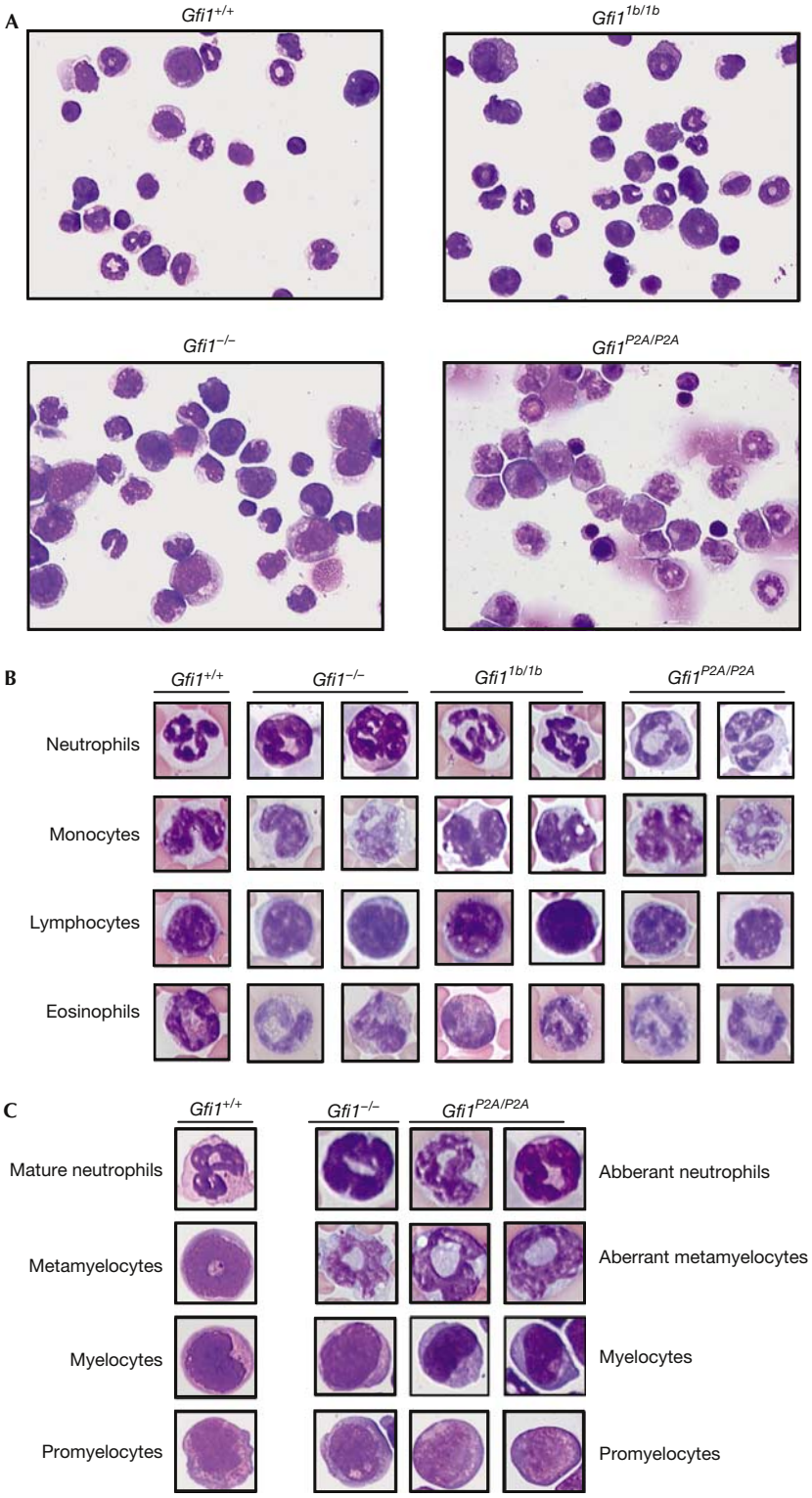


Fig 3 | Early stages in haematopoietic development are influenced by the repressor function of Gfi1. (**A**,**B**) Representative pictures of May–Grünwald–Giemsa-stained cytospins of bone marrow cells (after erythrocyte lysis; **A**) and blood smears (**B**) from the indicated mouse mutants ($n = 3$–8). Note atypical features of some monocytes in *Gfi1*$^{-/-}$ and *Gfi1*$^{P2A/P2A}$ mutants ($n = 4$–8). (**C**) Orderly granulocytic differentiation in *Gfi1*$^{+/+}$ bone marrow and accumulation of atypical monocytoid cells beyond the myelocyte stage in *Gfi1*$^{-/-}$ and *Gfi1*$^{P2A/P2A}$ mutants. The pictures were taken with a $\times 100$ objective and a $\times 10$ ocular ($n = 3$–8).

Fig 4 | Evaluation of inner ears of *Gfi1*$^{-/-}$, *Gfi1*$^{1b/1b}$ and *Gfi1*$^{P2A/P2A}$ mice. (**A**) Auditory brainstem response (ABR) thresholds of both *Gfi1*$^{-/-}$ and *Gfi1*$^{1b/1b}$ mice at age 3–3.5 months were determined to be over 100 dB at 8 kHz and over 90 dB at 16 and 32 kHz, whereas ABR thresholds of age-matched wild-type mice were within the normal range of hearing. $N = 2$ for wild type; $n = 4$ for all mutants. Error bars represent 1 standard error measured. (**B–E**) Cochlear epithelia from the mid-basal turn of P0 (**B**) wild type, (**C**) *Gfi1*$^{-/-}$, (**D**) *Gfi1*$^{1b/1b}$ and (**E**) *Gfi1*$^{P2A/P2A}$ mice. Cochlear epithelia were stained with an antibody for myosin VI (red) that labels the hair cells' cuticular plate and cytoplasm, and with phalloidin (green) to label filamentous actin at cell boundaries and in the stereocilia. (**F–I**) Cochlear epithelia from the mid-basal turn of P0 (**F**) wild type, (**G**) *Gfi1*$^{-/-}$, (**H**) *Gfi1*$^{1b/1b}$ and (**I**) *Gfi1*$^{P2A/P2A}$ mice. Cochlear epithelia were stained with an antibody for acetylated tubulin (green) that labels the hair cell kinocilia, pillar cells and various other tubulin-based structures in the cochlear sensory epithelium, and with phalloidin (red) to label filamentous actin at cell boundaries and in the stereocilia. IHC, inner hair cell; OHC, outer hair cell, Scale bar, 20 μM.

blood seemed to be slightly reduced in the *Gfi1*$^{1b/1b}$ knock-in mice as compared with the wild-type controls, and, in accordance with the fluorescence-activated cell sorting analysis, in the bone marrow a left shift towards more immature granulocytic precursors was observed (Fig 2C; supplementary Table 1 online). In addition, a few 'atypical monocytes', typical for *Gfi1* null mice, were also present in the *Gfi1*$^{1b/1b}$ knock-in mice (Fig 3A). Nevertheless, *Gfi1*$^{1b/1b}$ knock-in mice produced mature granulocytes in numbers that vastly exceeded those found in *Gfi1* null mice (Figs 2C,3A–C; supplementary Table 1 online). This indicated that the intermediary domain in Gfi1 is not required for the proper formation of mature, differentiated neutrophils, but may be required for proliferative expansion of granulocytic/monocytic cell types, which assures the high frequencies seen in wild-type mice.

The 'atypical monocytes' that emerge in *Gfi1* null cells and in *Gfi1*$^{P2A/P2A}$ mutants were clearly a heterogenous population, rarely resembling aberrant neutrophils, more frequently reminiscent of atypical metamyelocytes and sometimes evoking more immature granulocytic precursors (Fig 3C). These observations suggest that a loss of *Gfi1* or expression of a Gfi1 with a mutated SNAG domain maintains granulocytic maturation up to the myelocyte stage only and then leads to the development of aberrant forms that cannot be readily classified because the cells show features of both atypical metamyelocytes and atypical monocytes. The introduction of a *Gfi1b* gene was sufficient to largely rescue the loss of Gfi1, supporting the view that the intermediary domain of both proteins is less relevant for neutrophil differentiation, whereas an intact SNAG domain is essential.

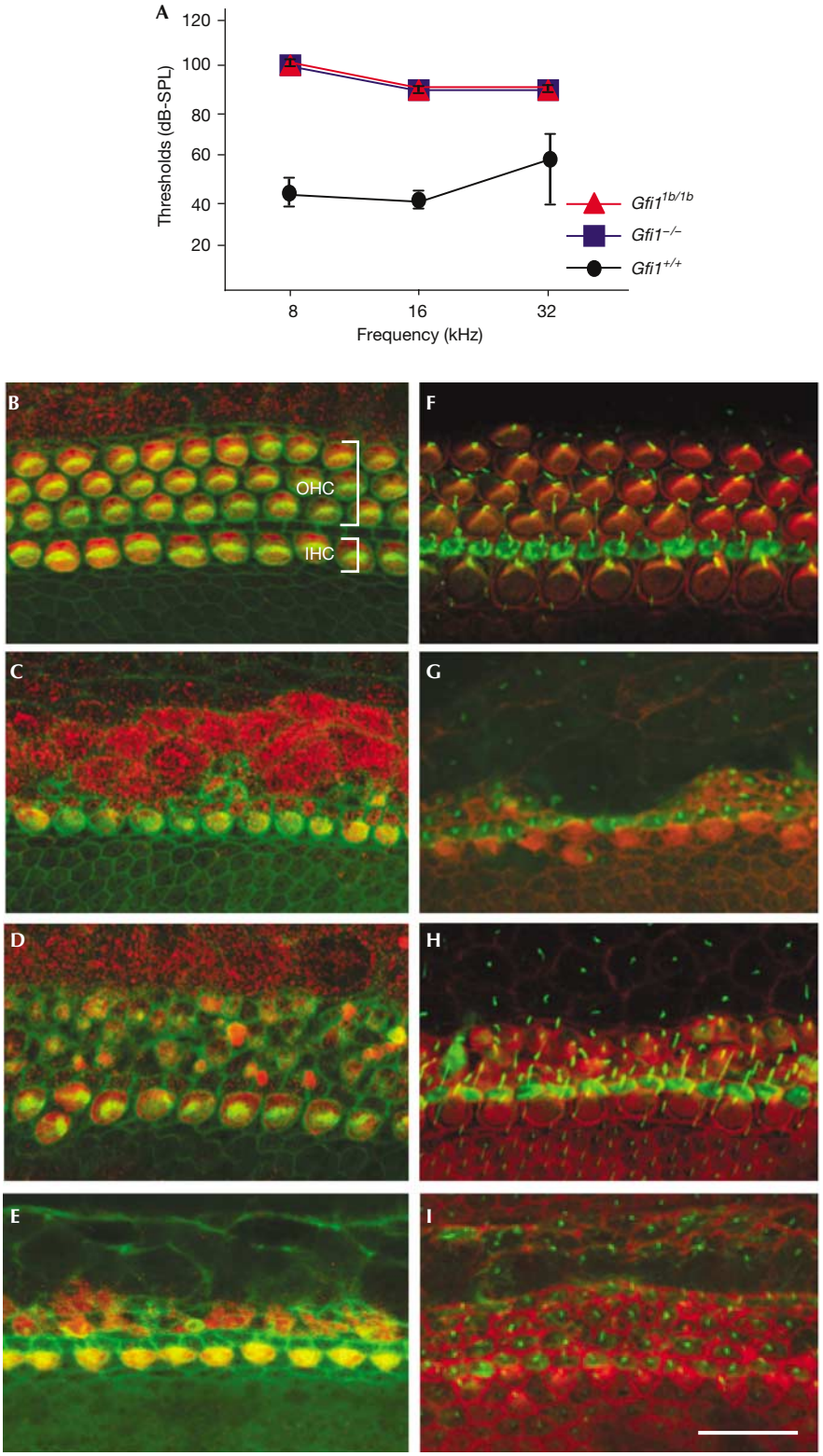
Gfi1 and Gfi1b functions diverge in inner ears

It has previously been described that *Gfi1* null mice show deafness and vestibular dysfunction (Wallis *et al*, 2003). Hearing loss in these animals is caused by late maturation defects and degeneration of inner ear hair cells beginning already before birth (Wallis *et al*, 2003; Hertzano *et al*, 2004). *Gfi1*$^{P2A/P2A}$ mice presented with the full Gfi1 knockout phenotype, including hearing loss, assessed by Preyer reflex and vestibular dysfunction (data not shown). Surprisingly, however, the inner ear defect could not be compensated for by Gfi1b, in contrast to all other Gfi1 deficiency phenotypes tested here. *Gfi1*$^{1b/1b}$ mice were clearly deaf, because they did not show a Preyer reflex, and no reaction to sound could be recorded by auditory brainstem response measurements in mice of 3–3.5 months of age (Fig 4A). They also showed an overt vestibular dysfunction manifested by head bobbing, an abnormal reaching response and a positive swimming test (data not shown), similar to *Gfi1*$^{-/-}$ mice. To exclude that this effect was due to a lack of expression of the *Gfi1*$^{1b/1b}$ knock-in allele, we isolated RNA from inner ears and tested Gfi1b expression by real-time PCR. Clearly, a substantial expression of Gfi1b noticeably higher than wild-type levels was detected (supplementary Fig 3 online), demonstrating the functionality of the knock-in allele. In addition, we had previously shown that a Gfi1:GFP knock-in allele that was constructed in an analogous manner to the Gfi1:Gfi1b knock-in allele was properly expressed in inner ear hair cells (Hertzano *et al*, 2004; Yücel *et al*, 2004), which would also exclude that a lack of Gfi1b expression could account for the observed phenotype in *Gfi1*$^{1b/1b}$ mice.

Whole-mount immunohistochemistry of inner ears from newborn mice stained with myosin VI, a hair cell marker (Fig 4B–E), or with an antibody for acetylated tubulin that labels hair cell kinocilia, pillar cells and other tubulin-based structures in the cochlear sensory epithelium (Fig 4F–I), demonstrated a typical pattern of a single row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs) in wild-type cochlear epithelia (Fig 4B,F). However, only the single row of IHCs was clearly seen in the cochlear epithelia from *Gfi1*$^{-/-}$, *Gfi1*$^{P2A/P2A}$ and *Gfi1*$^{1b/1b}$ mice (Fig 4C–E,G–I), and it appeared morphologically immature when compared with the wild-type littermates. The region where OHCs develop was disorganized in both *Gfi1*$^{1b/1b}$ and *Gfi1*$^{P2A/P2A}$ mutants. The hair cells appeared smaller, the stereociliary bundles were hardly identifiable and the overall myosin VI staining (a hair cell marker) was diffuse, suggesting a continuing degenerative process in that region. The phenotype in the OHC region seemed, however, more pronounced in the *Gfi1*$^{-/-}$ and *Gfi1*$^{P2A/P2A}$ mice than in the *Gfi1*$^{1b/1b}$ knock-in mice (Fig 4C–E,G–I), suggesting that the OHCs might degenerate at a slightly slower pace when Gfi1b is expressed instead of Gfi1. The results indicate clearly that Gfi1b cannot compensate for the loss of Gfi1 in the cochlear hair cells as it apparently can in haematopoietic cells. However, as both IHCs and OHCs of the *Gfi1*$^{1b/1b}$ mice seemed to be slightly less severely affected compared with the *Gfi1*$^{-/-}$ mice and as it has not been evaluated whether the Gfi1b knock-in allele is expressed with exactly the same spatiotemporal expression pattern as Gfi1 in the inner ear during fetal development, it cannot be entirely ruled out that some functions of Gfi1 that are necessary for hair cell survival can be taken over by Gfi1b. Despite such a potential partial compensation, Gfi1b was apparently not able to prevent hair cell death in newborn mice.

Speculation

Our results indicated a cell type-specific requirement of the intermediary domains in Gfi1 and Gfi1b. Until now, the only protein that interacts with Gfi1 through this intermediary domain is the STAT3 inhibitor PIAS3 (Rödel *et al*, 2000). We therefore



assessed whether Gfi1b is unable to interact with PIAS3, as a basis for the functional divergence of both proteins in inner ear hair cells. Indeed, co-immunoprecipitation experiments with extracts from cells expressing high levels of Gfi1b demonstrated that Gfi1b does not physically interact with PIAS3 (supplementary Fig 4 online). Because STAT3 is expressed in the same hair cell subtype

(OHC) as Gfi1 (Hertzano *et al*, 2004), it is possible that Gfi1, but not Gfi1b, could promote OHC differentiation or survival by sequestering PIAS3 and enhancing STAT3 signalling (Hertzano *et al*, 2004). Therefore, PIAS3 could be the factor responsible for mediating the cell type specificity of both Gfi1 and Gfi1b. However, several alternative possibilities exist, and it is also likely that the intermediary domain mediates the differential abilities of Gfi1 and Gfi1b to associate with specific cofactors through which accessibility to target genes or the ability to modify chromatin constituents is conferred in a cell type-specific manner.

METHODS

Generation of Gfi1:Gfi1b and Gfi1:Gfi1P2A knock-in mice. Gfi1:Gfi1b and Gfi1:Gfi1P2A knock-in mice were generated by modifying the previously described *Gfi1:GFP* knock-in construct (Yücel *et al*, 2004). The constructs were transfected in R1 ES cells and selection with G418 and ganciclovir was carried out. Mice were held under specific pathogen-free conditions in individually ventilated cages. All animal experiments were carried out according to the German Animal Protection Law and were carried out with licence granted by the Bezirksregierung Düsseldorf/NRW Germany (Nr.G022/02Z) and the Animal Care and Use Committee of Tel Aviv University (M-04-014).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

ACKNOWLEDGEMENTS

We thank I. Spratte, A. Warda, W. Wegrzyn and L. Mittelman for assistance, D. Bechtel for comments on the manuscript and P. Plessow and T. Civela for excellent animal care. This work was supported by the Deutsche Forschungsgemeinschaft grant Mo 435/18-1, 18-2, the 'Fonds der chemischen Industrie', the 'IFORES Program' of the University of Essen Medical School, European Commission FP6 Integrated Project EUROHEAR LSHG-CT-20054-512063 and US–Israel Binational Science Foundation (BSF) Grant 2003335.

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