A short Gfi-1B isoform controls erythroid differentiation by recruiting the LSD1–CoREST complex through the dimethylation of its SNAG domain

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Summary
Gfi-1B is a transcriptional repressor essential for the regulation of erythropoiesis and megakaryopoiesis. Here we identify Gfi-1B p32, a Gfi-1B isoform, as essential for erythroid differentiation. Gfi-1B p32 is generated by alternative splicing and lacks the two first zinc finger domains of the protein. Selective knock down of Gfi-1B p32 compromises erythroid differentiation, whereas its ectopic expression induces erythropoiesis in the absence of erythropoietin. Gfi-1B p32 isoform binds to Gfi-1B target gene promoters and associates with the LSD1–CoREST repressor complex more efficiently than the major Gfi-1B p37 isoform. Furthermore, we show that Gfi-1B includes a KSKK motif in its SNAG domain, which recruits the repressor complex only when dimethylated on lysine 8. Mutation of lysine 8 prevents Gfi-1B p32-induced erythroid development. Our results thus highlight a key role for the alternatively spliced Gfi-1B p32 isoform in erythroid development.

Key words: Gfi-1B, LSD1, Erythroid differentiation, Spliced variant, Transcriptional repressor, Isoform

Introduction
Hematopoiesis is dependent on the proper regulation of self-renewal, proliferation and differentiation of stem cells from which all hematopoietic lineages originate. These processes are tightly regulated and involve coordinated activation of specific genes and silencing of others. Changes in gene expression during hematopoietic differentiation are accompanied by epigenetic modifications, such as methylation of DNA or modification of histone tails (Reik, 2007).

The zinc finger protein Gfi-1B (growth factor independent 1B) is a sequence-specific transcriptional repressor known to recruit corepressor complexes to its target gene promoters. Gfi-1B plays a crucial role in the repression of gene transcription in hematopoietic stem cell (Khandanpour et al., 2010) and during erythroid and megakaryocytic development (Garcon et al., 2005; Osawa et al., 2002; Randrianarison-Huetz et al., 2010; Saleque et al., 2002; van der Meer et al., 2010). Gfi-1B is mainly expressed in cells of the erythroid and megakaryocytic lineages (Vassen et al., 2006b). We have recently demonstrated that Gfi-1B is highly expressed in the human common erythro-megakaryocytic progenitors (MEP) (Randrianarison-Huetz et al., 2010). Targeting of Gfi-1B in mice leads to embryonic lethality because of a lack of red blood cell production (Salezque et al., 2002) demonstrating the importance of Gfi-1B function in early stages of differentiation. In addition, it was suggested that Gfi-1B might play a role in oncogenesis: the human GFI1B gene was mapped at chromosomes 9q34.13, a region translocated to chromosome 22 generating the ‘Philadelphia chromosome’ responsible for tumorigenic process in chronic myeloid leukemia (CML) (Rodel et al., 1998).

Sequence-specific transcriptional repressors in eukaryotes recruit co-repressor complexes to their target gene promoters, leading to post-translational modifications of histones and changes in the chromatin status (Huang and Berger, 2008). The mechanisms involved in the specific recruitment of chromatin-modifying protein complexes to DNA sequences are still under debate. LSD1, a lysine-specific demethylase and component of the repressor complex LSD1–CoREST–HDAC (LCH complex) (Shi et al., 2004), is recruited to DNA through methylated histone (H3K4 or K9). However, it has been recently suggested that LSD1 can also be recruited to DNA through methylated non-histone DNA-binding proteins. Indeed, when p53, a DNA-binding tumor suppressor, is dimethylated on lysine (K) 370, it associates with LSD1 (Huang et al., 2007). Gfi-1B contains two domains: an N-terminal SNAG (Snail–Gfi1) repressor domain and six C-terminal zinc finger domains (Grimes et al., 1996; Zweidler-Mckay et al., 1996). It has been shown that the SNAG domain recruits the LCH complex to the Gfi-1B-targeted promoters (Saleque et al., 2007). We recently provided evidence that the recruitment of the repressor LCH complex by Gfi-1B evolves during erythroid differentiation (Laurent et al., 2009). At early erythroblast stages (pro- and basophilic erythroblasts), Gfi-1B regulates Myc expression through the recruitment of LSD1 and CoREST to the Myc promoter. At these stages of differentiation, Gfi-1B is also present at its own promoter but LSD1 is not. The absence of the LSD1–CoREST complex through the dimethylation of its SNAG domain...
complex at the GFI1B promoter allows GFI1B transcription at these early erythroblast stages, the chromatin remaining in an open configuration. By contrast, Gfi-1B and LSD1–CoREST complex bind to the GFI1B promoter at late stage of differentiation and GFI1B transcription is repressed. Thus, Gfi-1B may or may not recruit the LSD1–CoREST complex at Gfi-1B or Gfi-1B target gene promoters depending on the stage of differentiation.

Expression of a large number of genes encoding hematopoietic transcriptional factors gives multiple splicing variants producing various proteins that associate with different partners and thus probably have different biological activities during the differentiation process (Yamamoto et al., 2009). For instance, Myb (O’Rourke and Ness, 2008), GATA-1 (Calligaris et al., 1995), Fog (Snow and Orkin, 2009) and Oct4 (Wang and Dai, 2010) are expressed as different isoforms during hematopoietic development. Two isoforms of Gfi-1B were described in mouse bone marrow progenitors and erythroid cells (Osawa et al., 2002; Vassen et al., 2006a). Interestingly, Vassen et al. described a new splice variant of GFI1B in peripheral blood mononuclear cells (PBMC) of patients with myeloid leukemia. This splice variant encodes a Gfi-1B p32 isoform lacking the first two zinc finger domains (Vassen et al., 2009). Whether Gfi-1B p32 is expressed and functional in normal erythroid cells has not been determined.

In this work, we investigated whether Gfi-1B alternative splice variants are expressed in developing human erythroid cells and whether the isoforms generated by these variants contribute to the differentiation of these cells. We found that the expression of Gfi-1B p32, which was previously described to be highly expressed in patients with myeloid leukemia, is regulated during normal erythroid differentiation of normal CD34+ progenitors. Consistently, selective silencing of endogenous Gfi-1B p32 expression severely compromises erythroid differentiation of UT-7 cells, demonstrating its functionality. We further showed that this Gfi-1B p32 isoform efficiently recruits the LSD1–CoREST complex to Gfi-1B target gene promoters, and that such recruitment relies on the dimethylation of a lysine residue located at position 8 in its SNAG domain. Accordingly, mutation of K8 prevents Gfi-1B-p32-induced erythroid development. We conclude that regulation of Gfi-1B alternative splicing and methylation are essential for erythropoiesis.

Results

Three Gfi-1B isoforms are developmentally regulated during normal human erythropoiesis

Two Gfi-1B isoforms have been described in mouse bone marrow progenitors and erythroid cells (Osawa et al., 2002). Furthermore, an alternative spliced Gfi-1B transcript was recently described in peripheral blood mononuclear cells (PBMC) of patients with myeloid leukemia (Vassen et al., 2005). We asked whether such Gfi-1B isoforms can be detected in normal human erythroid cells and if so, whether their expression is regulated during erythroid differentiation. To address this question, we analyzed the kinetics of Gfi-1B expression at the protein level during the differentiation of CD34+ cells from umbilical cord blood. Immunoblot experiments performed with either a polyclonal (Laurent et al., 2009) or a monoclonal antibody against Gfi-1B revealed three Gfi-1B isoforms (Fig. 1A). The major band, previously referred to as Gfi-1B, migrated with an apparent molecular mass of 37 kDa (p37). In addition, two other bands were detected: an upper band with an apparent molecular mass of 39 kDa (p39) and a lower one of 32 kDa (p32). Interestingly, the expression level of all three isoforms showed important variations during erythroid differentiation without modification in their relative proportions. In particular, Gfi-1B p32 expression increased one day after induction of erythroid differentiation when the cells developed proerythroblast features, and the level was sustained at day 3 when the erythroblasts became mature (Fig. 1A) (Laurent et al., 2009; Randrianarison-Huetz et al., 2010). Analysis of Gfi-1B expression in a pluripotent cell line, UT-7, which can differentiate into mature erythroblasts in response to erythropoietin (EPO) (Goncalves et al., 1998), showed the presence of the same three forms of Gfi-1B (Fig. 1B).

We conclude that human hematopoietic cells express three Gfi-1B isoforms, the levels of which are regulated during erythropoiesis.

To identify whether these Gfi-1B isoforms resulted from alternative mRNA splicing, we analyzed Gfi-1B mRNA expression using conventional RT-PCR. Because an alternative splicing of exon IX, which encodes the two first Gfi-1B zinc fingers, was previously identified in PBMCs of patients with myeloid leukemia (Vassen et al., 2009), we focused on this region of Gfi-1B transcripts. Three fragments were amplified when using primers covering a region from exon VIII to exon X (primers S and AS; Fig. 1C). These three fragments were further characterized using sets of primers internal to this region. The largest fragment (fragment 1) contained part of the intronic sequence between exons IX and X, and probably did not correspond to a cDNA. The fragment of middle size (fragment 2) corresponded to the amplification of the three exons (VIII, IX and X), and the shortest fragment (fragment 3) did not contain exon IX, in agreement with what was reported for patients with myeloid leukemia (Vassen et al., 2009) (Fig. 1C). To determine whether this short Gfi-1B mRNA variant corresponded to the p32 isoform identified by immunoblot, we designed a specific siRNA corresponding to the junction between Gfi-1B exon VIII and X. Immunoblot experiments showed that transfection of this siRNA into UT-7 cells had no effect on Gfi-1B p37 expression but strongly decreased the amount of Gfi-1B p32 (Fig. 1D), suggesting that this short isoform does indeed result from the removal of exon IX and thus lacks the first two zinc finger domains of the transcription factor. Therefore our data strongly suggest that normal differentiating erythroid cells strongly express a p32 Gfi-1B variant previously reported in bone-marrow cells from patients with acute and chronic myeloid leukemia. This prompted us to focus on this isoform.

Gfi-1B p32 is essential for erythroid differentiation

Because Gfi-1B p32 is endogenously expressed in erythroid precursors, we next asked whether it is indeed required for erythroid differentiation in vivo. To address this question, we compared the consequences of silencing Gfi-1B p32 variant alone, using the specific Gfi-1B p32 siRNA described above, with the consequences of silencing Gfi-1B with previously published siRNA (Garcon et al., 2005) or short hairpin RNA (shRNA) of all the Gfi-1B variants (Randrianarison-Huetz et al., 2010). Complete silencing of Gfi-1B or silencing only the p32 variant induced the same effects in UT-7 cells: a substantial reduction in the percentage of differentiated cells 2, 3 and 4 days after EPO treatment in comparison with cells transfected with a control siRNA (47, 49 and 44% inhibition after p32 knock down and 72, 71 and 50% after knock down of all the
Gfi-1B RNAs, respectively (Fig. 2A). Cells from the UT-7 5.3 clone proliferate in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) without EPO and do not proliferate in the presence of EPO alone. Thus, as expected, whatever siRNA was used, UT-7 cells did not proliferate in the presence of EPO (Fig. 2B). These results indicate that the function of endogenous Gfi-1B p32 cannot be compensated for by Gfi-1B p37, whose expression levels were not affected by the siRNA used (Fig. 1D). We conclude that endogenous Gfi-1B p32 is required for EPO-induced erythroid differentiation.

Overexpression of Gfi-1B p32 or Gfi-1B p37 induces erythroid differentiation in the absence of EPO

These results prompted us to investigate further the respective role of Gfi-1B p37 and p32 during erythroid development. To address this question, we compared the effects of Gfi-1B p37 or p32 forced expression on the induction of erythroid
differentiation of UT-7 cells in the absence of EPO. We confirmed our recently published results in primary erythroid progenitors (Garcon et al., 2005; Randrianarison-Huetz et al., 2010) and showed that overexpression of the full-length Gfi-1B p37 in UT-7 cells reduced cell expansion (Fig. 3A) and induced erythroid differentiation in the absence of added EPO (Fig. 3B). This equally applied to Gfi-1B p32. Indeed, Gfi-1B cDNA lacking exon IX when ectopically expressed in UT-7 cells induced a decrease in cell expansion (Fig. 3A) and an increase in hemoglobin synthesis (Fig. 3B) and GPA expression (not shown) in the absence of EPO.

Analysis of Gfi-1B protein levels revealed that ectopic expression of the complete human GFI1B cDNA (Gfi-1B p37) in UT-7 cells increased the protein level of Gfi-1B p37 and also the level of Gfi-1B p32, compared with non-infected cells (2.5 ± 0.7 times; Fig. 3C). Similar results were obtained when expressing mouse Gfi-1B p37 in UT-7 cells (supplementary material Fig. S1). By contrast, when the cDNA encoding the Gfi-1B p32 isoform was over-expressed in UT-7 cells, it induced a decrease in cell expansion (Fig. 3A) and an increase in hemoglobin synthesis (Fig. 3B) and GPA expression (not shown) in the absence of EPO.

**Gfi-1B p32 associates with LSD1–CoREST**

Gfi-1B is known to repress transcription through its interaction with the chromatin-modifying enzyme complex LSD1–CoREST–HDAC (LCH complex). Does this Gfi-1B function also apply to Gfi-1B p32? To investigate this point, we infected UT-7 cells with retroviral vectors transducing FLAG-tagged Gfi-1B p37 or p32 cDNAs and GFP. GFP-positive cells were sorted, cultured in the presence of EPO and used for co-immunoprecipitation assays. We observed that anti-FLAG antibody immunoprecipitated both LSD1 and CoREST in FLAG-tagged-p37- or FLAG-tagged-p32-expressing cells (Fig. 4). Noticeably, FLAG-tagged Gfi-1B p32 interacted more efficiently with LSD1 and Co-REST than FLAG-tagged Gfi-1B p37 (lane 7 vs lane 3), although both constructs were expressed at similar levels (input lane 5 vs 1). We further confirmed these associations in LSD1 immunoprecipitation assays. Again, we found stronger interactions between LSD1 and Gfi-1B p32 than Gfi-1B p37 (lane 6 vs 2). Similar results were obtained in the absence of EPO stimulation (supplementary material Fig. S2), suggesting that Gfi-1B association with LSD1–CoREST is not EPO dependent. Intriguingly, FLAG-tagged Gfi-1B p32 and p37 were detected as two species recognized by both anti-Gfi-1B (not shown) and anti-FLAG antibodies (see the asterisk in Fig. 4). This could reflect the presence of a proteolytic degradation process in the N-terminal part of both Gfi-1B isoforms, the FLAG sequence being cloned at the C-terminal part of the Gfi-1B cDNA. However, only the full-length Gfi-1B p37 and p32 isoforms co-immunoprecipitated with LSD1, suggesting that LSD1 binds to the N-terminus of Gfi-1B, as expected from the results of Saleque et al. (Saleque et al., 2007).

Hence, Gfi-1B p32 can interact more strongly with chromatin-modifying enzymes than Gfi-1B p37, suggesting that this Gfi-1B shorter isoform has an important contribution to endogenous Gfi-1B function. This finding is fully consistent with the strict requirement of Gfi-1B p32 for erythroid development.

**Interaction of Gfi-1B p32 with LSD1 requires its dimethylation on K8**

So far, our results show that the Gfi-1B p32 isoform is essential for erythroid differentiation and efficiently interacts with the LSD1–CoREST complex. We next determined how Gfi-1B p32
interacts with LSD1. Interestingly, the DNA-binding non-histone protein p53 was shown to interact directly with LSD1 in vivo through its dimethylated lysine, K370, contained in a KSKK sequence (Huang et al., 2007). Because our in silico analysis showed that the SNAG domain of Gfi proteins contained the same peptide motif KSKK as p53 (Fig. 5A), we hypothesized that dimethylation on K8 of Gfi-1B p32 isoform was involved in its association with LSD1. To test this hypothesis, we incubated peptides corresponding to the SNAG Gfi-1B domain dimethylated or not on K8 or K10 (K8me2 and K10me2; Fig. 5B) with cellular extracts from UT-7 cells, and analyzed the pulled down proteins by immunoblotting. Remarkably, we observed that LSD1 and CoREST associated with the SNAG peptide dimethylated on K8, but barely interacted with the K10me2 peptide or with the unmethylated one (Fig. 5B). We conclude that dimethylation of Gfi-1B on its K8 residue promotes its association with LSD1–CoREST.

To further strengthen this finding, we expressed Gfi-1B p32 mutated or not on its K8 residue into UT-7 cells and analyzed the ability of these Gfi-1B proteins to co-immunoprecipitate with LSD1 and CoREST. Mutation of K8 abolished the association.
between Gfi-1B p32 and the chromatin-modifying enzymes (Fig. 5C), suggesting that dimethylation of K8 in the SNAG domain of Gfi-1B is indeed responsible for its interaction with LSD1–CoREST. Accordingly, when performing DNA-affinity precipitation experiments using an oligonucleotide containing the consensus Gfi-1B-binding site and nuclear extracts from transfected HEK293 cells, we observed that although wild-type and K8A mutated Gfi-1B 32 kDa isoform, GFP-positive cells were sorted and subjected to EPO stimulation. After 5 days, cell lysates were prepared and immunoprecipitated with LSD1 or FLAG antibody. Immunoprecipitated proteins were analyzed by western blotting using LSD1, CoREST and Gfi-1B antibodies. Antibody against IgG was used as control. The asterisk indicates the same degraded form of Gfi-1B p37 or p32 as is seen in Fig. 4. (D) Binding of the 32 kDa Gfi-1B isoform to the consensus Gfi-1B binding site. HEK293 cells were not transfected (NT) or transfected with plasmid containing the GFP sequence and the coding sequence for either the wild-type (WT p32) or the FLAG-tagged K8A mutated (K8A p32 c-FLAG) 32 kDa Gfi-1B isoform. The concentration of the wild-type remained constant, whereas the concentration of the mutated form increased (ratio of 1:8 in comparison with the WT isoform). 48 hours after transfection, oligonucleotide pull down experiments were performed. Cell lysates from transfected cells were incubated with biotinylated oligonucleotide carrying the wild-type (WT) or mutated (Mut) consensus Gfi-1B binding site. Bound proteins were analyzed by western blotting using Gfi-1B, FLAG and LSD1 antibodies.

Dimethylation of Gfi-1B p32 is essential for LSD1 recruitment in vivo to Gfi-1B target gene promoters

Having shown that Gfi-1B p32 requires dimethylation on K8 to recruit the LSD1–CoREST protein complex to its DNA-binding sites in vitro, we investigated, by chromatin immunoprecipitation, whether this protein modification was required for recruitment of LSD1–CoREST to target gene promoters in vivo. Gfi-1B target genes in erythroid cells have been identified in comparative transcriptomic analyses performed on MEP and CD34+ wild-type cells and cells in which GFI1B was knocked down (Randrianarison-Huetz et al., 2010). There were 60 genes upregulated in both the Gfi-1B-depleted MEP and CD34+ cell populations and Gfi-1B was found to bind to the promoter of 12 of these 60 genes in chromatin immunoprecipitation and sequencing (ChIP-seq) experiments (C. A.-S., E. S., J. C. Bryne, E. de Boer, M. Stevens, W. van Ijcken, B. Lenhard and F. Grosveld, unpublished data). Among these 12 genes, six of them displayed a similar expression pattern to that of Gfi-1B during erythroid development (MGEA5, CSDE1, GABPB2, HIF1B, ITSN1 and CMYC;
Endogenous Gfi-1B and LSD1 bound to these six promoters in differentiated erythroid cells in vivo (supplementary material Fig. S4), so we performed ChIP experiments to determine whether Gfi-1B p32 was present on these promoters in cells overexpressing wild-type Gfi-1B p32 or the K8A mutant. We found that wild-type Gfi-1B p32 was recruited in vivo to these six promoters as well as to the Gfi-1B promoter (Fig. 6A). Because overexpression of Gfi-1B p32 leads to a decrease in endogenous Gfi-1B p37 protein expression (Fig. 4D), the ChIP results reflect mainly the DNA binding activity of Gfi-1B p32. In contrast to wild-type Gfi-1B p32, the K8A mutant was not detected at Gfi-1B target promoters nor was it recruited to LSD1. We conclude that, in contrast to what was observed in vitro by oligonucleotide pull down (Fig. 5D), the K8A p32 isoform does not bind to Gfi-1B target gene promoters in vivo.

Similarly, ChIP experiments showed that LSD1 recruitment to Gfi-1B target gene promoters paralleled the binding of Gfi-1B p32, LSD1 being weakly detected when the Gfi-1B p32 K8A mutant was used (Fig. 6B). These data strongly suggest that methylation of the Gfi-1B 32 kDa isoform on K8 is responsible for LSD1–CoREST recruitment to target gene promoters in vivo.

**Dimethylation of Gfi-1B p32 is required for erythroid development**

To investigate whether dimethylation of Gfi-1B p32 on K8 is required for erythroid differentiation, we compared the ability of the wild-type and K8A mutated Gfi-1B p32 isoforms to induce erythroid differentiation without EPO in UT-7 cells. Interestingly, western blot analysis showed that overexpression of wild-type p32 inhibited p37 expression as described above (Fig. 3D), but overexpression of the K8A mutant did not (Fig. 7A). Although overexpression of the Gfi-1B p32 isoform resulted in a substantial increase in the percentage of benzidine-positive cells from day 1 to day 7 upon transduction in the absence of EPO (as shown in Fig. 2A), overexpression of the K8A mutated p32 isoform did not induce erythroid differentiation (Fig. 7B). We conclude that methylation on K8 in the SNAG domain of Gfi-1B p32 is essential for its activity during erythroid development.

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**Fig. 6.** The methylated 32 kDa Gfi-1B isoform binds to Gfi-1B target gene promoters in vivo. UT-7 cells were infected with bi-cistronic retroviruses containing the GFP and the coding sequences of either wild-type or K8A mutated Gfi-1B p3. GFP-positive cell populations were sorted and cultured for 2 days in the presence of EPO. ChIP experiments were performed with Gfi-1B (A) or LSD1 (B) antibodies. Gfi-1B target gene promoters were amplified with specific primers.

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**A**

|       | Relative fold enrichment |
|-------|--------------------------|
| Gfi-1B |                           |
| MGEA5 |                           |
| CSDE1 |                           |
| Gabpa2 |                          |
| HIF1b |                           |
| Intersect-1 |                     |
| Myc   |                           |
| GAPDH |                           |
| B2m   |                           |

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**B**

|       | Relative fold enrichment |
|-------|--------------------------|
| Gfi-1B |                           |
| MGEA5 |                           |
| CSDE1 |                           |
| Gabpa2 |                          |
| HIF1b |                           |
| Intersect-1 |                     |
| Myc   |                           |
| GAPDH |                           |
| B2m   |                           |

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**WT**  **K8A**
Together, our data show that developing erythroid cells express a short isoform of Gfi-1B, the repressor function of which relies on dimethylation of its K8 residue, and it is required for erythropoiesis.

**Discussion**

In this study, we provide new insights into the mechanisms involved in the transcriptional function of Gfi-1B and show a key role of one Gfi-1B isoform in this function.

We detected three Gfi-1B isoforms whose expression levels are regulated during erythropoiesis and showed that the Gfi-1B p32 isoform that lacks the two first zinc finger domains of the protein, found in patients with myeloid leukemia (Vassen et al., 2009), is expressed and functional in normal erythroid cells. Although we identified a transcript that encodes the Gfi-1B p32 isoform, we did not find any corresponding to Gfi-1B p39, suggesting that this Gfi-1B isoform results from post-translational modifications. Gfi-1B p37 and p32 might undergo ubiquitination similar to that described for Gfi-1 (Martiejn et al., 2007). Further analysis should be performed to determine the precise nature and role of the Gfi-1B p39 isoform.

Alternative splicing is considered as one of the most powerful biological mechanisms that convey diversification of gene function without a corresponding increase in gene number (Parnsley et al., 2007). Often, alternative splicing is cell or stage-specific: for example, changes in the pattern of Myb alternative RNA splicing occurred in a lineage- and differentiation-specific manner upon normal hematopoietic differentiation (O’Rourke and Ness, 2008). Surprisingly, the expression patterns of the three Gfi-1B protein isoforms described in this paper are similar during erythroid development, suggesting that they have complementary functions in erythropoiesis.

We further show that, whereas Gfi-1B p37 and p32 contain the KSKK motif in their SNAG domain, the Gfi-1B p32 isoform interacts more efficiently with LSD1 than the full-length p37 isoform. Several recent studies have suggested that relative minor changes in the functional domains of proteins such as the transcriptional activation domain of Myb (O’Rourke and Ness, 2008) or truncation of the N-terminal sequence of Fog (Snow and Orkin, 2009) can dramatically affect their activity in the regulation of hematopoiesis. It would be interesting to determine whether the absence of the two first zinc fingers domains plays a role in the affinity of Gfi-1B to LSD1 or other protein partners. It has been shown that the deletion of these two zinc fingers does not modify the ability of Gfi-1B to bind DNA or its repressive function (Grimes et al., 1996; Vassen et al., 2009). However, the lack of these protein domains might modify the quaternary structure of Gfi-1B, improving the post-translational modification of the SNAG domain and thus its association with chromatin-modifying enzymes. Alternatively, competitive binding of, yet undefined, Gfi-1B partners might be impaired or facilitated with the zinc-finger-deleted p32 isoform, resulting in a stabilized Gfi-1B-p32–LSD1 interaction. Fine mapping of the interaction domains between Gfi-1B and its different binding partners combined with structural data could be used in future studies to test this hypothesis.

Our findings indicate that dimethylation of the Gfi-1B p32 isoform is required for the recruitment of LSD1 and subsequently for its function in erythroid differentiation. These results underlie the role of post-translational modifications (PTMs) in the recruitment of repressive complexes by non-histone proteins. When the p32 isoform mutated at the methylation site is overexpressed, no Gfi-1B binding to Gfi-1B target gene promoter occurred and no erythroid differentiation was induced.

Recently, Snail, another protein containing a similar SNAG domain, has been described to recruit LSD1 to Snail target gene promoters and especially to the E-cadherin promoter (Lin et al., 2010). The formation of the Snail–LSD1–CoREST ternary complex is crucial for the stability and function of Snail protein. In that study, the authors suggested that the SNAG domain of Snail resembles a histone H3-like structure and functions as a ‘hook’ to recruit the LSD1–CoREST complex to its targeted gene chromatin. We suggested that methylation of K8 in the sequence KSKK present in the SNAG domain of Gfi-1B (similar to the methylation of the K370 of the KSKK sequence of p53) is responsible for LSD1 recruitment. Importantly, the sequences of the two SNAG domains (those of Gfi-1B and those of Snail) are similar for the seven N-terminal amino acids (MPRSFLV) of the domain but differ in the C-terminal part of the SNAG sequence.
amplified using the following primers:

To generate the splice variant expression plasmid, the Gfi-1B p32 cDNA was cloned using the TOPO-PCR cloning kit (Invitrogen, Carlsbad, USA). Oligo(dT)-primed cDNAs were synthesized from total RNAs using Superscript II reverse transcriptase (Invitrogen) and amplified by PCR using the following thermal cycling program: 95 °C for 90 seconds, 40 cycles of 5 seconds at 95 °C, 20 seconds at 60 °C and 10 seconds at 72 °C, followed by a 5 minute extension time at 72 °C. The primer sequences used are described in supplementary material Table S1.

Transfection of siRNA in UT-7 cells

siRNAs targeting the splice variant lacking exon IX of the human Gfi-1B gene were used to knock down the Gfi-1B p32 variant. Only one set of oligoribonucleotides overloading the junction between exon VIII and exon X could be designed and was potentially efficient in silencing the splice variant lacking exon IX. The sequence of the sense primer was 5'-GUGCAACAAGGAGCGCAGC(dTT)-3' and the antisense primer 3'-GUGCAACAAGGAGCGCAGC(dTT)-3'. Double-strand RNAs were transfected with Amaxa nucleofector (Amaxa Biosystems, Lonza, Basel, Switzerland) following the manufacturer’s instructions. UT-7 cells (5 x 10^6) were suspended in 100 µl nucleofector solution (VCA-1003). Oligoribonucleotides were added at 300 or 400 nM. The program T20 was used. For erythroid differentiation, 24 hours after electroporation cells were stimuluated with EPO in FCS-supplemented z-MEM. Transfection efficiency was determined by co-transfecting a CDNA encoding GFP. 48 hours after transfection of the oligoribonucleotides 80% of the cells were GFP positive.

Preparation of nuclear extracts

For nuclear extract preparation, cells were washed once with phosphate-buffered saline (PBS) and incubated for 10 minutes at 4°C in buffer A (10 mM HEPES, pH 7.6, 3 mM MgCl2, 10 mM KCl, 5% glycerol, 0.5% NP-40) containing 1 mM Na2VO4, 20 mM NaF, 1 mM sodium pyrophosphate, 25 mM β-glycerophosphate and protease inhibitors (Roche Diagnostics). After centrifugation, nuclear pellets were resuspended in buffer A containing 300 mM KCl.

Oligonucleotide and peptide pull-down assays

For oligonucleotide pull-down assays, complexes from nuclear extracts of 10^7 cell were mixed with 4 µg double-strand biotin-labeled oligonucleotide at 4°C for 1 hour and then pelleted using streptavidin-agarose beads (Amersham Biosciences, Freiburg, Germany). Beads were then washed three times with buffer A (see above) and resuspended in 1 x Laemmli buffer.

For peptide pull-down assays, complexes from nuclear extracts of 10^7 cell were mixed with 200 or 500 ng biotin-labeled peptide at 4°C for 1 hour and then pelleted using streptavidin-agarose beads (Amersham Biosciences). Beads were then washed three times with buffer A and resuspended in Laemmli buffer.

Western blot analysis and antibodies

Samples were subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Filters were blocked overnight in Tris-buffered saline (TBS)–0.05% Tween 20 with 5% skimmed milk, and incubated with the appropriate antibody. Membranes were washed four times in TBS–TWEEN 20 and incubated for 1 hour with the appropriate peroxidase-conjugated secondary antibody. The primary antibodies used were as follows: Gfi-1B (D3G2; Cell Signaling, Beverly, MA, USA); LSD1 (ab-17721; Abcam, Cambridge, UK); CoREST (07-455; Upstate, Lake Placid, NY, USA); FLAG M2 (Sigma, Saint Louis, USA); β-actin (Sigma) and β-tubulin (Santa Cruz, Santa Cruz, CA, USA). The homemade polyclonal antibody against human Gfi-1B has been described previously (Laurent et al., 2005).

For immunoprecipitation assays, nuclear extracts from 10^7 cell were precluated by addition of 2 µg normal IgG for 2 hours. After incubation with 50 µl protein G beads and centrifugation, 2 µg anti-FLAG M2 antibody (Sigma, Saint Louis, USA) or control IgG were added to the supernatant. The day after, complexes were pelleted using 50 µl protein G beads. Beads were then washed three times with buffer A and mixed in Laemmli buffer.

Cloning of the splice variant, retroviral production and infection

To generate the splice variant expression plasmid, the Gfi-1B p32 CDNA was amplified using the following primers: GFI1B sense 5’-TGTTGGGTGGTGCAACTCAGAA-3’, GFI1B antisense 5’-CAGGAGAATCTGCAACGGCTGGCAGG-3’ and GFI1B antisense C-FLAG 5’-TGTAATT-GTGATTTGTCGTCATCGTCTTTGTAGTCCTTGAGATTGTGCTG-3’ and cloned using the TOPO-PCR cloning kit (Invitrogen, Carlsbad, USA). PCR products were sequenced and subcloned, after EcoRI digestion, into the Migr recombination vector, described previously (Garcon et al., 2005) those the GFP sequence under the control of the EF1α promoter. The K8A (lysine to alanine) mutation of the p32 Migr–Gfi-1B was generated using the QuickChange Site-directed Mutagenesis Kit (Stratagene, Santa Clara, CA, USA). The retroviral construct containing the full-length Gfi-1B p37 coding sequence and the products of the retroviral vectors were released. UT-7.5.3 cells were infected twice with viral supernatants and GFP-positive cells were sorted 48 hours after infection.

RT-PCR assay

Oligo(dT)-primed cDNAs were synthesized from total RNAs using Superscript II reverse transcriptase (Invitrogen) and amplified by PCR using the following thermal cycling program: 95 °C for 90 seconds, 40 cycles of 5 seconds at 95 °C, 20 seconds at 60 °C and 10 seconds at 72 °C, followed by a 5 minute extension time at 72°C. The primer sequences used are described in supplementary material Table S1.
Chromatin immunoprecipitation

UT-7 cells harvested on different days after EPO stimulation were fixed, lyzed and sonicated as described previously (Laurent et al., 2009). Immunoprecipitations were performed following the Upstate protocol (www.upstate.com) using a human Gfi-1B antibody (prepared in the laboratory), LSD1 antibody (ab-17721, Abcam) and control rabbit IgG (Santa Cruz). PCR was performed on immunoprecipitated DNA using specific primers of Gfi-1B target gene promoters (see supplementary material Table S1).

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