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Dynamic changes in transcription factor complexes during erythroid differentiation revealed by quantitative proteomics

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During erythroid differentiation, β-globin gene expression is regulated by the locus control region (LCR). The transcription factor NF-E2p18/MafK binds within this region and is essential for β-globin expression in murine erythroleukemia (MEL) cells. Here we use the isotope-coded affinity tag (ICAT) technique of quantitative mass spectrometry to compare proteins interacting with NF-E2p18/MafK during differentiation. Our results define MafK as a 'dual-function' molecule that shifts from a repressive to an activating mode during erythroid differentiation. The exchange of MafK dimerization partner from Bach1 to NF-E2p45 is a key step in the switch from the repressed to the active state. This shift is associated with changes in the interaction of MafK with co-repressors and co-activators. Thus, our results suggest that in addition to its role as a *cis*-acting activator of β-globin gene expression in differentiated erythroid cells, the LCR also promotes an active repression of β-globin transcription in committed cells before terminal differentiation.

Erythroid differentiation leads to the production of mature red blood cells from pluripotent hematopoietic stem cells. A key event during terminal differentiation of erythroid cells is the activation of β-globin gene transcription, leading to hemoglobin synthesis. In both human and murine β-globin loci, several clustered β-like globin genes are arranged sequentially in the order in which they are expressed during development. The high level of expression of these genes in erythroid cells is dependent on the LCR, which consists of five nuclease hypersensitive sites (HSs) spread over 20–30 kilobases (kb) of DNA and located 10–60 kb upstream of the β-like globin genes[1–5]. These HSs are formed in committed erythroid cells before the transcription of the β-globin genes, indicating that the β-globin locus chromatin structure is relatively 'open' in these cells[6]. HSs form as a result of the binding of specific transcription factors to this region of the β-globin locus. The β-globin LCR contains numerous binding sites for both erythroid-specific and ubiquitous transcription factors, including CACC motifs (recognized by Kruppel-like proteins and by the Sp family of proteins), E boxes, GATA-1 motifs and MAREs (Maf recognition elements)[7,8]. However, the molecular mechanism by which the LCR and associated factors activate transcription of the β-globin genes upon terminal erythroid differentiation is still unclear[9,10].

Structure-function analyses have shown that the tandem MARE in 5′ HS2 of the LCR is crucial for β-globin gene expression[11–18]. MAREs are recognized by the transcriptional activation complex NF-E2, which comprises the hematopoietic-specific subunit p45 and the more ubiquitously expressed small Maf (sMaf) protein p18/MafK[19–23]. To examine the role of NF-E2p18/MafK[21] (subsequently referred to as MafK) in β-globin expression, we have identified and compared proteins associated with MafK before and after terminal erythroid differentiation, using ICAT quantitative proteomics[24–26]. We did this analysis in MEL cells[27–30], which are blocked at an early (proerythroblast) stage in the adult erythroid lineage and can be efficiently induced to undergo terminal erythroid differentiation by exposure to DMSO, resulting in an exit from the cell cycle, a 100-fold activation of adult β-major globin gene transcription and hemoglobin synthesis[31–33]. We find that the exchange of MafK-dimerization partner is a key step in the switch from the silent to the active state. This shift is associated with changes in the interaction of MafK with several co-repressors and co-activators, before and after differentiation, respectively.

RESULTS

Identification of MafK-associated proteins

MEL cells were induced to differentiate by exposure to DMSO for 4 d, and nuclear extracts[34] were prepared from both proliferating and differentiating cells. MafK-associated proteins were then purified by a single-step immunoprecipitation (IP) and differentially labeled with

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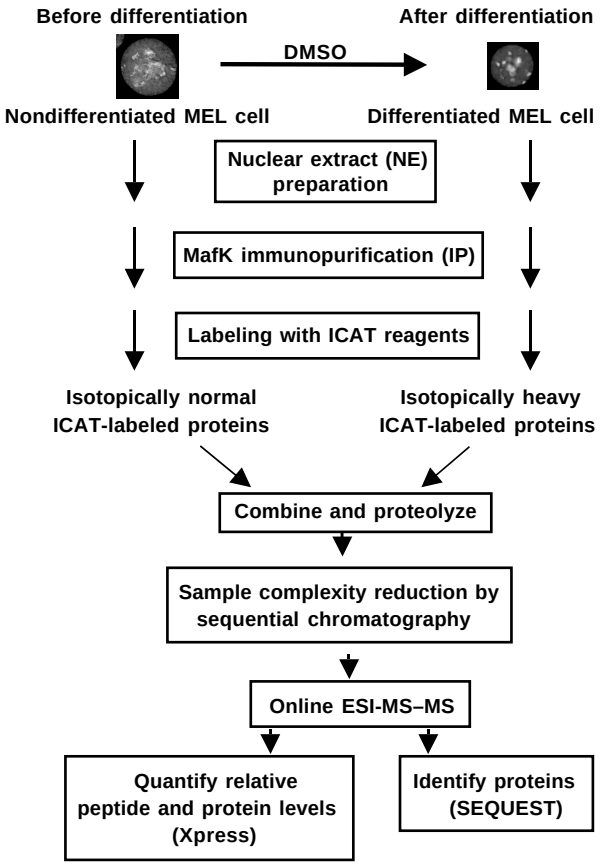

isotopically heavy (after differentiation) or normal (before differentiation) forms of the cysteine-reactive ICAT reagents (**Fig. 1**). Labeled proteins were then combined and proteolyzed. After avidin affinity purification, labeled peptides were analyzed by microcapillary reverse-phase liquid chromatography (μLC) electrospray ionization (ESI) tandem mass spectrometry (MS–MS)[35] (**Fig. 1**). The identity of the proteins was determined using the SEQUEST algorithm[36]. Relative protein abundances were calculated from the ratios of the signal intensities of identified ICAT-labeled peptides using Xpress[35]. As a negative control, mock IPs were done in parallel, from both differentiated and nondifferentiated nuclear extracts. The significance of identification of each protein was estimated using a statistical model (see Methods[37,38]).

Using this method, we identified 103 proteins as possible MafK-interacting proteins and quantified 79 (76.7%) of these (see Methods). Representative proteins were subsequently confirmed by independent experiments (see below). As equal amounts of MafK were detected in the two eluted fractions (before and after erythroid differentiation) by both quantitative mass spectrometry and western blot analysis (**Fig. 2a**), the ICAT ratios (see Methods) of the other proteins could be used directly to estimate if they were preferentially associated with MafK before or after differentiation. Based on their abundance ratios, MafK-associated proteins were separated into two categories: (i) proteins that associate with MafK primarily before erythroid differentiation (46 total; partial list, **Table 1**) and (ii) proteins that associate with MafK primarily after erythroid differentiation (25 total; partial list, **Table 2**). A complete list of differentially associated MafK-interacting proteins is presented in **Supplementary Tables 1** and **2** online. Three proteins that were not quantified (owing to a low signal-to-noise ratio) were placed in these tables based on the ratio(s) obtained for other subunit(s) of the same complex. Additional potential MafK-interacting proteins were placed in **Supplementary Tables 3** and **4** online if their ICAT ratios were either close to 1 or undetermined, respectively. In essence, it is not possible to determine (unless assessed by an independent method) whether these proteins interact with MafK both before and after differentiation, or if their differential association with MafK is masked by a low signal-to-noise ratio.

Notably, MafK interacts with a largely different subset of proteins before and after induction of differentiation (compare **Tables 1** and **2**), suggesting that this protein functions differently in proerythroblasts and differentiated erythroid cells.

Figure 1 Quantitative proteomics analysis of MafK-associated proteins purified from MEL cells before and after erythroid differentiation.

MafK exchanges its partner upon erythroid differentiation

We found that MafK interacts preferentially with the basic leucine zipper (bZIP) protein Bach1 (ref. 39) before differentiation (**Table 1** and **Fig. 2a**) when the β-globin gene is not active. After differentiation, when the β-globin gene is transcribed, MafK interacts preferentially with another bZIP protein, p45 (**Table 2** and **Fig. 2b**), forming the erythroid-specific complex NF-E2 (ref. 20). These results are in agreement with a gel-filtration analysis of MEL nuclear extracts showing that the majority of MafK coelutes in a fraction containing Bach1 but not p45 before differentiation, whereas after differentiation the peaks of MafK and p45 coelute in a fraction containing little Bach1 (**Fig. 3**).

To confirm that the exchange of MafK-dimerization partner also occurs *in vivo* in the endogenous β-globin locus during terminal

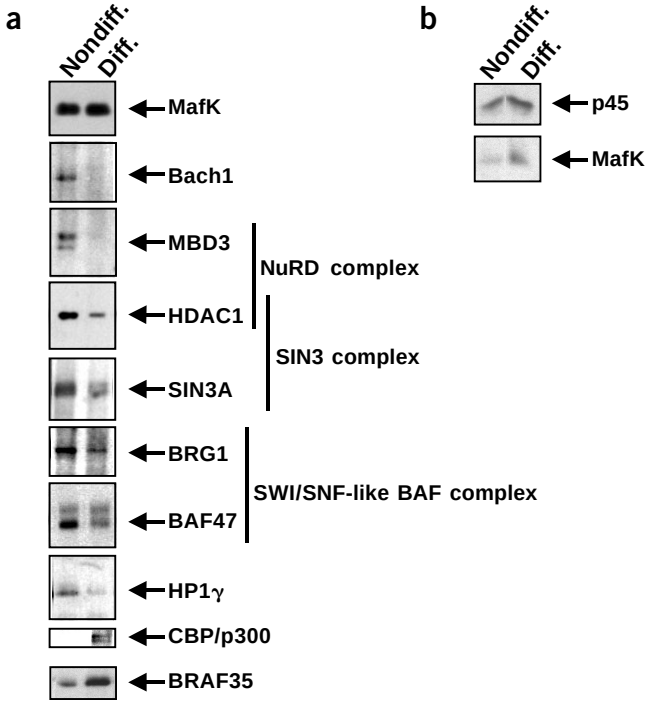

Figure 2 Western blot analysis of MafK-associated proteins purified before and after MEL cell differentiation. (**a**,**b**) Fractions eluted from a MafK (**a**) and a p45 (**b**) immunopurification. Nondiff., nondifferentiated. Diff., differentiated.

Table 1 Partial list of MafK-associated proteins before erythroid differentiation

Protein	MW (kDa)[a]	ICAT ratio ± s.d.[b]	No.[c]	Identification probability (%)[d]
Transcription				
Sequence-specific DNA-bound factors				
Bach1	81.4	2.7 ± 0.4	3	100
Basal machinery				
RNA polymerase II RPB1 subunit	217.2	2.0 ± N.D.[e]	1	96
Cofactors				
HET/SAF-B	96.7	5.6 ± N.D.[e]	1	99
HP1α and/or γ	19.7	2.0 ± 0.3	2	100
NuRD/Mi2 complex				
Mi2β homolog	94[f]	1.7 ± 0.3	3	100
MBD3	32.2	5.6 ± N.D.[e]	1	100
HDAC1	55.1	1.1 ± 0.2[g]	2	100
HDAC2	55.3	1.8 ± N.D.[e]	1	99
RbAp46	47.8	2.7 ± 0.6	4	100
RbAp48	51.8	2.0 ± 0.2	2	100
MTA1 and/or MTA3	79.3	2.6 ± N.D.[e]	1	76
BAF and/or PBAF complex (SWI/SNF-type)				
BAF190/BRG1	177.9	1.2 ± N.D.[e]	1	100
BAF53a/ARP7	47.4	1.3 ± 0.1	2	100
BAF155/SRG3	123.3	1.2 ± 0.3	2	100
BAF47/SNF5/INI1	44.1	1.1 ± 0.2[g]	2	100
Similar to hBAF170	132.7	N.D.[e]	1	99
Chromosome condensation and/or segregation				
Rad50	153.5	1.8 ± 0.1	4	100
Cohesin complex				
Rad21 homolog	72	1.3 ± N.D.[e]	1	65

[a]Molecular mass. [b]Average of the abundance ratio obtained from several peptides' ions corresponding to the indicated protein (Methods) followed by the standard deviation. [c]Number of identified cysteine-containing peptide ions. [d]Calculated as described in Methods. [e]N.D., not determined. [f]Partial sequence. [g]The preferential association of these proteins with MafK before differentiation was confirmed by western blot (**Fig. 2**). The discrepancy between the ratios obtained by ICAT measurements and western blot for these proteins was due to a high signal-to-noise ratio and/or to the slightly more stringent wash conditions of the IP analyzed by western blot.

erythroid differentiation, we performed chromatin immunoprecipitation (ChIP) experiments (Methods). Three different regions of the β-globin locus were analyzed: 5′ HS2 of the β-globin LCR, which contains a tandem of MafK-binding sites (MAREs); the embryonic stage–specific Ey-globin gene promoter, which is inactive in adult lineage MEL cells both before and after differentiation and contains no canonical MAREs; and the adult β-major globin gene promoter, which is activated during erythroid differentiation and contains no canonical MAREs (**Fig. 4a**). Consistent with our previous results[33], we found that MafK is bound to the β-globin LCR (HS2) and β-globin promoter both before and after differentiation, although it binds more stably after differentiation. More importantly and in agreement with the mass spectrometry results, Bach1 is bound to HS2 only before differentiation, whereas p45 is recruited to HS2 and the β-globin promoter after differentiation (**Fig. 4b**). Neither MafK nor p45 binds the Ey-globin promoter before or after differentiation, demonstrating the binding specificity of these factors to the LCR and the β-major globin promoter. The presence of MafK and p45 (both subunits of the NF-E2 complex) at the active β-globin promoter after differentiation is probably a result of protein-protein interaction[40], although a low-affinity DNA-binding site cannot be ruled out[41].

MafK-partner exchange correlates with changes in cofactors

Quantitative proteomics and western blot analyses revealed that several previously characterized co-repressor complexes, including the NuRD/Mi2, SIN3 and SWi/SNF-type complexes, associate preferentially with MafK before differentiation (**Table 1** and **Fig. 2a**). Indeed, all seven subunits (Mi2β, MBD3, HDAC1, HDAC2, RbAp46, RbAp48 and MTA1/MTA3) of the ATP-dependent chromatin remodeling and histone deacetylase transcriptional co-repressor complex NuRD[42] were identified as preferentially associated with MafK before differentiation. Because the SIN3 co-repressor complex shares four components (HDAC1, HDAC2, RbAp46 and RbAp48) with NuRD/Mi2 (ref. 43), and because a nonquantitative proteomics analysis revealed an interaction between MafK and the SIN3-associated protein 18 (SAP18) (data not shown), we used western blotting to investigate the SIN3A-MafK interaction during erythroid differentiation. We found that SIN3A is associated with MafK predominantly before erythroid differentiation (**Fig. 2a**). Thus before differentiation, MafK is associated with both the NuRD and the SIN3 transcriptional co-repressor complexes. Surprisingly, four subunits (BRG1, BAF53a, BAF155 and BAF47) of a SWI/SNF-like BAF chromatin remodeling complex[44] were also identified as MafK-interacting proteins before differentiation (**Table 1** and **Fig. 2a**). Although such remodeling complexes are thought to be involved in transcriptional activation[45], recent evidence suggests that they also have an important role in the repression of transcription[46]. Several other proteins implicated previously in transcriptional repression were also associated with MafK before erythroid differentiation. These include the transcriptional co-repressor HET/SAF-B (**Table 1** and **Supplementary Note** online) and the heterochromatin proteins 1 (HP1) (**Table 1** and **Fig. 2a**), which, in addition to their role in heterochromatic gene silencing[47], can be recruited to euchromatic genes by protein-protein interactions to mediate gene repression[48–50]. In summary, before the induction of differentiation, when the adult β-globin gene is inactive, MafK interacts predominantly with cofactors implicated transcriptional repression.

After differentiation, the exchange of MafK-dimerization partner from Bach1 to p45 correlates with the preferential association of transcriptional co-activator complexes with MafK. Using the ICAT strategy, we identified two subunits of the *trans*-activation

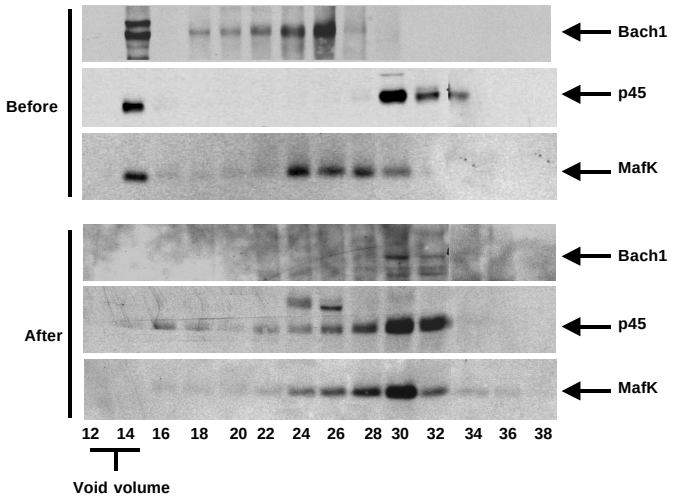

Figure 3 Gel-filtration analysis of MEL cell nuclear extracts before and after differentiation. Fractions collected after gel filtration are indicated. Fractions 14 and below correspond to the column void volume.

Table 2 Partial list of MafK-associated proteins after erythroid differentiation

Protein	MW[a] (kDa)	ICAT ratio ± s.d.[b]	No.[c]	Identification probability (%)[d]
Transcription				
Sequence-specific DNA-bound factors				
NF-E2 p45 and/or NRF2 and/or NRF3	41.6	1.9 ± N.D.[e]	1	47
Sp2 and/or 4	82	1.9 ± N.D.[e]	1	99
ZBP-89	88.7	14.3 ± N.D.[e]	1	70
Cofactors				
Ldb-1	42.8	1.4 ± 0.1	3	100
p75	59.7	1.7 ± N.D.[e]	1	100
Similar to hRHII/Gu α	93.6	1.6 ± 0.1	4	100
TIP co-activator complex				
TIP48	51.1	N.D.[e]	1	98
TIP49	50.2	2.0 ± 0.0	2	100
Architectural proteins				
BRAF35	35.9	2.1 ± 0.0	3	100
HMG2	24	1.2 ± N.D.[e]	1	10
Chromosome condensation and/or segregation				
Cohesin complex				
SmcB	143.2	1.5 ± 0.0	2	100

[a]Molecular mass. [b]Average of the abundance ratio obtained from several peptides' ions corresponding to the indicated protein followed by the standard deviation from several quantified ions. [c]Number of identified cysteine-containing peptide ions. [d]Calculated as described in Methods. [e]N.D., not determined.

domain–interacting protein (TIP) complex (TIP48 and TIP49) (**Table 2**), both of which have ATPase and helicase activities[51]. This complex is an essential co-activator of c-myc-dependent *trans*-activation[52]. Our results suggest a similar role for this complex in NF-E2-dependent activation of transcription. Although the acetyltransferase co-activator CBP/p300 was not identified in our ICAT analysis, it has been reported that p45 interacts with CBP/p300 through its *trans*-activation domain, and that this interaction is important for transcriptional activation[53,54]. Thus, we examined the association of CBP/p300 with MafK during MEL cell differentiation by western blot analysis. Consistent with our observation that MafK interacts with p45 predominantly after differentiation, we found that CBP/p300 interacts with MafK after, but not before, differentiation (**Fig. 2**). Several other proteins implicated in the co-activation of transcription were also found by quantitative mass spectrometry to interact with MafK after erythroid differentiation. These include the Lim domain–binding protein 1 (LDB1) subunit of the hematopoietic-specific transcriptional co-activator complex SCL (stem cell leukemia)[55,56], the general transcriptional co-activator p75 (ref. 57) and the c-jun co-activator RNA helicase RHII/Gu α[58] (**Table 2**). In summary, after the induction of terminal differentiation, when the β-globin gene is transcribed at high levels, MafK interacts predominantly with cofactors implicated in transcriptional activation.

Having identified a large number of MafK-associated cofactors, we next examined whether any of these proteins are differentially recruited to the β-globin locus during erythroid differentiation. Using ChIP analysis, we tested for the presence of one representative subunit from several of the identified complexes at three different locations within the β-globin locus (**Fig. 4a**) both before and after differentiation. Consistent with our IP data, the proteins MBD3 (NuRD complex), BRG1 (SWI/SNF-like BAF complexes) and SIN3A (SIN3 complex) are associated with the β-globin LCR (HS2) before differentiation (**Fig. 4b**). After differentiation, CBP and the LDB1 subunit of the SCL complex[55] replace the co-repressors within the same specific region of the β-globin locus (**Fig. 4b**). These co-activators are also concomitantly recruited to the promoter of the β-major globin gene (**Fig. 4b**). Together, these results suggest that multiple complexes can associate with the β-globin locus through MafK, indicating that multiple forms of MafK complexes exist. It remains unclear whether these different MafK complexes are present in the same locus at any given time, though it is possible, as the LCR contains several MafK-binding sites (MAREs)[8] (**Fig. 4a**). Alternatively, the cycling of factors and/or an ordered recruitment of factors to the locus could also explain the large number of bound cofactors.

Thus, using ChIP analysis we have confirmed that the identified cofactors are recruited to the β-globin locus in agreement with their respective ICAT abundance ratios, validating this experimental approach for the identification of proteins involved in β-globin gene regulation.

DISCUSSION

Although both Bach1 and p45 were known to be dimerization partners for MafK[20,30,59], a temporal regulation of these interactions had not been described; thus, it was unclear how these multiple dimerization partners function in β-globin gene regulation. Here we suggest that the role of the MafK–Bach1 heterodimer is to establish a repressed transcriptional state within the β-globin locus before differentiation. This view is compatible with previous observations that Bach1 represses transcription of a reporter gene in a MARE-dependent manner[60] and is present in large amounts at the earliest stages of hematopoiesis, before the expression of the β-globin genes[30,61]. Thus, our results provide evidence that, in addition to its previously defined role as a positive effector of β-globin gene activation after differentiation, the LCR also promotes an 'active' repression of transcription in committed cells before differentiation. To our knowledge, this is the first demonstration that the LCR can function as a *cis*-acting repressor of β-globin gene transcription in erythroid cells before terminal differentiation.

Notably, before differentiation, histone modifications associated with euchromatin (such as histone H3 acetylation) are present throughout the β-globin gene locus[33,40,62,63]. Thus the locus is already in an 'open' chromatin conformation in committed but not yet differentiated cells. Differentiation is associated with a two- to three-fold increase in histone H3 acetylation at the β-globin promoter[33,40,62,63], which is consistent with the concomitant decrease in HDAC and increase in CBP/p300 association with MafK after induction of differentiation. Although this relatively moderate increase in histone acetylation may have an important role in transcriptional activation, acetylation of other MafK-associated transcription factors and/or MafK itself, in addition to histones, may also be involved in effecting this change in transcription state[64]. Moreover, the change in the association of the chromatin remodeling complex with MafK that accompanies differentiation may be essential for the switch between repressed and activated states, for example by modifying the position and/or the structural state of nucleosomes in the β-globin locus. Consistent with this hypothesis, a differentiation-associated change in the nucleosome positioning at the β-globin promoter has been reported in MEL cells[65].

On the basis of these results, we propose a model in which, before differentiation, MafK–Bach1 heterodimers recruit co-repressor complexes (including NuRD, SIN3 and SWI/SNF-like BAF) that generate a transcriptionally repressive, but not heterochromatic, chromatin structure within the β-globin locus. Upon erythroid differentiation, an exchange of MafK-binding partner (replacement of Bach1 by the transcriptional activator p45, which may be initiated by the heme-mediated displacement of Bach1 from DNA[66]) leads to the formation of the activator complex NF-E2 on the β-globin LCR. We propose that the exchange in MafK-binding partner is a key event in the switch from a repressed to an active state of β-globin gene transcription, leading to a depletion of co-repressor complexes from and the recruitment of co-activators (CBP, TIP and SCL complexes) to the LCR and generating a transcriptionally permissive (active) structure within the β-globin locus (**Fig. 5**). The association between the exchange of heterodimerization partner, differential recruitment of co-repressors and co-activators, and differential gene activity is reminiscent of the Myc-Max-Mad transcription factor network[67].

Furthermore, many other proteins identified in this study that had not been implicated previously in the regulation of transcription of the β-globin gene (**Supplementary Tables 1–4** online) may also have important roles in this process (**Supplementary Note** online). These include DNA-binding transcription factors (Sp2 and ZBP-89) (**Table 2**) and high-mobility group architectural proteins (HMG1, HMG2 and BRAF35) (**Table 2**, **Fig. 2a** and **Supplementary Table 3** online) that could be essential in the formation of an 'enhancesome' type of *trans*-activator complex on the β-globin LCR after differentiation (**Supplementary Note** online). Moreover, the identified subunits of the cohesin molecular motor (**Tables 1** and **2** and **Supplementary Table 3** online) are promising candidates for mediating 'long-range' interactions (both negative and positive, before and after differentiation, respectively) between the LCR and the distant β-globin genes (**Supplementary Note** online).

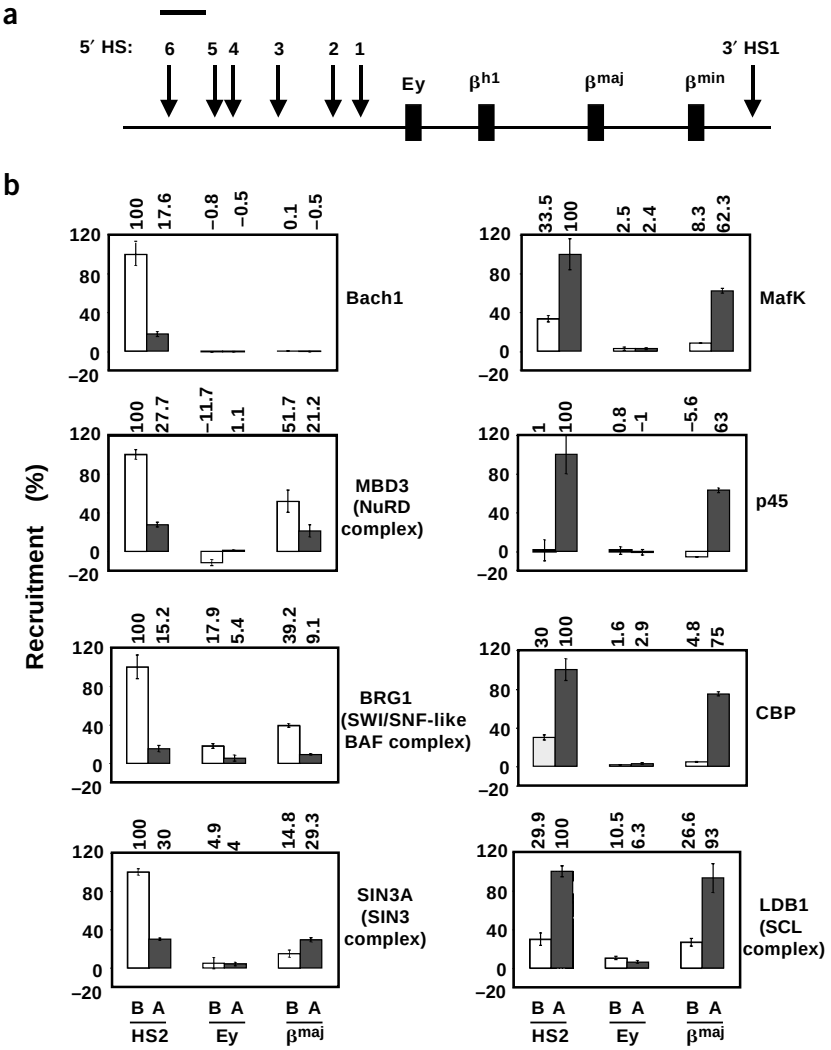

Figure 4 Analysis of the occupancy of the β-globin locus by MafK-interacting proteins during MEL cell differentiation. (**a**) Schematic of the murine β-globin locus. Globin genes are represented by black boxes; the seven DNase I hypersensitive sites (HSs) are marked by vertical arrows. Bar, 10 kb. (**b**) ChIP experiments. B, before differentiation (white bars); A, after differentiation (filled bars). HS2, hypersensitive site 2 of the β-globin LCR. Ey, embryonic globin promoter. β^{maj}, adult β-major globin promoter. Recruitment (%) represents the relative binding of a given protein to the indicated DNA region (HS2, Ey or β^{maj}) as normalized to the highest binding observed (fixed at 100%).

The use of the ICAT technique has revealed, for the first time, the complexity and dynamic nature of the interaction between MafK and other cellular proteins during erythroid differentiation. This in turn has further clarified the role of the LCR in the regulation of β-globin gene expression. As the expression of many other genes and loci may be similarly controlled by the dynamic association of protein complexes and their binding to regulatory regions, the strategy described here may provide a general approach for studying differentiation and development in other cell types.

METHODS

Immunoprecipitation. MafK-specific rabbit polyclonal antibodies (Santa Cruz Biotechnology, sc-477) were crosslinked on a protein A–Sepharose resin (Pharmacia) with 20 mM (final concentration) dimethylpimelimidate (Sigma). Nuclear extracts[34] (NEs) were incubated with the MafK antibody–bound resin at 4 °C for 12 h. Proteins bound to protein A–Sepharose–MafK antibody were then washed with IP buffer (25 mM Tris, pH 7.9, 5 mM $MgCl_2$, 10% (v/v) glycerol, 0.1% (v/v) NP40, 0.3 mM DTT and protease inhibitors) containing either 0.3 M KCl (IP used for the mass spectrometry analysis) or 0.5 M KCl (IP used for the western blot analysis) and equilibrated with IP buffer containing 100 mM KCl. Antibody-bound proteins were then eluted with 6 M urea for 2 h at 37 °C. A mock IP under the same conditions was done in parallel using normal rabbit IgG (Santa Cruz Biotechnology) instead of the MafK antibody. Eluted proteins were then either analyzed by mass spectrometry or separated on SDS-PAGE, transferred to nitrocellulose membrane and probed with the indicated antibody. Antibodies used for western blot: MafK (sc-477), Bach1 (A1-5, rabbit polyclonal antibody raised against a GST-Bach1 (residues 174–415) fusion protein), MBD3 (sc-9402), HDAC1 (Upstate Biotechnology, 05-614), SIN3A (sc-767), BRG1 (ref. 68), BAF47 (BD Transduction), HP1γ (gift from P. Chambon[69]), CBP/p300 (Upstate Biotechnology, 05-267) and BRAF35 (Upstate Biotechnology, 05-641).

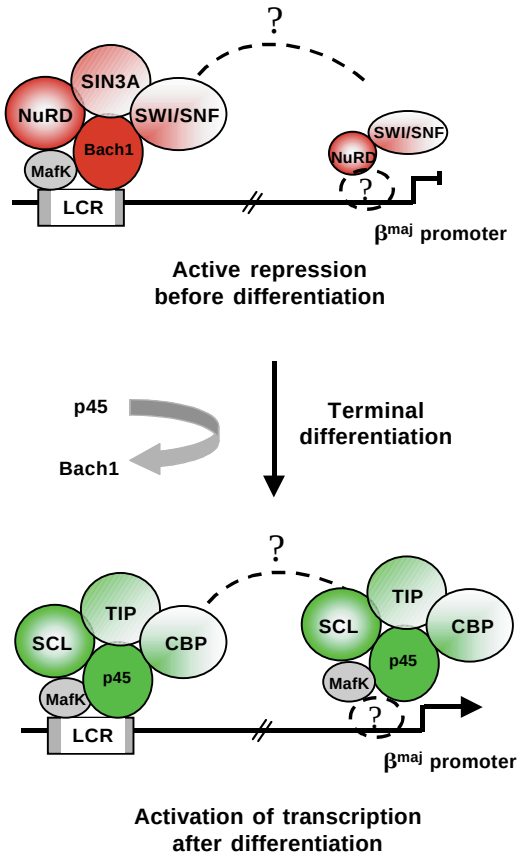

Figure 5 The exchange of MafK-heterodimerization partner leads to the activation of transcription of the adult β-major globin (β^{maj}) gene during terminal erythroid differentiation in MEL cells. Before erythroid differentiation, a heterodimer composed of the sMaf protein MafK and the repressor Bach1 recruits three types of transcriptional co-repressor complexes with chromatin remodeling and deacetylase activities (NuRD, SIN3A and SWI/SNF) to the LCR, leading to transcriptional repression of the β-major globin gene. Upon erythroid differentiation, an exchange of MafK-binding partner occurs: Bach1 is replaced by the transcriptional activator p45. This exchange leads to the removal of the co-repressor complexes from the LCR and the recruitment of co-activators (including the acetyltransferase CBP, the DNA helicase complex TIP and the hematopoietic-specific complex SCL) to the LCR, as well as to the β^{maj} promoter. This, in turn, leads to transcriptional activation of the β-globin gene. It is not yet clear whether the differentiation-associated binding of co-activators to the LCR and to the β-major promoter are dependent events.

The p45 IP was done in the same way as the MafK IP (see above) except that the antibody-bound proteins were eluted using an excess of the corresponding epitope peptide. The p45 antibody used in this IP is a monoclonal antibody (5A10) raised against a peptide (residues 78–100) corresponding to the human p45 protein. This antibody also immunoprecipitates the murine p45. Eluted proteins were transferred to nitrocellulose membrane and probed with the MafK (sc-477) and p45 (sc-291) antibodies.

ICAT labeling of MafK-associated proteins and preparation of peptides for μLC-MS–MS. Fractions eluted from the MafK IPs (each containing ~3 μg total protein) were concentrated to 25 μl each in Nanosep 3 K centrifugal devices (Pall Corporation) and treated essentially as described[26].

Preparation of the mock peptides for μLC-MS–MS. Fractions eluted from the mock IPs were treated similarly to the MafK IP eluted fractions except that the ICAT labeling reactions were replaced by a treatment with 10 mM iodoacetamide at room temperature for 20 min to alkylate the cysteines. Proteins were proteolyzed, and the resulting peptides were purified on a MCX mixed bed cation exchange cartridge (Waters Oasis MCX, 1 ml). After washing successively with Buffer A (5 mM KH_2PO_4, pH 3, 25% (v/v) CH_3CN) and 80% (v/v) CH_3CN, 0.4% (v/v) acetic acid, peptides were eluted with 10% (v/v) NH_4OH, 90% (v/v) methanol and further treated as described[26].

μLC-MS–MS and data analysis. μLC-MS–MS as described[26] was done twice independently using half of the samples in each case. The probability of each peptide (and protein) identification was estimated using an empirical statistical model based on a number of criteria including SEQUEST search scores and the number of tryptic termini of peptides[37,38]. All the MS–MS spectra were manually inspected for the match of the major product ions with theoretically predicted product ions from the database-matched peptides. The reconstructed ion chromatograms, which allow the relative quantification of peptide pairs, were also visually inspected as described[70]. An ICAT ratio for each identified protein was then calculated by averaging the abundance ratios of several peptide ions where possible. The standard deviation between those ratios was also calculated. The relative error of quantifications was estimated at ≤22% for 88% of the proteins with ≥2 quantified peptide ions. A threshold of 1.2 of the abundance ratio was used to guide the identification of proteins that were enriched either before or after differentiation. This value was determined empirically by examination of the abundance ratios obtained for MafK and for the 'nonspecific' proteins (those present in both the MafK fractions and the mock fractions). MafK, whose abundance did not vary before or after differentiation, had a ratio of 1.0 and the nonspecific proteins, for the most part, had ratios <1.2. Because of the variation of the signal-to-noise ratios among different peptides, the abundance ratios represent only tendency for enrichment and not absolute enrichment values among different proteins. Unless confirmed by western blotting, proteins detected in the mock fractions were considered nonspecific and eliminated from this analysis.

Gel filtration of MEL cell nuclear extracts. Proteins present in NEs[34] prepared from MEL cells before and after differentiation were resolved by size-exclusion chromatography using a Superose 6 column (Amersham Pharmacia) on an FPLC AKTA system following the instructions of the manufacturer (Amersham Pharmacia). Collected fractions were analyzed by western blot using antibodies against MafK (sc-477), p45 (sc-291) and Bach1 (Al.5).

Chromatin immunoprecipitation. ChIP experiments were done essentially as described[71] with some modifications. Briefly, MEL cells were crosslinked for 30 min at room temperature with 1% (w/w) final formaldehyde. Antibodies (see sources below) were coupled to Dynabeads with protein A (for rabbit antibodies) or with protein G (for goat antibodies) (Dynal) for 2 h at room temperature in IP buffer containing 100 mM KCl. Resin-bound antibodies were then washed extensively with sonication buffer (50 mM HEPES, pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% (w/v) Triton X-100, 0.1% (v/v) sodium deoxycholate, 0.1% (w/v) SDS and protease inhibitors) and equilibrated with 1 ml sonication buffer containing 2 μg sonicated λ DNA and 1 mg ml^{-1} ovalbumin, before incubation with the precleared chromatin overnight at 4 °C. Each experiment was carried out at least twice independently. Antibodies: normal rabbit IgG (sc-2027), normal goat IgG (sc-2028), MafK (sc-477), p45 (sc-291), Bach1 (Al-5), MBD3 (sc-9402), BRG1 (ref. 68), SIN3A (sc-767), CBP (sc-369) and LDB1 (sc-11198).

Real-time PCR and data analysis. Each fraction (inputs and elutions) was analyzed by real-time PCR using the ABI Prism 7700 sequence detector following the instructions of Applied Biosystems for TaqMan probes. Each real-time PCR experiment was done in duplicate at 50 °C for 2 min and 94 °C for 10 min, followed by 40 cycles at 94 °C for 20 s, 55 °C for 20 s and 72 °C for 30 s. Data were collected at 72 °C and analyzed using the 'standard curve' method (ABI). The standard curve was obtained using mouse genomic DNA and was linear (correlation coefficient >0.985 minimum). The enrichment of the target sequence was calculated by dividing the amount of target sequence in the elution fraction by the amount in the input fraction. The value obtained for the mock ChIP (using normal rabbit or goat IgG) was then subtracted from the value obtained for the specific antibody in each experiment. All enrichments are indicated as a percentage of the highest enrichment obtained for each antibody. Primers and TaqMan fluorescent probes were selected using ABI Primer Express, and were all obtained from Integrated DNA Technology (IDT). The primers and probes

were as follows: 5′ HS2: primers, 5′-CAGAGGAGGTTAGCTGGGCC and 5′-CAAGGCTGAACACACCCACA; probe, 5′-6FAM-AGGCGGAGTCAATTCTCTACTCCCCACC-BHQ-1. The generated 69-bp amplicon comprises positions –9805 to –9736 with the transcription start site from the Ey-globin promoter being the +1 position. Ey promoter: primers, 5′-CTTCAAAGAATAATGCAGAACAAAGG and 5′-CAGGAGTGTCAGAAGCAAGTACGT; probe, 5′-6JOE-ATTGTCTGCGAAGAATAAAAGGCCACC-ACTT-BHQ-1. The generated 101-bp amplicon comprises positions –77 to +24. β-major promoter: primers, 5′-CTGCTCACACAGGATAGAGAGGG and 5′-GCAAATGTGAGGAGCAACTGATC; probe, 5′-6FAM-AGCCAGGGCAGAGCATATAAGGTGAGGT-BHQ-1. The generated 79-bp amplicon comprises positions 28930–29009.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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