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NFE2 regulates transcription of multiple enzymes in the heme biosynthesis pathway

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During erythroid differentiation, cooperative activities of erythroid transcription factors coordinate transcript levels of genes required for correct maturation¹. These genes include the eight heme biosynthetic enzymes that are sequentially upregulated to ensure augmented heme synthesis essential for the formation of hemoglobin.

The transcription factor nuclear factor erythroid 2 (NFE2) plays a pivotal role in erythroid maturation and, together with other erythroid transcription factors such as EKLF and GATA1¹, controls the transcription of erythroid-specific genes including β-globin². Furthermore, NFE2 is necessary for the transcriptional activation of two heme biosynthetic enzymes, porphobilinogen deaminase (PBGD)³ and ferrochelatase (FECH)⁴.

This lead us to investigate the role of NFE2 in the regulation of the remaining heme biosynthetic enzymes. 

*In silico* analysis revealed potential NFE2 consensus sites within promoter and upstream regions of five enzymes besides PBGD and FECH: aminolevulinic acid dehydratase (ALAD), uroporphyrinogen III synthase (UROS), uroporphyrinogen III decarboxylase (UROD), coproporphyrinogen oxidase (CPOX) and protoporphyrinogen oxidase (PPOX) (Fig. 1A). Chromatin immunoprecipitation (ChIP) assays using K562 cells⁵ confirmed *in vivo* binding of NFE2 to the UROS erythroid promoter at the -27 bp binding site (Fig. 1B, second row). NFE2 also bound the sequence between bp -452 and bp -183 of the UROD promoter, encompassing two predicted consensus sites at bp -391 and bp -311 (Fig. 1B, third row). Finally, we observed strong NFE2 binding in the sequence containing the -5161 bp site of the CPOX promoter (Fig. 1B, fourth row). The other sites investigated were not bound by NFE2. This suggests that UROS, UROD and CPOX constitute novel direct NFE2 target genes and raises the question whether NFE2 activity is required for transcription of these genes.
To investigate this, we depleted human erythroleukemia (HEL) cells of NFE2 by transduction with a lentiviral construct containing either an shRNA against NFE2 (shNFE2) or a scrambled RNA as a negative control. Western blot analysis demonstrated a strong reduction in NFE2 protein expression after transduction with shNFE2 (Fig. 1C). The expression levels of ALAD, PBGD, UROS, UROD and CPOX were determined by quantitative real-time PCR (Fig. 1D). mRNA expression of both UROS and CPOX was decreased by 60% and 25%, respectively, in HEL cells silenced for NFE2 expression. UROD expression showed a slight, but reproducible reduction, which did not reach statistical significance. As expected, ALAD mRNA levels remained unchanged while the mRNA levels of PBGD, which is a known NFE2 target, were reduced by approximately 40% in HEL cells following NFE2 knockdown. These data demonstrate that NFE2 activity is required for full expression of UROS and CPOX and contributes to UROD expression, confirming these enzymes as novel NFE2 target genes. Consistent with this decrease in mRNA levels, HEL cells silenced for NFE2 displayed a reduction in overall cellular heme content by 20% as determined by a microfluorometric assay (Fig. 1E). These data underscore the critical role of NFE2 in controlling heme biosynthesis.

Because UROS expression was most strongly reduced upon NFE2 knockdown, we wished to further delineate the effect of NFE2 on the UROS promoter. We therefore cloned either a region encompassing the proximal 1438 bp of the UROS promoter (UROS-1438), which includes both sites at bp -27 and at bp -984, or a fragment encoding 790 bp (UROS-790), which only includes the site at bp -27, into luciferase reporter gene vectors (Fig. 2A). The resulting constructs were transfected into HEK293T cells in the presence or absence of expression vectors encoding NFE2 and MafG as previously described. Cotransfection of both NFE2 and MafG with the UROS-1438 construct resulted in a 2.5-fold increase in UROS-promoter-driven luciferase activity (Fig. 2B). Cotransfection of NFE2 and MafG with the UROS-790 construct likewise achieved a 2.2-fold increase, showing that sequences upstream of bp -790 do not contribute to the transactivation of the UROS promoter by NFE2 (Fig. 2C). Mutation of the NFE2 binding site at bp -27 completely abrogates NFE2 transactivation of the UROS promoter (Fig. 2D), identifying this site as the NFE2 target sequence. These data demonstrate that NFE2 is able to directly transactivate the UROS erythroid promoter and corroborate the ChIP assays, demonstrating NFE2 binding to this sequence in vivo (Fig. 1B).

Taken together, our results demonstrate that NFE2 modulates the heme biosynthesis pathway in erythroid cells at multiple steps and point to the existence of a novel regulatory pathway (Fig. 2E). A critical step during erythropoiesis is the upregulation of the erythroid isoform of the first heme biosynthetic enzyme, 5-aminolevulinic acid synthase 2 (ALAS2).
followed by a sequential upregulation of the remaining enzymes. Mouse erythroleukemia cells transduced with an antisense RNA against ALAS2 show decreased levels of NFE2, suggesting that ALAS2 expression positively regulates NFE2 expression. Thus, the initial induction of erythroid heme synthesis by activation of ALAS2 will result in increased NFE2 activity. Furthermore, hemin induction increases the activity of NFE2. NFE2 cooperates with other erythroid transcription factors that regulate the expression of heme biosynthetic enzymes, such as EKLF and GATA1. Together, this will lead to transcriptional activation of successive enzymes in the pathway, with NFE2 contributing particularly to the activation of PBGD and FECH and as shown here of UROS and potentially to UROD and CPOX (Fig. 2E).

For hemoglobin synthesis, globin proteins and heme molecules are needed at a fixed stoichiometric ratio. NFE2 is a crucial regulator of β-globin transcription in erythroid cells. Regulation of both pathways by a common transcription factor such as NFE2 provides an attractive possibility of fine-tuning hemoglobin synthesis and avoiding both an excess production of individual components and an accumulation of possibly toxic metabolites.

Based on our data, we propose that NFE2 regulates the transcription of several downstream enzymes of the heme biosynthesis pathway after the initial induction by activation of ALAS2. Thereby, NFE2 may establish a positive feedback loop (Fig. 2E) that secures a constant supply of both heme and globin for the assembly of hemoglobin and ensures coordinated levels of synthesis of both proteins during erythroid differentiation.

**Authorship and Disclosures**

LR designed and performed experiments, collected, analyzed and interpreted data, generated figures and wrote the manuscript.

TSS and JW designed experiments and analyzed data.

HLP designed experiments, interpreted data and wrote the manuscript.

The authors declare no possible conflict of interest.
References


**Figures and Figure Legends**

**Figure 1:** (A) Predicted NFE2 consensus sites in the promoters of heme biosynthetic enzymes. (B) Chromatin immunoprecipitation of K562 cells was performed with either an NFE2 antibody or an unrelated IgG control, as indicated. Precipitated DNA was amplified using primers covering the predicted binding sites shown in A. In the lane marked “Input”, a 1:25 dilution of the input DNA was used. As an additional control, a genomic region of myogenin known not to interact with NFE2 was amplified (bottom panel). (C-E) HEL cells were treated with a lentiviral construct containing either shRNA against NFE2 (shNFE2) or scrambled RNA as negative control. (C) Western blot analysis of NFE2 protein expression. Equal protein loading was assessed by reprobing with an antibody against GAPDH. (D) mRNA expression of ALAD, PBGD, UROS, UROD, CPOX and the housekeeping gene β-2-microglobulin (β-2-M) was quantified by qRT-PCR. Expression levels of the heme biosynthetic enzymes were normalized to β-2-M expression and the expression in cells treated with scrambled RNA set to 1. Values are reported as fold change shRNA versus scrambled RNA treated cells and represent the means and standard error of the mean of four individual experiments measured in duplicates. *p< 0.05; **p< 0.01; ***p< 0.001 one sample t-test. (E) Heme content of the cells was determined by measuring the fluorescence at an excitation wavelength of 400 nm and an emission wavelength of 662 nm. Values are depicted relative to heme content in cells treated with scrambled RNA, which was set to one. The results represent the means and standard error of the mean of two independent transfections with five independent measurements each. * p< 0.05 paired t-test.

**Figure 2:** (A) Schematic representation of the UROS erythroid promoter and sequences inserted into reporter gene constructs. The constructs include bp -1438 to bp 105 (UROS-1438) and bp -790 to bp 105 (UROS-790), respectively, of the erythroid promoter. NFE2 sites predicted by *in silico* analysis and investigated by ChIP are indicated by open boxes. (B-C) The constructs were transfected into HEK293T cells together with expression plasmids for MafG, NFE2 or both as indicated. 24 hours after transfection, luciferase activity was determined. Results were normalized for transfection efficiency by cotransfection of a Tk-driven Renilla luciferase reporter gene vector and for background luminescence of the empty pGL3 luciferase vector as previously reported. Values are given as fold activation with NFE2 and MafG compared to an internal calibrator consisting of the reporter constructs transfected in the presence of MafG expression vectors alone, which was set to 1. Results represent the means and standard deviation of at least three independent experiments, each performed in duplicate. * p< .05 one-way Mann Whitney test. (D) By site-directed mutagenesis, a reporter construct containing a mutated NFE2 site at bp -27 (UROS-790mut-27) was generated. The constructs were co-transfected into HEK293T
cells together with expression plasmids for NFE2 and MafG. Bar graphs represent the mean and standard deviation of at least three independent experiments, each performed in duplicate. * p< .05 one-way Mann Whitney test.

(E) Proposed model of regulation of heme synthesis by NFE2
Figure 1:

A

B

C

D

E
Figure 2:

A Erythroid promoter

Exon 2A

-984

-27

UROS -1438

UROS -790

B

UROS -1438

fold activation

NFE2

MafG

- +

- +

* +

C

UROS -790

fold activation

NFE2

MafG

- +

- +

* +

D

UROS -1438

-984

-27

UROS -790

-27

UROS -790mut-27

-27

fold activation

*+

E

ALAS2

ALAD

PBGD

UROS

UROD

CPOX

PPOX

FECH

HEME

α-/β-globin

HEMOGLOBIN