Nur77 variants solely comprising the amino-terminal domain activate hypoxia-inducible factor-1α and affect bone marrow homeostasis in mice and humans

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Gene targeting via homologous recombination can occasionally result in incomplete disruption of the targeted gene. Here, we show that a widely used Nur77-deficient transgenic mouse model expresses a truncated protein encoding for part of the N-terminal domain of nuclear receptor Nur77. This truncated Nur77 protein is absent in a newly developed Nur77-deficient mouse strain generated using Cre-Lox recombination. Comparison of these two mouse strains using immunohistochemistry, flow cytometry, and colony-forming assays shows that homologous recombination–derived Nur77-deficient mice, but not WT or Cre-Lox–derived Nur77-deficient mice, suffer from liver immune cell infiltrates, loss of splenic architecture, and increased numbers of bone marrow hematopoietic stem cells and splenic colony–forming cells with age. Mechanistically, we demonstrate that the truncated Nur77 N-terminal domain protein maintains the stability and activity of hypoxia-inducible factor (HIF)-1, a transcription factor known to regulate bone marrow homeostasis. Additionally, a previously discovered, but uncharacterized, human Nur77 transcript variant that encodes solely for its N-terminal domain, designated TR3β, can also stabilize and activate HIF-1α. Meta-analysis of publicly available microarray data sets shows that TR3β is highly expressed in human bone marrow cells and acute myeloid leukemia samples. In conclusion, our study provides evidence that a transgenic mouse model commonly used to study the biological function of Nur77 has several major drawbacks, while simultaneously identifying the importance of nongenomic Nur77 activity in the regulation of bone marrow homeostasis.

In response to inflammatory stimuli and other danger signals, the bone marrow can give rise to a wide range of specialized immune cells. The production of such immune cells is tightly regulated at each step of differentiation from hematopoietic stem and progenitor cell (HSPC)5 to functional leukocyte (1). HSPCs reside in specific areas of the bone marrow, termed the HSPC niche, where their survival is supported by low oxygen tension and concomitant induction of the transcription factor hypoxia-inducible factor (HIF)-1α (2). Tight control of HIF-1 expression and activity is crucial for both bone marrow homeostasis and the biological function of HSPCs (3). Elevated expression of this factor may enhance HSPC mobilization from the bone marrow into the circulation (4).

Nur77 (Nr4a1) is a transcription factor belonging to the NR4A nuclear receptor family. Together with its family members Nur1 (Nr4a2) and NOR-1 (Nr4a3), it regulates a wide variety of biological processes, including inflammation, metabolism, and apoptosis (5). Like other nuclear receptors, Nur77 consists of three protein domains: the N-terminal domain, the DNA-binding domain (DBD), and the ligand-binding domain (LBD). Nur77 is known to play an important role in various aspects of hematopoiesis. For example, the differentiation of Ly6Cε+ patrolling monocytes from more naive myeloid progenitors crucially depends on Nur77, as Nur77-deficient mice essentially lack this subset of monocytes (6). Additionally, a dual deficiency of Nur77 and its family member NOR-1 leads to rapid development of acute myeloid leukemia (AML), with bone marrow differentiation being heavily skewed toward myeloid progeny (7). Finally, Nur77-deficient mice suffer from spontaneous, systemic inflammation with age characterized by excessive immune cell infiltration of various peripheral organs (8).

Whole-body Nur77-deficient mice generated via homologous recombination (B6;129S2-Nr4a1tm1Jmi/J, JAX stock no. 006187) have been used in a plethora of studies to investigate the biological functions of Nur77. However, the gene-targeting

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This article contains Table S1 and Figs. S1–S4.

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strategy originally used to generate these mice disrupts neither the transcriptional nor the translational start site of the Nr4a1 gene locus (9). Instead, a neomycin resistance gene (Neo9) cassette is inserted ~300 bp downstream of the Nr4a1 translational start site. Although such a targeting strategy could leave the Nr4a1 gene locus nonfunctional, previous reports have shown that disrupting gene loci downstream of the translational start site can lead to the production of truncated but biologically active proteins (10, 11).

Here, we show that Nur77-deficient mice generated via homologous recombination (referred to as classical Nur77-KO mice) develop bone marrow, liver, and spleen abnormalities with age, which are absent in similarly aged WT mice or a newly generated Cre-Lox system–derived Nur77-deficient mouse line (referred to as complete Nur77-KO mice). Classical Nur77-KO mice still express part of the N-terminal domain of Nur77, which is not present in complete Nur77-KO mice. We show that this truncated Nur77 protein can stabilize and activate HIF-1 in vitro. In vivo, classical Nur77-KO mice maintain the expression of HIF-1 and its target genes in the bone marrow at WT levels, whereas complete Nur77-KO mice do not. Given that both classical Nur77-KO and complete Nur77-KO mice lack Nur77 transcriptional activity, these mice have altered expression of bone marrow mobilization genes. Finally, we show that the truncated N-terminal domain protein may be relevant in human disease, because a hitherto uncharacterized transcript variant of human Nur77, also encoding solely for the truncated N-terminal domain of full-length Nur77 protein, is expressed in human bone marrow and AML samples, as opposed to the full-length Nur77 protein.

Results

Classical Nur77-KO mice, but not WT or complete Nur77-KO mice, exhibit liver immune cell infiltrates and loss of splenic architecture at old age

It has previously been reported that the classical Nur77-KO mice, generated via homologous recombination, display systemic inflammation and immune cell infiltrates in liver and spleen upon aging (8). We decided to investigate whether this phenotype is a true hallmark of Nur77 deficiency by comparing WT and the classical Nur77-KO mice with the newly developed complete Nur77-KO mice, in which exons 2–4 of the Nr4a1 gene are removed using cytomegalovirus promoter-driven Cre-Lox recombinase. We confirmed the presence of liver immune cell infiltrates in our 6.5-month-old classical Nur77-KO mice (Fig. 1A). Additionally, we found that these infiltrates consist of macrophages (IBA1–positive), T-cells (CD3-positive), and B-cells (B220-positive; Fig. 1B). Interestingly, these liver infiltrates were not present in age-matched WT or complete Nur77-KO mice (Fig. 1A). Similarly, immunohistochemical assessment of the spleen revealed a loss of splenic architecture in aged classical Nur77-KO mice, but not in aged WT or complete Nur77-KO mice (Fig. 1C). This altered spleen morphology was accompanied by a lower intensity staining for T-cells (CD3), B-cells (B220), and macrophages (IBA-1) in classical Nur77-KO mice when compared with both WT and complete Nur77-KO mice, suggesting that spleens of classical Nur77-KO mice contain more naive immune cell progenitors (Fig. 1C).

In response to infections and other inflammatory stimuli, the spleen and liver may function as emergency sites of extramedullary hematopoiesis (12). We therefore speculated that the immune cell liver infiltrates and splenic disorganization observed exclusively in classical Nur77-KO mice might arise from abnormalities in their bone marrow and hematopoiesis. There were no differences in the total number of bone marrow cells between either 3-month-old or 6.5-month-old WT, classical Nur77-KO, and complete Nur77-KO mice (Fig. S1C). However, bone marrow from classical Nur77-KO mice did give rise to significantly more bone marrow–derived macrophages than either WT or complete Nur77-KO bone marrow, suggesting that the bone marrow in classical Nur77-KO mice is skewed toward myeloid progeny (Fig. S1D). Furthermore, classical Nur77-KO mice had significantly more HSPCs, defined as lineage-negative (Lin−), Sca1-positive (Sca1+), and ckit-positive (cKit+) cells (LSK cells), in the bone marrow than either WT or complete Nur77-KO mice at 6.5 months of age (Fig. S1E). Moreover, we observed that spleens isolated from classical Nur77-KO mice gave rise to significantly more HSPC-derived colonies than spleens from either WT or complete Nur77-KO mice did (Fig. S1F). There were no significant differences in splenic LSK cell counts or the colony-forming capacity of bone marrow between the three mouse strains (Fig. S1, E and F).

Together, these results suggest that the liver immune cell infiltrates and splenic disorganization observed exclusively in classical Nur77-KO mice are due to aging-induced irregularities in their bone marrow compartment, leading to increased seeding of peripheral tissues, such as the spleen and liver, with hematopoietic progenitor cells.

Classical Nur77-KO mice express part of the N-terminal domain of full-length Nur77

We were intrigued by the phenotypic differences between aged classical Nur77-KO and complete Nur77-KO mice and decided to investigate a possible cause for this discrepancy in more detail. Classical Nur77-KO mice were originally generated by insertion of a Neo9 cassette in exon 2 of the Nr4a1 locus (9). This gene-targeting strategy leaves both the transcriptional and translational start sites of the Nr4a1 gene locus entirely intact (Fig. 2A and Fig. S2A). It has previously been shown that such gene-targeting strategies can lead to the production of aberrant mRNA and protein products from the targeted gene locus (10, 11). Therefore, we assessed whether classical Nur77-KO mice still produce mRNA and protein from the Nr4a1 gene locus. Nur77 mRNA expression was measured using primer sets targeting either exons 3 and 4, which are present in WT but absent in both Nur77-KO mouse strains, or targeting exon 2, which is present in WT and classical Nur77-KO mice but absent in complete Nur77-KO mice. In bone marrow cells and in bone marrow–derived macrophages
Figure 1. Classical Nur77-KO mice, but not WT or complete Nur77-KO mice, exhibit liver immune cell infiltrates and loss of splenic architecture at old age. A, hematoxylin and eosin (H&E) staining showing liver morphology of 6.5-month-old WT, classical Nur77-KO, and complete Nur77-KO mice. The asterisks indicate immune cell infiltrates present exclusively in classical Nur77-KO mice. B, representative immunohistochemical characterization of immune cell infiltrates in livers of 6.5-month-old classical Nur77-KO mice. C, morphological and immunohistochemical characterization of spleens from 6.5-month-old WT, classical Nur77-KO, and complete Nur77-KO mice. For B and C, CD45 staining was used to detect leukocytes, IBA-1 staining for macrophages, CD3 staining for T cells, and B220 staining for B cells. All stainings were performed on consecutive sections. Representative images of four mice per group are shown. Scale bar, 500 μm. See also Fig. S1.
Figure 2. Classical Nur77-KO mice express part of the N-terminal domain of full-length Nur77. A, schematic overview of the mouse Nur77 (Nr4a1) gene locus and resulting RNA and protein products in WT, classical Nur77-KO, and complete Nur77-KO mice. Nr4a1 exons are numbered E1–E7. TSS, transcriptional start site; START, translational start site; STOP, translational termination codon; NTD, N-terminal domain. B and C, Nr4a1 (Nr4a1) mRNA expression in bone marrow and LPS-stimulated BMDMs from WT, classical Nur77-KO, and complete Nur77-KO mice using primers targeting either Nr4a1 exon 3–exon 4 (B) or exon 2 (C). D, immunoprecipitated Nur77WT and Nur77(1–117) protein in untreated or 16-h LPS-treated BMDMs from WT, classical Nur77-KO, and complete Nur77-KO mice detected by WB. Numbers on the left, molecular mass in kDa. E, Western blotting for Nur77 protein expression in RAW264.7 cells transiently overexpressing Myc-Nur771-117 protein. Numbers on the left, molecular mass in kDa. For B and C, data are shown as mean ± S.D. (error bars) (n = 3–4). p values were calculated using one-way ANOVA (for bone marrow) or two-way ANOVA (for BMDMs; significance indicated for comparison of WT versus complete Nur77-KO and classical Nur77-KO versus complete Nur77-KO) with the Tukey post hoc test. *, p < 0.05; **, p < 0.01; ***, p < 0.001. See also Fig. S2. AU, arbitrary units; n.d., not determined.
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(BMDMs) stimulated with lipopolysaccharide (LPS; a known inducer of Nur77 expression (13)), cells from classical Nur77-KO mice did not produce detectable Nur77 mRNA when using a primer set targeting exons 3 and 4 (Fig. 2B) but had high Nur77 mRNA expression when using the primer set targeting exon 2 (Fig. 2C). Importantly, neither primer set could detect Nur77 mRNA in cells from complete Nur77-KO mice, whereas both primer sets detected Nur77 mRNA in cells from WT mice (Fig. 2, B and C). Similar results were found for spleen and liver mRNA of these three mouse lines (Fig. S2B).

To assess whether the Nur77 mRNA produced in classical Nur77-KO mice is translated into protein, we used a combination of Nur77 N-terminal domain–specific antibodies for immunoprecipitation (IP) and Western blotting (WB) to detect endogenous Nur77 protein as described previously (14). Full-length Nur77 protein was successfully detected in WT BMDMs stimulated with LPS (Fig. 2D, left). Neither classical Nur77-KO nor complete Nur77-KO BMDMs expressed full-length Nur77 protein, but we did observe a smaller protein of ~21 kDa exclusively in LPS-stimulated classical Nur77-KO BMDMs (Fig. 2D, right). Notably, this smaller protein (referred to as Nur77(1–117) here) was not expressed in BMDMs of WT or complete Nur77-KO mice (Fig. 2D). We hypothesized that Nur77(1–117) may be the product of the aberrant mRNA expressed in classical Nur77-KO mice, which encodes for the first 115 amino acids of full-length Nur77 plus 2 amino acids from the subsequent Neo cassette (Fig. S2A). We cloned the putative coding domain sequence of the aberrant mRNA expressed in classical Nur77-KO mice and overexpressed it in RAW264.7 macrophages, which also resulted in a single band of ~21 kDa (Fig. 2E). Together, these results show that an aberrant Nur77 mRNA and protein is produced from the endogenous Nr4a1 gene locus exclusively in classical Nur77-KO mice.

The cellular localization of full-length Nur77 is tightly regulated via two nuclear localization signals in its DBD and three nuclear export signals in the LBD (15), all of which are absent in the Nur77(1–117) protein (Fig. 2A). Therefore, we assessed the cellular localization of Nur77(1–117) protein in RAW264.7 macrophages using immunofluorescence (IF) microscopy, which showed that overexpressed full-length Nur77 is contained entirely in the nucleus, whereas Nur77(1–117) is present throughout the cell (Fig. S2C). Consistently, IF microscopy using two different Nur77 N-terminal domain–specific antibodies showed that BMDMs from classical Nur77-KO mice expressed a protein, which is localized throughout the cell, while BMDMs from complete Nur77-KO mice did not (Fig. S2D). Taken together, these results show that the aberrant Nur77 mRNA produced in classical Nur77-KO mice is translated into a protein that encodes for part of the N-terminal domain of Nur77 and that this protein is localized in both the nucleus and cytoplasm of the cell.

**Nur77(1–117) increases HIF-1α transcriptional activity, and HIF-1α protein and target gene expression are elevated in bone marrow of classical Nur77-KO mice**

Having established that classical Nur77-KO mice express part of the Nur77 N-terminal domain, we next investigated possible mechanisms that may be responsible for the bone marrow and spleen phenotypes observed exclusively in these mice. It has previously been shown that classical Nur77-KO mice have significantly reduced numbers of circulating Ly6C<sup>lo</sup> monocytes (6). We speculated that a loss of these patrolling monocytes plays a role in the immune cell infiltrates observed in these mice. However, both classical Nur77-KO and complete Nur77-KO mice showed a significant decrease in circulating Ly6C<sup>lo</sup> monocytes and an increase in circulating Ly6C<sup>hi</sup> monocytes (Fig. S3A). It is therefore highly unlikely that altered differentiation of Ly6C<sup>lo</sup> monocytes is the cause of the spleen and liver abnormalities observed exclusively in classical Nur77-KO mice.

The increased presence of hematopoietic progenitor cells and differentiated leukocytes in peripheral tissues of classical Nur77-KO mice (Fig. 1) could also be indicative of increased HSPC mobilization from the bone marrow in these mice. HSPC mobilization can be induced by various inflammatory cytokines and chemokines (16), including the chemokine stromal cell–derived factor-1 (SDF-1α) (17). Nur77 is known to repress SDF-1α expression in macrophages (18, 19), and we have previously reported that pro-atherosclerotic Ldr<sup>−/−</sup> mice that receive a transplantation of classical Nur77-KO bone marrow have higher circulating SDF-1α levels than mice that received WT bone marrow (19). However, we now report that circulating SDF-1α plasma levels are not changed between unchallenged WT, classical Nur77-KO, and complete Nur77-KO mice at either 3 or 6.5 months old (Fig. S3B). Similarly, expression of the inflammatory cytokine genes Il6 and Tnf was unchanged in spleens and livers of 6.5-month-old WT, classical Nur77-KO, and complete Nur77-KO mice (Fig. S3C). As such, altered levels of the above-mentioned cytokines are probably not the cause of the phenotypes observed in classical Nur77-KO mice.

In addition to induction by external cytokines, HSPC mobilization is also tightly regulated by various signaling pathways and transcription factors. One such transcription factor is HIF-1α, which plays an important role in survival of HSPCs (3). Pharmacological stabilization of HIF-1α protein has been shown to significantly enhance HSPC mobilization (4). Interestingly, it has also been shown that Nur77 can stabilize HIF-1α protein by inhibiting MDM2-induced degradation and that the N-terminal domain of Nur77 by itself is sufficient for this stabilization to occur (20). Therefore, we considered the possibility that the N-terminal domain–encoding Nur77(1–117) protein expressed in classical Nur77-KO mice regulates HIF-1α stability and thereby causes the bone marrow, spleen, and liver phenotypes observed in these mice. Consistent with this hypothesis, lentiviral overexpression of Nur77(1–117) protein in BMDMs from complete Nur77-KO mice resulted in increased HIF-1α protein expression in response to the hypoxia mimetic cobalt chloride (Fig. 3A). Overexpressing either full-length Nur77 or Nur77(1–117) protein in RAW264.7 macrophages also significantly increased HIF-1 transcriptional activity in response to cobalt chloride, deferoxamine (DFO), or hypoxic growth conditions (Fig. 3B). These results demonstrate that Nur77(1–117) overexpression can increase HIF-1α protein stability and HIF-1 transcriptional activity in vitro.

**In vivo**, we found that HIF-1α protein expression in the bone marrow was similar between WT and classical Nur77-KO mice,
Figure 3. Nur77(1–117) increases HIF-1α transcriptional activity, and HIF-1α protein and target gene expression are elevated in bone marrow of classical Nur77-KO mice. A, Western blotting for HIF-1α protein in complete Nur77-KO BMDMs overexpressing Nur77(1–117) protein or GFP as a control and left untreated or stimulated with 100 μM CoCl₂ for 6 h. β-Actin was used as loading control. Numbers on the right, molecular mass in kDa. Numbers at the bottom, fold change (FC) in β-actin–normalized HIF-1α band intensity relative to unstimulated GFP-transduced cells. B, luciferase reporter assay for HIF-1α transcriptional activity in RAW264.7 transiently overexpressing full-length Nur77 or Nur77(1–117) protein and left untreated or stimulated with 100 μM CoCl₂, 100 μM DFO or cultured at 1% O₂ (hypox.). GFP was included as a negative control. HRE, hypoxia response element. C, Western blotting for HIF-1α protein in bone marrow of 6.5-month-old WT, classical Nur77-KO, and complete Nur77-KO mice. β-Actin was used as loading control. Numbers on the right, molecular mass in kDa. The bar graph shows quantification of HIF-1α band intensity normalized to β-actin band intensity. D, mRNA expression of HIF-1α target genes in bone marrow of 6.5-month-old WT, classical Nur77-KO, and complete Nur77-KO mice. E and F, mRNA expression of bone marrow mobilization-inducing (E) or retention-inducing (F) genes in bone marrow of 6.5-month-old WT, classical Nur77-KO, and complete Nur77-KO mice. For B–F, data are shown as mean ± S.D. (error bars) (n = 3 for B and C, n = 4 for D–F). p values were calculated using two-way (B) or one-way (C–F) ANOVA with Tukey post hoc test. *, p < 0.05; **, p < 0.01; ***, p < 0.001 for comparisons indicated by horizontal lines or versus WT (D). ns, not significant. See also Fig. S3.
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whereas complete Nur77-KO mice had significantly less HIF-1α when compared with classical Nur77-KO mice (Fig. 3C). This result is consistent with the idea that HIF-1α is stabilized by a nongenomic function of full-length Nur77 in WT mice and by Nur77(1–117) in classical Nur77-KO mice, but not stabilized in complete Nur77-KO mice. Moreover, the lower level of HIF-1α protein in complete Nur77-KO mice was accompanied by decreased expression of several established HIF-1 target genes in the bone marrow, whereas the expression of these same genes was unchanged between WT and classical Nur77-KO mice (Fig. 3D). Finally, we found that gene expression of two chemokine receptors important for the induction of HSPC mobilization, Cxcr4 and Csf3r (21, 22), was significantly higher in bone marrow of both classical Nur77-KO and complete Nur77-KO mice compared with WT mice (Fig. 3E), revealing a Nur77-mediated genomic function by suppression of these genes. On the other hand, gene expression of Egfr and Vcam1, two factors that promote HSPC retention in the bone marrow (23, 24), was significantly lower in both classical Nur77-KO and complete Nur77-KO mice compared with WT mice (Fig. 3F).

These results show that Nur77(1–117) can stabilize and activate HIF-1α both in vitro and in vivo. Combined with the altered expression of several genes that regulate bone marrow mobilization in both classical Nur77-KO and complete Nur77-KO mice when compared with WT mice, we speculate that the bone marrow, liver, and spleen phenotypes observed exclusively in classical Nur77-KO mice are caused by an imbalance between the genomic action of Nur77 as a transcription factor versus its nongenomic action as a stabilizer of HIF-1α.

A transcript variant of human NUR77 encoding solely for its N-terminal domain (TR3β) has similar functions as the Nur77(1–117) protein expressed in classical Nur77-KO mice

Our findings for Nur77(1–117) may have implications beyond the transgenic mouse models described here, because a transcript variant of human NUR77 encoding for the entire NUR77 N-terminal domain was previously detected and deposited in the GenBankTM database (GenBankTM accession number D85245; Ensembl ENST00000548232). To date, no further characterization of this Nur77 variant has been reported. The transcript variant, designated TR3β, shares a large part of NR4A1 exon 2 with full-length Nur77, but has a distinctive transcriptional start site, exon structure, and 3′-UTR (Fig. 4A and Fig. S4A). Using a primer set targeting these unique features, TR3β mRNA expression was detected in various human tissues and cells (Fig. 4B). Furthermore, we found that TR3β mRNA expression, like full-length NUR77 mRNA, is induced by LPS stimulation in human monocyte–derived macrophages (Fig. 4B and Fig. S4B) and the THP-1 monocyte cell line (Fig. S4C).

Similar to the mouse Nur77(1–117) protein, the TR3β transcript variant lacks all nuclear localization signals and nuclear export signals present in full-length NUR77. Consistently, overexpressed TR3β protein, like Nur77(1–117), localized throughout the cell (Fig. 4C). Using a similar Nur77-specific antibody IP and Western blotting approach that we used to detect Nur77(1–117) protein, we identified a band of 77 kDa (full-length NUR77) and a band of ~34 kDa in LPS-stimulated K562 bone marrow cells (Fig. 4D). This latter band closely corresponds with the predicted molecular mass of 33.8 kDa for TR3β based on its primary amino acid sequence. Furthermore, TR3β overexpression resulted in both increased hypoxia-induced HIF-1α protein expression (Fig. 4E) and higher HIF-1 transcriptional activity (Fig. 4F). Finally, both TR3β and Nur77(1–117) protein reduced the activation of a Nur77 response element (NurRE)-driven luciferase reporter construct by full-length Nur77, indicating that these two truncated Nur77 proteins can reduce the transcriptional activity of full-length Nur77 (Fig. 4G). Taken together, these results show that the human Nur77 transcript variant TR3β has cellular localization and HIF-1α–activating properties similar to those of the murine Nur77(1–117) protein and also inhibits the transcriptional activity of full-length human Nur77.

The human NUR77 transcript variant TR3β is highly expressed in bone marrow and associated cancers

Because TR3β has similar effects as the mouse Nur77(1–117) protein, and classical Nur77-KO mice exhibit splenic and bone marrow abnormalities, we next investigated whether TR3β is expressed differentially in human bone marrow pathologies. To do so, we explored publicly available gene expression data sets. Interestingly, the Affymetrix Human Genome U133 Plus 2.0 Array contains multiple probes for products of the NR4A1 gene locus, one of which specifically targets the unique 3′-UTR of TR3β (probe ID: 210226_at; Fig. 4A). This allowed us to perform a meta-analysis of all publicly available microarray data sets using the Affymetrix Human Genome U133 Plus 2.0 Array with the NEBION Genevestigator software suite. From this meta-analysis, we found that full-length NUR77 mRNA is most highly expressed in various tissues in which it is known to play an important role, such as heart (25), lung (26), and adrenal gland (27) (Fig. 5A, left). When looking at the types of cancers with the highest expression of NUR77, a mix of endocrine tumors and lymphomas were found (Fig. 5A, right). On the other hand, TR3β mRNA is most highly expressed in a set of tissues distinct from full-length Nur77, which most notably includes a number of bone marrow cell types (Fig. 5B, left). Moreover, 4 of the 10 cancer types with the highest average expression of TR3β were various subtypes of AML (Fig. 5B, right). Consistent with this latter observation, pathway analysis on a set of 800 genes whose expression most strongly correlated with that of TR3β showed enrichment of genes known to be involved in AML, such as RUNX1T1, SOST2, PIK3CB, and KRAS (Fig. 5D; genes indicated by red dots). On the other hand, genes that strongly correlated in expression with full-length Nur77 were predominantly associated with coagulation, infection, and breast cancer pathways, but not AML (Fig. 5C). Together, these results indicate that the human Nur77 transcript variant TR3β is strongly associated with high bone marrow expression and AML.

Discussion

Nur77 regulates various aspects of hematopoiesis and related pathologies, such as monocyte differentiation (6) and AML (7). Although its actions as a transcription factor are established in
Figure 4. A transcript variant of human NUR77 encoding solely for its N-terminal domain (TR3β) has similar functions as the Nur77(1–117) protein expressed in classical Nur77-KO mice.

A, schematic overview of the human NUR77 (NR4A1) gene locus and resulting RNA and protein products. The TR3β transcript variant has a different transcriptional start site and encodes for the N-terminal domain of full-length NUR77. Exons are numbered E1–E7. E1* is the alternative first exon of TR3β. TSS, transcriptional start site; START, translational start site; STOP, translational termination codon; NTD, N-terminal domain; DBD, DNA-binding domain; LBD, ligand-binding domain. Triangles indicate binding sites for full-length NUR77-specific (202340_x_at) and TR3β-specific (210226_at) microarray probes in the Affymetrix Human Genome U133 Plus 2.0 Array.

B, TR3β (Exon 1* – Exon 2) mRNA expression in different human tissues and monocyte-derived macrophages stimulated with 10 ng/ml LPS for the times indicated.

C, cellular localization of TR3β protein detected by IF microscopy using mouse-myc antibody in transiently transfected HeLa cells. Scale bar, 5 μm.

D, immunoprecipitated full-length NUR77 (NUR77FL) and TR3β endogenous protein in untreated or 6–24-h LPS-treated K562 cells detected by WB. Numbers on the left, molecular mass in kDa. IgG, Immunoglobulin G. Numbers at the bottom, fold change in β-actin–normalized HIF-1α band intensity relative to control (GFP-transfected) cells.

E, Western blotting for HIF-1α protein in RAW264.7 cells overexpressing full-length NUR77, Nur77(1–117), or TR3β and cultured in 1% O2 conditions for 24 h. GFP overexpression was included as control. Numbers on the right, molecular mass in kDa. Numbers at the bottom, fold change in β-actin–normalized HIF-1α band intensity relative to control (GFP-transfected) cells. *p < 0.05; **p < 0.01; ***p < 0.001 versus control samples.

F, luciferase reporter assay for NUR77 transcriptional activity in HeLa cells transiently overexpressing full-length NUR77, Nur77(1–117), and/or TR3β. GFP overexpression was included as a control. For B, F, and G, data are shown as mean ± S.D. (error bars) (n = 3). p values were calculated using one-way (B) or two-way (F and G) ANOVA with a Tukey post hoc test. *, p < 0.05; **, p < 0.01; †, p < 0.001 versus control samples. ns, not significant; aa, amino acids; AU, arbitrary units. See also Fig. S4.
Figure 5. The human NUR77 transcript variant TR3β is highly expressed in bone marrow and associated cancers. A and B, mRNA expression of human full-length NUR77 (A) or TR3β (B) in different human tissues and cancers from analysis of publicly available microarray data sets that use the Affymetrix Human Genome U133 Plus 2.0 Array. Bars shaded red indicate bone marrow and AML-related hits. C and D, top 800 genes that most strongly correlate (positively or negatively) in mRNA expression with human full-length NUR77 (C) or TR3β (D) from analysis of publicly available microarray data sets that use the Affymetrix Human Genome U133 Plus 2.0 Array. Bar graphs show the three most highly enriched KEGG pathways for these correlating genes. Red dots indicate correlated genes that are involved in the AML pathway.
these processes, relatively little is known about the role of non-
genomic Nur77 activity in the bone marrow. In that regard, the
fact that the classical Nur77-KO mouse model still expresses
part of the Nur77 N-terminal domain gave us insight into the
nongenomic (N-terminal domain–mediated) and genomic
actions of Nur77 in the regulation of bone marrow homeostasis.

Although the production of aberrant mRNAs and truncated
proteins in transgenic mice due to suboptimal gene targeting
strategies are an established phenomenon (10, 11), the resulting
mRNAs are not always translated and may be degraded by the
cell’s internal quality-control mechanisms, such as nonsense-
mediated RNA decay (NMD) (30). The NMD pathway is
strongly induced by the presence of premature termination
codonts (PTCs): translational stop signals that precede the final
exon–exon junction in the nascent mRNA that are bound by
 exon junction complexes. Although the Nur77(1–117)–
encoding mRNA produced in classical Nur77-KO mice con-
tains an in-frame PTC directly following Nur77 exon 2, this
mRNA may escape NMD for two reasons: 1) there are no
intronic sequences, and therefore no exon–exon junctions or
exon junction complexes, between Nur77 exon 2 and the PTC;
2) the NeoR cassette contains a polyadenylation sequence (9),
which is a strong trigger for transcriptional termination (31).
In fact, this second point provides a plausible explanation as to
why the mRNA produced in classical Nur77-KO mice cannot
be detected by primers targeting Nur77 exons 3 and 4, namely
that these latter exons are simply not transcribed. Moreover,
the presence of strong polyadenylation sequences derived
from the NeoR cassette in the mRNA produced in classical
Nur77-KO mice may make it extremely stable, which is consis-
tent with the observation that the expression of this aberrant
mRNA stays elevated for longer periods of time following LPS
stimulation of classical Nur77-KO BMDMs (Fig. 2C).

Based on the data presented here, we propose that the hema-
topoietic phenotypes observed exclusively in aged classical
Nur77-KO mice (namely increased LSK cells in the bone mar-
row, immune cell infiltrates in the liver, loss of splenic architec-
ture, and increased numbers of HSPC-derived colonies in the
spleen) are caused by an imbalance between the genomic action
of Nur77 as a regulator of genes involved in bone marrow mobil-
ization (which is absent in these mice) and its nongenomic
action as a stabilizer of HIF-1α (which is still present). This
imbalance in Nur77 and HIF-1 signaling is not present in com-
plete Nur77-KO mice, which may explain their apparent lack
of overt hematopoietic phenotypes compared with classical
Nur77-KO mice (Fig. 6). The phenotype of classical Nur77-KO
mice may be further aggravated by the increased stability of the
Nur77(1–117)– encoding mRNA, leading to higher Nur77(1–
117) expression for longer periods of time (as was observed in
LPS-stimulated macrophages).

Nur77 has previously been shown to stabilize HIF-1α protein
by inhibiting the expression of the E3 ligase MDM2 and thereby
its subsequent degradation of HIF-1α. The N-terminal domain
of Nur77 by itself is shown to be sufficient for this stabilization
to occur, and the extracellular signal–regulated kinase (ERK)
pathway is critically involved (20). We propose that Nur77(1–
117) may stabilize HIF-1α in a similar fashion via ERK and
MDM2.

A case could also be made that the phenotypes observed in
classical Nur77-KO mice are a less severe form of the rapid-
onset AML phenotype observed in Nur77 and NOR-1 double-
knockout mice, which display splenomegaly, skewing of HSPCs
toward myeloid progeny, and severe bone marrow defects
within 21 days (7). We observed that classical Nur77-KO bone
marrow gives rise to more macrophages than WT or complete
Nur77-KO mouse bone marrow (Fig. S1D). We also occasion-
ally observe severe splenomegaly in older classical Nur77-KO
mice, with spleens of up to 2 g in 8.5-month-old mice (data not
shown). Finally, the hematopoietic phenotypes in classical
Nur77-KO mice described here only develop with old age and
are seemingly absent in younger mice. It could therefore be
speculated that the proposed imbalance in bone marrow Nur77

Figure 6. Nuclear receptor Nur77 variants solely comprising the N-terminal domain activate HIF-1α and affect bone marrow homeostasis in mice and humans. Shown is a graphical summary.
Nur77 variants regulate HIF-1α and bone marrow homeostasis

and HIF-1 signaling does not necessarily trigger the phenotypes by itself, but instead lowers the threshold for exaggerated reactions to other stimuli that can induce HSPC mobilization and proliferation, such as pathogenic infections or aging itself (29). Whereas we primarily focus on differences in hematopoietic biology between classical Nur77-KO and complete Nur77-KO mice and the regulation of HIF-1α signaling, the N-terminal domain of Nur77 interacts with a variety of other transcription factors, coregulators, and kinases (5). It may therefore be worthwhile to revisit other previously reported phenotypes of classical Nur77-KO mice. In that regard, we already show here that both Nur77-KO mouse models display decreased circulating Ly6Clo monocytes (Fig. S3A), which seems to be dependent on Nur77 transcriptional activity.

For this study, the existence of a microarray probe specific for TR3β mRNA and a plethora of publicly available expression data sets were crucial for determining the differences in tissue and tumor expression patterns between full-length Nur77 and TR3β, especially because a natural form of TR3β does not exist in mice. These observations lend credence to the idea that the human TR3β transcript variant exists and that it has a biological function distinct from that of full-length Nur77. Moreover, our analyses suggest a role for TR3β in AML and KRAS signaling (Fig. 5, B and D). Future research could therefore focus on a possible interplay between TR3β, HIF-1α, and KRAS signaling and its functional consequences in AML, especially because cross-talk between HIF-1α and KRAS signaling has previously been reported to play a role in colon cancer (32). We also show that TR3β can reduce the transcriptional activity of full-length Nur77 (Fig. 4G). It could therefore be speculated that high expression of this transcript variant may inhibit the tumor-suppressive and homeostasis-promoting functions of full-length Nur77, thereby predisposing bone marrow cells to pathologies such as AML.

Experimental procedures

Mice

All animal breeding, care, and procedures were approved by the institutional animal ethics committee of the University of Amsterdam (reference no. DBC102827), in accordance with both institutional and European directive 2010/63/EU guidelines.

B6;129S2-Nr4a1tm1mlj/mj mice were purchased from the Jackson Laboratory (JAX stock no. 006187). These transgenic mice were originally generated by insertion of a phosphoglycerate kinase promoter–driven neomycin resistance (Neo) cassette into the coding region of exon 2 of the Nr4a1 gene (9). These mice are referred to as classical Nur77-KO mice in this work.

Mice in which exons 2–4 of the Nr4a1 locus are flanked by loxp sites (Nur77fl/fl) were generated in collaboration with H. Ichinose (Tokyo Institute of Technology) and were described elsewhere (33). C57Bl6/j mice overexpressing Cre recombinase under control of the human cytomegalovirus minimal promoter (CMV-cre mice) were purchased from the Jackson Laboratory (stock no. 006054). Nur77fl/fl mice and CMV-cre mice were crossed to generate CMV-cre:Nur77fl/fl mice. These mice were subsequently bred to lose the CMV-cre allele while remaining deficient for Nur77. These mice are referred to as complete Nur77-KO mice in this work.

For all experiments, male 6–8-week-old, 3-month-old, or 6.5-month-old mice were used. Mice were euthanized by intraperitoneal injection of a lethal dose of ketamine (238 mg/kg) plus xylazine (102 mg/kg) and subsequent orbital bleeding. Before tissue harvest, mice were perfused with ice-cold PBS.

All animal studies have been performed and analyzed in a blinded manner.

BMDM generation and culture

Bone marrow cells were flushed from femurs and tibias of 6–8-week-old WT, classical Nur77-KO, and complete Nur77-KO mice and differentiated to macrophages by culturing in RPMI medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 15% L929-conditioned medium for 7 days. In all experiments with LPS stimulation, a final concentration of 100 ng/ml LPS (Fluka) was used.

Cell line culture

The RAW264.7 mouse macrophage cell line was cultured in Dulbecco’s modified Eagle’s medium high-glucose medium with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (all from Gibco). HEK293T and HeLa human cell lines were cultured in Dulbecco’s modified Eagle’s medium high-glucose medium with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The THP-1 and K562 cell lines were cultured in RPMI 1640 medium with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Histology and immunohistochemistry

Livers and spleens were fixed in 4% paraformaldehyde (Roth), embedded in paraffin, and subsequently sectioned and mounted on StarFrost glass slides (Thermo Scientific). Tissue morphology was assessed by hematoxylin and eosin staining (Sigma). For immunohistochemistry, sections were deparaffinized, rehydrated, treated with 1% H2O2 (Merck), and subjected to heat-induced epitope retrieval. Sections were stained for leukocytes (anti-CD45; eBioscience catalog no. 14-0451), macrophages (anti-IBA-1; WAKO catalog no. 019-19741), T cells (anti-CD3; Pharmingen catalog no. 550367), and B cells (anti-B220; Southern Biotech catalog no. 1665-09). For anti-CD45, a biotin-conjugated antibody (DAKO catalog no. E0468) was used for signal amplification. Poly-HRP IgG conjugate (ImmunoLogic catalog no. DPVR110HRP) was used as a secondary antibody, and immunostaining was detected using 3,3′-diaminobenzidine (DAB; ImmunoLogic). Nuclei were counterstained with hematoxylin. Sections were visualized using a Zeiss Axioshot microscope and QWin version 3 software (Leica Microsystems).

Reverse transcription–quantitative PCR

Total RNA from primary single cell suspensions or cultured cells was isolated using TRIreagent (Sigma) according to the manufacturer’s instructions. RT-PCR was performed on 1 μg of RNA using the iScript cDNA synthesis kit (Bio-Rad). Quantita-
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Cloning and vector construction

The pCMV-myc-Nur77 vector has been described previously (18). For the pCMV-myc-Nur77(1–117) vector, the N-terminal domain of Nur77 (residues 1–115 plus 2 residues from the Neo8 cassette) was amplified from genomic DNA of classical Nur77-KO mice by PCR using the primers 5′-CCCCT-GTATTCAAGCTCAATATGGA-3′ (forward) and 5′-TTA-ATTGATGGTGAGCCGTAAGGGTACG-3′ (reverse) and a PCR program of 30-s denaturing at 94 °C, 30-s annealing at 63 °C, and 30-s extension at 72 °C for 35 cycles. The amplification product was subsequently ligated into the pGEM-T Easy vector (Promega) and transferred into the pCMV-myc vector (Clontech) using EcoRI digestion to generate the construct. For the pCMV-myc-TR3β vector, the TR3β coding domain sequence was amplified from genomic DNA of human SMCs by PCR using the primers 5′-CTAGCGAATTCCACCCTGTATC-3′ (forward) and 5′-GATCGCTCGAGGAAGTTAACTG-3′ (reverse) and a touch-down PCR program of 30-s denaturing at 95 °C, 45-s annealing starting at 67 °C and decreasing by 1 °C per cycle to a minimum of 55 °C, followed by a 2 min 30-s extension at 68 °C for a total of 32 cycles. The amplification product was digested with EcoRI and Xhol and ligated into the pCMV-myc vector.

Immunofluorescence microscopy

RAW264.7 or HeLa cells were seeded on uncoated glass coverslips and transiently transfected with pCMV-myc-Nur77, pCMV-myc-Nur77(1–117), or pCMV-myc-TR3β expression vectors using Lipofectamine LTX (RAW264.7) or Lipofectamine 2000 (HeLa) according to the manufacturer’s instructions. After 24 h, cells were fixed with 4% (w/v) paraformaldehyde in PBS and subsequently permeabilized with 0.5% (v/v) Triton X-100. Protein expression was detected using mouse anti-Myc (Santa Cruz Biotechnology, Inc.) and Alexa Fluor 488–conjugated goat anti-mouse (Molecular Probes) antibodies. Nuclei were visualized using Hoechst 33258 (Invitrogen) staining.

Luciferase assays

RAW264.7, HeLa, or HEK293T cells were transiently transfected with 4xNurRE-luc or 5xHRE-luc firefly luciferase reporter constructs and the pRluc-N3 Renilla luciferase reporter construct as an internal control using Lipofectamine LTX (RAW264.7) or Lipofectamine 2000 (HeLa and HEK293T) according to the manufacturer’s instructions. After 24 h, cells were stimulated with 100 µM cobalt chloride (CoCl2), 100 µM deferoxamine (DFO) or incubated at 1% O2 in a H35 HypoxiStation (Don Whitley Scientific) for the times indicated. Luciferase assays were performed using the Dual-Luciferase reporter assay system on a Glomax multidetection system (both from Promega) according to the manufacturer’s instructions.

Nur77 immunoprecipitations

For detection of endogenous Nur77 proteins, BMDMs were lysed in IP lysis buffer (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10% (w/v) glycerol, 1% (w/v) IGEPAH CA-630) supplemented with phosphatase inhibitors (10 mM NaF, 1 mM Na2VO4) and complete protease inhibitor mixture (Roche Applied Science). Lysates were precleared for 1 h at 4 °C with protein A/G–agarose beads (Alpha Diagnostic), followed by incubation overnight with 2 µg of rabbit-anti-Nur77 antibody (Santa Cruz Biosciences catalog no. sc-5569). The next day, complexes were precipitated using protein A/G–agarose beads, washed three times in IP lysis buffer, and eluted by boiling in Laemmli sample buffer. Immunoprecipitated Nur77 protein was subsequently detected by Western blotting.

HIF-1α protein isolation from bone marrow

To detect HIF-1α protein in the bone marrow, femurs of 6.5-month-old mice were rapidly harvested after euthanasia and directly flushed with 2× Laemmli sample buffer (120 mM Tris-HCl (pH 6.8), 20% (w/v) glycerol, 4% (w/v) SDS, 200 mM DTT). HIF-1α protein was subsequently detected by Western blotting.

Western blotting

Protein samples were separated on 10 or 12% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked using 5% nonfat milk for 1 h and subsequently incubated with rabbit anti-HIF-1α (Novus Biologicals catalog no. NB100-449) or mouse-anti-Nur77 (Pharmingen catalog no. 554088) primary antibodies overnight. Protein bands were detected by incubating membranes with appropriate HRP-conjugated secondary antibodies for 1 h at room temperature, followed by visualization using Supersignal West Femto ECL (Thermo Scientific) and detection using an ImageQuant LAS 4000 imager (GE Healthcare). Equal loading of samples was confirmed by reprobing blots with antibodies against mouse-anti-tubulin (Cedarlane catalog no. CLT9003) or rabbit anti-β-actin (Cell Signaling catalog no. 4970).

Flow cytometry

Whole blood was collected in EDTA tubes (BD Bioscience). Spleens were passed through a 70-µm nylon strainer (Falcon) to obtain single cell suspensions. Blood and splenic cells were subsequently treated with erythrocyte lysis buffer (Roche Applied Science). Bone marrow, blood, and splenic cells were suspended in PBS + 1% FCS and blocked with anti-CD16/CD32 antibody (BD Biosciences catalog no. 553142). To assess Ly6c± and Ly6c+ monocyte populations, the following monoclonal antibodies were used (all from Invitrogen): CD19-FITC (catalog no. 11-0193), CD3e-FITC (catalog no. 11-0033), Ly6g-FITC (catalog no. 11-5931), CD11b-PE-Cy7 (catalog no. 25-1152-80), CD11b-APC (catalog no. 17-0112), and Ly6c-PE (catalog no. 12-5932). 7-Aminoactinomycin D was used as via-
bility staining. Acquisition was performed on a FACSComp II flow cytometer (BD Biosciences). Ly6c monocytes are given as a percentage of CD11b⁺ CD115⁺ monocytes. To assess LSK cell populations, the following monoclonal antibodies were used (all from Invitrogen): CD19-FITC (catalog no. 11-0193), CD3e-FITC (catalog no. 11-0033), Ly6g-FITC (catalog no. 11-5931), cKit-APC (catalog no. 17-1172), and Sca-1-PE (catalog no. 12-5981). 7-Aminoactinomycin D was used as viability staining. Acquisition was performed on a CytoFLEX flow cytometer (Beckman Coulter). LSK cells are given as a percentage of lineage-negative (Lin⁻; negative for CD19, CD3e, and Ly6g staining) cells.

**Colony-forming unit assay**

Mouse bone marrow and splenic single cell suspensions were seeded in MethoCult GF M3434 medium (STEMCELL Technologies) according to the manufacturer’s instructions. For each mouse, 30,000 total bone marrow cells or 200,000 total splenic cells were seeded in duplicate wells. The total number of myeloid colonies per mouse was counted using an inverted light microscope, on day 8 for bone marrow and day 12 for spleen cells.

**Data analysis**

Statistical analyses were performed using GraphPad Prism software. Normal distribution of data was tested with the Kolmogorov–Smirnov normality test. Data are presented as mean ± S.E. p values were calculated using Student’s t test, one-way ANOVA, or two-way ANOVA with either Bonferroni or Tukey post hoc correction as indicated in the figure legends. A p value <0.05 was considered statistically significant. Flow cytometry data were analyzed using FlowJo software. Meta-analysis of publicly available microarray data sets using the Affymetrix Human Genome U133 Plus 2.0 Array was performed using NEBION Genevestigator software (28).

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