

NMD is essential for hematopoietic stem and progenitor cells and for eliminating by-products of programmed DNA rearrangements

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Nonsense-mediated mRNA decay (NMD) is a post-transcriptional surveillance process that eliminates mRNAs containing premature termination codons (PTCs). NMD has been hypothesized to impact on several aspects of cellular function; however, its importance in the context of a mammalian organism has not been addressed in detail. Here we use mouse genetics to demonstrate that hematopoietic-specific deletion of *Upf2*, a core NMD factor, led to the rapid, complete, and lasting cell-autonomous extinction of all hematopoietic stem and progenitor populations. In contrast, more differentiated cells were only mildly affected in *Upf2*-null mice, suggesting that NMD is mainly essential for proliferating cells. Furthermore, we show that UPF2 loss resulted in the accumulation of nonproductive rearrangement by-products from the *Tcrb* locus and that this, as opposed to the general loss of NMD, was particularly detrimental to developing T-cells. At the molecular level, gene expression analysis showed that *Upf2* deletion led to a profound skewing toward up-regulated mRNAs, highly enriched in transcripts derived from processed pseudogenes, and that NMD impacts on regulated alternative splicing events. Collectively, our data demonstrate a unique requirement of NMD for organismal survival.

[*Keywords:* Hematopoietic stem and progenitor cells; T-cell development; nonsense-mediated mRNA decay; programmed DNA rearrangements; alternative splicing; pseudogenes]

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Nonsense-mediated mRNA decay (NMD) is part of a larger network of RNA surveillance pathways, which ensure that only mRNAs with proper coding potential become available for protein synthesis. Specifically, the NMD machinery recognizes mRNAs with premature termination codons (PTCs) and mediates their degradation. If left available for translation, PTC-containing (PTC⁺) mRNAs pose a latent threat to the cell as they may encode proteins with potential dominant-negative properties.

The NMD pathway was initially considered as a guardian against newly arising nonsense mutations ei-

ther at the DNA level or as a consequence of erroneous transcription or mRNA processing (for recent reviews, see Conti and Izaurralde 2005; Isken and Maquat 2007). More recently, global expression profiling of tissue culture cells subjected to siRNA-mediated knockdown of key NMD components demonstrated that even nonmutated genes are regulated by NMD, suggesting that the NMD pathway actively participates in regulation of normal gene expression (Mendell et al. 2004; Rehwinkel et al. 2005; Wittmann et al. 2006). Of particular importance are the T-cell receptor (TCR) and immunoglobulin (Ig) genes, which undergo programmed DNA rearrangements. Here, two-thirds of the V(D)J recombination events are predicted to be nonproductive by generating a PTC, and the resulting PTC⁺ transcripts were shown to be stabilized both by inhibition of protein synthesis and

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by knockdown of known NMD components (at least in the context of PTC⁺ reporter constructs), thereby strongly suggesting that their clearances are facilitated by NMD (Carter et al. 1995; Li and Wilkinson 1998; Wang et al. 2002). Finally, the NMD pathway has also been proposed to mute genomic noise arising from transcription of pseudogenes and transposable elements as well as retroviruses (Mitrovich and Anderson 2005; Weischenfeldt et al. 2005).

The process of NMD is conserved throughout evolution and several of the mammalian NMD factors, including the core factors UPF1, UPF2, and UPF3A/B have conserved orthologs in lower eukaryotes (for review, see Chang et al. 2007). Depletion or mutation of the UPF proteins abolishes NMD in all eukaryotes studied, and the UPFs are considered to constitute the functional core of the NMD machinery (Perlick et al. 1996; Lykke-Andersen et al. 2000). In mammalian cells, PTCs are distinguished from stop codons by their position upstream of exon-exon junctions, which are marked during splicing by the deposition of the exon junction complex (EJC). When a ribosome is stalled on a PTC, UPF1 is recruited together with release factors (eRF1 and eRF3) and SMG-1 (Kashima et al. 2006). If an EJC complex is placed downstream from the PTC, UPF2 acts as a molecular bridge between the stalled ribosome and the EJC, through its interaction with UPF1 and UPF3A/B, respectively (Czaplinski et al. 1998; Singh et al. 2007). Subsequent recruitment of additional NMD components including SMG-5 and SMG-7 leads, through a series of UPF1 phosphorylation and dephosphorylation events, to the disruption of the stalled ribosome-mRNA complex and finally to degradation of the PTC⁺ mRNA in cytoplasmic processing bodies (P-bodies) (Fukuhara et al. 2005; Chang et al. 2007).

In addition to the classical linear NMD pathway, there are also data supporting UPF2-independent and UPF3-independent alternative branches of the NMD pathway (Gehring et al. 2005; Chan et al. 2007).

To establish the importance of the UPF proteins for the viability of mammalian cells, these factors have been depleted individually using RNAi-mediated gene silencing. Whereas depletion of UPF2 in human tissue culture had no effect on cellular proliferation, loss of UPF1 resulted in cell cycle arrest and activation of the DNA damage response pathways (Azzalin and Lingner 2006b). In the mouse, *Upf1* knockout led to early embryonic lethality, and attempts to generate embryonic stem (ES) cell lines from *Upf1*-null blastocysts were unsuccessful (Medghalchi et al. 2001). Loss-of-function studies have also been performed in lower eukaryotes including yeast, *Caenorhabditis elegans*, and *Drosophila melanogaster* (Pulak and Anderson 1993; He et al. 1997; Metzstein and Krasnow 2006). In the latter organism, both *Upf1* and *Upf2* were found to be essential for larval viability, a finding that is corroborated by the cell cycle arrest installed by depletion of either UPF1 or UPF2 in *Drosophila* Schneider cells (Rehwinkel et al. 2005). In contrast, depletion of any of the UPF proteins had no major impact on cell viability in either yeast or *C. elegans*.

These studies suggest that NMD factors have gained increased importance during the course of evolution, either due to the acquisition of additional functions or due to a change in the repertoire of regulated transcripts toward a more prominent role in post-transcriptional gene regulation.

The hematopoietic system is an attractive experimental model to address issues concerning stem cell function, lineage commitment, and responses to external stimuli. At the apex of the hematopoietic hierarchy resides the long-term hematopoietic stem cell (LT-HSC), a rare cell type present in the bone marrow (BM). In addition to their unique ability to self-renew throughout life, these cells give rise to all the cells of the blood system, through a series of defined differentiation steps. The capacity to specifically identify, isolate, and characterize LT-HSCs, their immediate multipotent descendants, and downstream lineage-committed progenitors has yielded unprecedented insights into issues of stem cell self-renewal, lineage relationships, and lineage commitment, as well as into the molecular pathways governing these processes (for review, see Kondo et al. 2003; Buzas-Vidas et al. 2007; Iwasaki and Akashi 2007). However, the importance of RNA surveillance, and in particular NMD, has yet to be analyzed within the hematopoietic system.

To determine the importance of NMD in the context of a mammalian organism, we generated a conditional mouse knockout of *Upf2* and used the hematopoietic system as a model to test the functional consequences of disrupting the NMD pathway. In these mice, ablation of UPF2 led to the fast, complete, and lasting extinction of the hematopoietic stem and progenitor compartment. In contrast, differentiated cells were only mildly affected, suggesting a particular important role of NMD in proliferating cells. Deletion of *Upf2* within the T-cell compartment was associated with the accumulation of PTC⁺ *Tcrb* transcripts in immature T-cells. In striking contrast, PTC⁺ *Tcrb* transcripts did not accumulate in mature *Upf2*-null T-cells, suggesting that it is the specific accumulation of PTC⁺-containing *Tcrb* transcripts and not the general loss of NMD activity that is deleterious for resting T-cells. Finally, at the molecular level, we could show the importance of NMD in suppression of pseudogene expression and as a modulator of alternative splicing (AS).

Results

To test the role of NMD in the development and maintenance of the hematopoietic system, we targeted the locus encoding one of the essential NMD factors, UPF2. Given that the *Upf2* gene spans >20 exons in a >200-kb region on chromosome 2, we adopted a targeting strategy where, by flanking exons 2 and 3 with *loxP* sites, we could inactivate *Upf2* in a conditional manner (Fig. 1A). Correct targeting of transfected ES cells was verified by Southern blotting, and following germline transmission, the resulting *Upf2*^{neostop/+} mice were used to generate the conditional *Upf2*^{fl} allele by in vivo removal of the

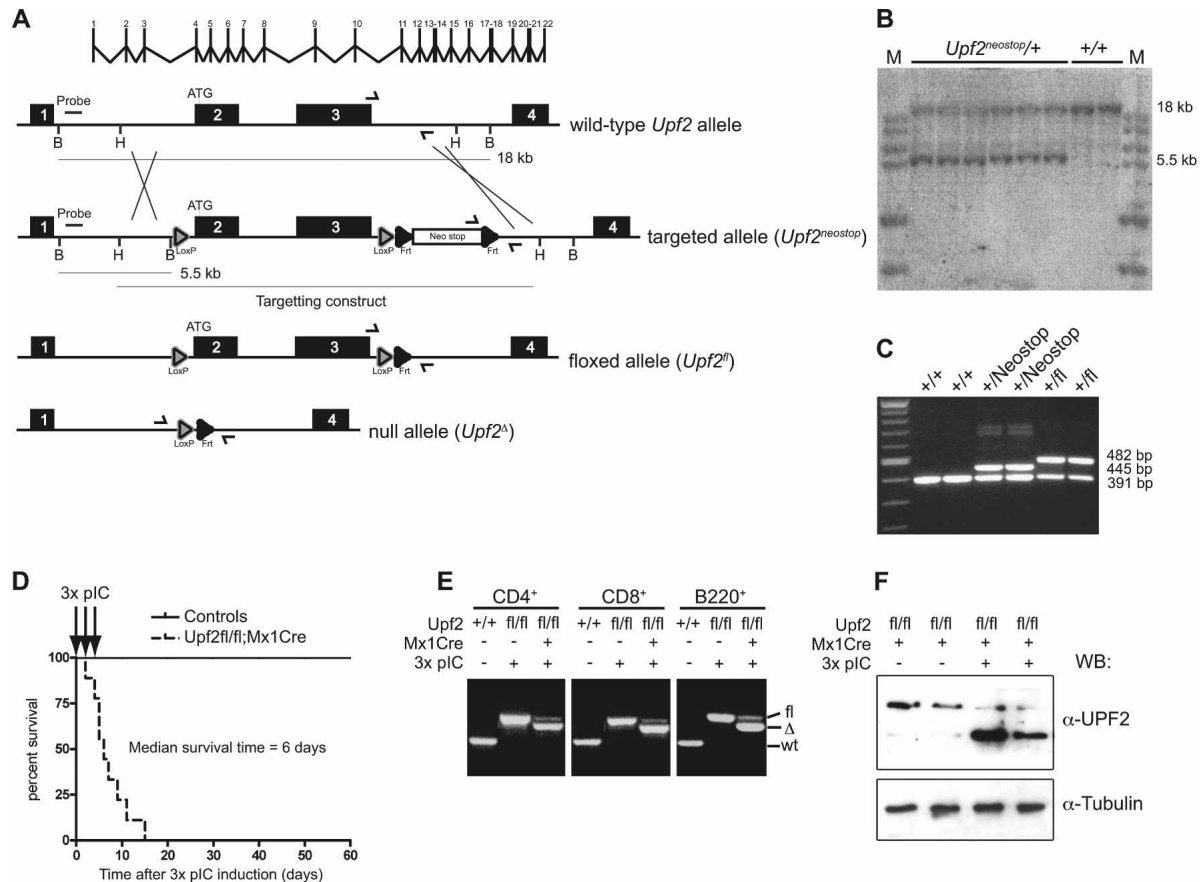


Figure 1. Mx1Cre-mediated conditional deletion of UPF2 leads to rapid mortality. (A) The genomic structure of the >200-kb *Upf2* locus is depicted. The targeting strategy involved flanking exons 2 (containing the initiating ATG) and 3 with *loxP* sites. Removal of the FRT-flanked Neo cassette by crossing with *FLP* mice resulted in the generation of the conditional *Upf2^{fl}* allele. The conditional *Upf2^{fl}* allele is converted to the recombined *Upf2^Δ* allele by the Cre recombinase. Primers used for genotyping of the various *Upf2* alleles are indicated. (B) Southern blot verification of correctly targeted ES cells. DNA was digested with EcoRI and probed with a probe 5' to the sequence within the targeting construct. (C) PCR genotyping of *Upf2^{fl/+}* crossed to *Flp* transgenics. (D) pIC injections were performed at days 0, 2, and 4 and led to the rapid death of *Upf2^{fl/fl};Mx1Cre* ($N = 9$), whereas no mortality was observed in any of the different pIC-injected controls (all other genotypes $N = 25$). (E) PCR genotyping of MACS-purified CD4⁺, CD8⁺, and B220⁺ splenocytes isolated from pIC-injected *Upf2^{fl/fl};Mx1Cre* mice (day 6) and relevant controls. Recombination levels were >85%. Given the fast clearance of cells upon UPF2 truncation, we were unable to assess recombination levels in the BM. (F) Western blot analysis of lysates from B220⁺ *Upf2^{fl/fl};Mx1Cre* splenocytes (day 6 after the first pIC injection) demonstrates the expression of a truncated UPF2 form in recombined cells.

Frt-flanked Neostop cassette (Fig. 1B,C). Cre-mediated recombination of the *Upf2^{fl}* allele resulted in the generation of the *Upf2^Δ* allele, which would lead to either ablation of UPF2 protein or to the generation of a truncated form of UPF2 initiating at one of several downstream ATGs. We predicted that such a truncated UPF2 protein would be functionally inactive as it would have deleted several domains of functional importance including the N-terminal part, which is required for UPF1 interaction and NMD (Supplemental Fig. S1A).

To test the effect of *Upf2* deletion on embryonic development, we intercrossed *Upf2^{Δ/+}* heterozygotes and isolated embryos at distinct developmental time points. No homozygous *Upf2^{Δ/Δ}* embryos could be detected at embryonic day 9.5 (E9.5), whereas genotyping of E3.5 blastocysts revealed the expected Mendelian ratio (Supplemental Table S1). These findings show that

UPF2, like UPF1 (Medghalchi et al. 2001), is essential for early embryonic development in the mouse.

Conditional ablation of UPF2 in the hematopoietic system

In order to probe the role of NMD during hematopoietic development, we crossed the *Mx1Cre* transgene onto our *Upf2^{fl/+}* line. In this model, expression of Cre recombinase can be induced within the entire hematopoietic system upon activation of the *Mx1Cre* transgene either by administering interferon directly or through a dsRNA-induced interferon response (Kuhn et al. 1995). Ablation of UPF2 within the hematopoietic compartment was accomplished by injecting *Upf2^{fl/fl};Mx1Cre* mice three times with polyinosinic-polycytidylic acid (pIC) at days 0, 2, and 4. This procedure led to the death of all

Upf2^{fl/fl};Mx1Cre animals within 10 d from the first pIC injection (Fig. 1D), whereas no mortality was observed in any of the pIC-injected control animals (*Upf2^{fl/fl}*, *Upf2^{+/+}*, *Upf2^{+/+};Mx1Cre*). Analysis of the BM from pIC-injected *Upf2^{fl/fl};Mx1Cre* mice at day 6 (i.e., 2 d after the last pIC injection) revealed an almost complete absence of nucleated cells (Fig. 2E). Similarly, flow cytometric analysis demonstrated a strong reduction of all major lineages of the BM including hematopoietic progenitors (HPCs; c-Kit⁺) (Fig. 2A), granulocytes (Mac-1⁺Gr-1⁺) (Fig. 2B) and B-cells (B220⁺) (Fig. 2C). The only cells present in the BM after pIC injection of *Upf2^{fl/fl};Mx1Cre* mice were CD71⁺Ter119⁺ erythroid cells (Fig. 2D). The finding that the latter cells accumulate in pIC-injected controls at the expense of CD71⁺Ter119⁺ cells suggests that their accumulation is independent of UPF2 depletion but is a consequence of the induced interferon response (Fig. 2D; Supplemental Fig. S1C). Consistent with the rapid loss of hematopoietic cells in the BM, depletion of UPF2 was accompanied by severe anemia (Fig. 2G). The possibility that interferon (induced by pIC) synergizes with the loss of UPF2 to mediate the strong hematopoietic phenotype is rendered highly unlikely by the opposite effects that pIC injection has on control and *Upf2^{fl/fl};Mx1Cre* HPCs (Fig. 2F).

Collectively, these findings demonstrate that UPF2 is essential for the maintenance of the hematopoietic compartment within the BM. In contrast, we could not detect any changes in the numbers of peripheral B- and T-cells in neither spleen nor lymph nodes, suggesting that resting lymphocytes are resistant to acute deletion of UPF2, at least at an early time point (data not shown).

The recombined Upf2^Δ allele is recessive and encodes a truncated form of UPF2

In order to verify efficient recombination of the *Upf2^{fl}* allele, we isolated CD4⁺, CD8⁺, and B220⁺ splenocytes from pIC-injected *Upf2^{fl/fl};Mx1Cre* mice. Allele-specific PCR demonstrated the efficient conversion (>85%) to the recombined *Upf2^Δ* allele in splenocytes in as little as 6 d after the first pIC injection (Fig. 1E). At the protein level, we detected a truncated ~120-kDa form of UPF2 in recombined cells, demonstrating that translation of mRNA derived from the *Upf2^Δ* allele initiates at a downstream ATG (Fig. 1F). A protein of similar size was also observed in nonrecombined cells, suggesting that it may be a natural isoform of UPF2 initiating at codon 403. We next tested whether the 120-kDa UPF2 displayed any dominant-negative or gain-of-function properties. Expression of the 120-kDa UPF2 were found to interfere neither with NMD in transfected HeLa cells nor with hematopoietic homeostasis in pIC-injected *Upf2^{fl/fl};Mx1Cre* mice (Supplemental Fig. S1B,C). These findings demonstrate that it is the loss of full-length UPF2 and not the expression of the 120-kDa isoform that promotes the strong hematopoietic phenotype observed in pIC-injected *Upf2^{fl/fl};Mx1Cre* mice.

UPF2 is necessary for maintenance of the hematopoietic system in a cell-autonomous manner

The *Mx1Cre* transgene is also expressed in nonhematopoietic cells, including liver and intestine (Kuhn et al. 1995). Therefore, in order to test the intrinsic effects of deleting *Upf2* on hematopoiesis, we took advantage of the CD45.1/CD45.2 system to generate BM chimeras by transplanting *Upf2^{fl/fl};Mx1Cre* BM (CD45.2) into lethally irradiated wild-type recipients (CD45.1) and vice versa (Fig. 3A). [CD45.1 and CD45.2 are alloantigens of CD45. They are used to distinguish cell populations of different origin in transplantation experiments.] pIC injection was initiated 16 wk after transplantation and was found to be fatal for all wild-type recipients transplanted with *Upf2^{fl/fl};Mx1Cre* BM (Fig. 3A). In contrast, only half of the chimeras (two out of four) generated by transplantation of wild-type BM died within our 3-wk observation period and did so by nonhematopoietic phenotypes (Fig. 3A; data not shown). The hematopoietic phenotype of the *Upf2^{fl/fl};Mx1Cre* BM chimeras was indistinguishable from that of *Upf2^{fl/fl};Mx1Cre* animals. At day 9 after initiation of pIC injection, the few surviving *Upf2^{fl/fl};Mx1Cre* BM chimeras were highly anemic (Fig. 3B) and had essentially no nucleated cells within their BM, including granulocytes and progenitors (c-Kit⁺) (Supplemental Fig. S2A; data not shown). Interestingly, UPF2 deletion in the *Upf2^{fl/fl};Mx1Cre* BM chimeras was associated with the rapid and almost complete loss of the HSC-containing LSK (Lin⁻, Sca-1⁺, c-Kit⁺) compartment (Fig. 3C). At this time point, control *Upf2^{fl/fl}* BM chimeras also displayed reduced numbers of nucleated cells, and the Sca-1 expression on LSK-HSCs was somewhat affected. This was attributed to the transient and mobilizing effect pIC have on HPCs (Fig. 2F), as no differences were present between uninjected and pIC-injected *Upf2^{fl/fl}* BM chimeras when interrogated at 3 wk post-pIC (data not shown). These findings demonstrate that UPF2 is necessary for maintenance of the BM in a cell-autonomous manner and that the pronounced lethality observed upon its deletion can be ascribed to its importance in maintaining hematopoietic homeostasis including the essential HSC compartment.

In order to follow the effects of UPF2 deletion over a longer time span and avoid the complications of analyzing the hematopoietic system during the strong interferon response, we generated BM chimeras by transplanting lethally irradiated CD45.1 recipients with a 1:9 ratio of wild-type (CD45.1/2) and *Upf2^{fl/fl};Mx1Cre* (CD45.2) BM cells. In this setting, the presence of a small wild-type BM fraction ensures the survival of the BM chimeras and also provides a competitive approach to evaluate the consequences of UPF2 ablation. Following engraftment, these mice were treated with three consecutive pIC injections, and the contributions from donor and recipient populations were followed in the peripheral blood (PB). In contrast to the control cohort, transplanted under identical conditions using *Upf2^{fl/fl}* (CD45.2) test cells, ablation of full-length UPF2 led to a rapid decline in overall donor derived CD45.2 cells in PB (Fig. 3D).

Analysis of peripheral B-cells and granulocytes 12 wk after UPF2 ablation revealed a fivefold and 25-fold reduction, respectively, of the *Upf2^{fl/fl};Mx1Cre* cell contribu-

tion to these two lineages (Fig. 3E). As granulocytes need to be continuously supplied from the BM, in contrast to the longer-living B-cells, these findings demonstrate that

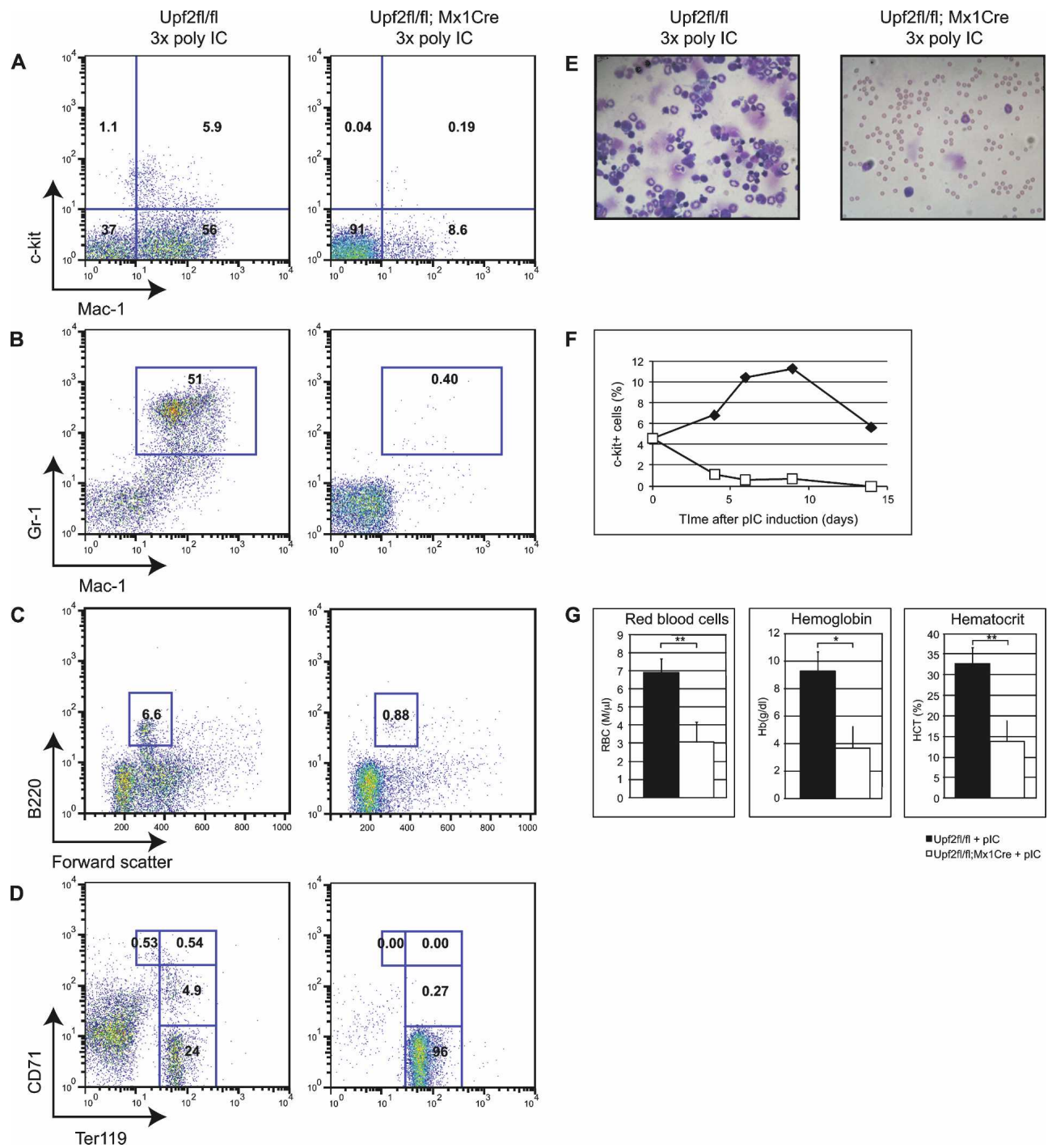


Figure 2. Deletion of full-length UPF2 leads to BM breakdown. (A–D) FACS analysis of BM cells from pIC-injected *Upf2^{fl/fl}* and *Upf2^{fl/fl};Mx1Cre* mice (analyzed at day 6 after initiation of pIC injections). BM cells were stained with directly conjugated antibodies against progenitors (c-Kit⁺), granulocytes (Mac1⁺Gr1⁺), B-cells (B220⁺), and erythroid cells (CD71⁺Ter119⁺). Immature erythroid cells are CD71⁺Ter119⁺ and gradually lose their CD71⁺ expression during maturation. (E) Cytospins of the same BM cells as described above. (F) Quantification of c-Kit⁺ HPC progenitors (as assayed by FACS) in the BM of control *Upf2^{fl/fl}* (black diamonds) and *Upf2^{fl/fl};Mx1Cre* mice (white squares) following pIC injection. (G) Erythroid parameters in the PB of the pIC-injected *Upf2^{fl/fl}* (black bars) and *Upf2^{fl/fl};Mx1Cre* mice (day 6 after the first pIC injection). Parameters were measured using a Hemavet 950FS. (*) *P* < 0.05; (***) *P* < 0.01.

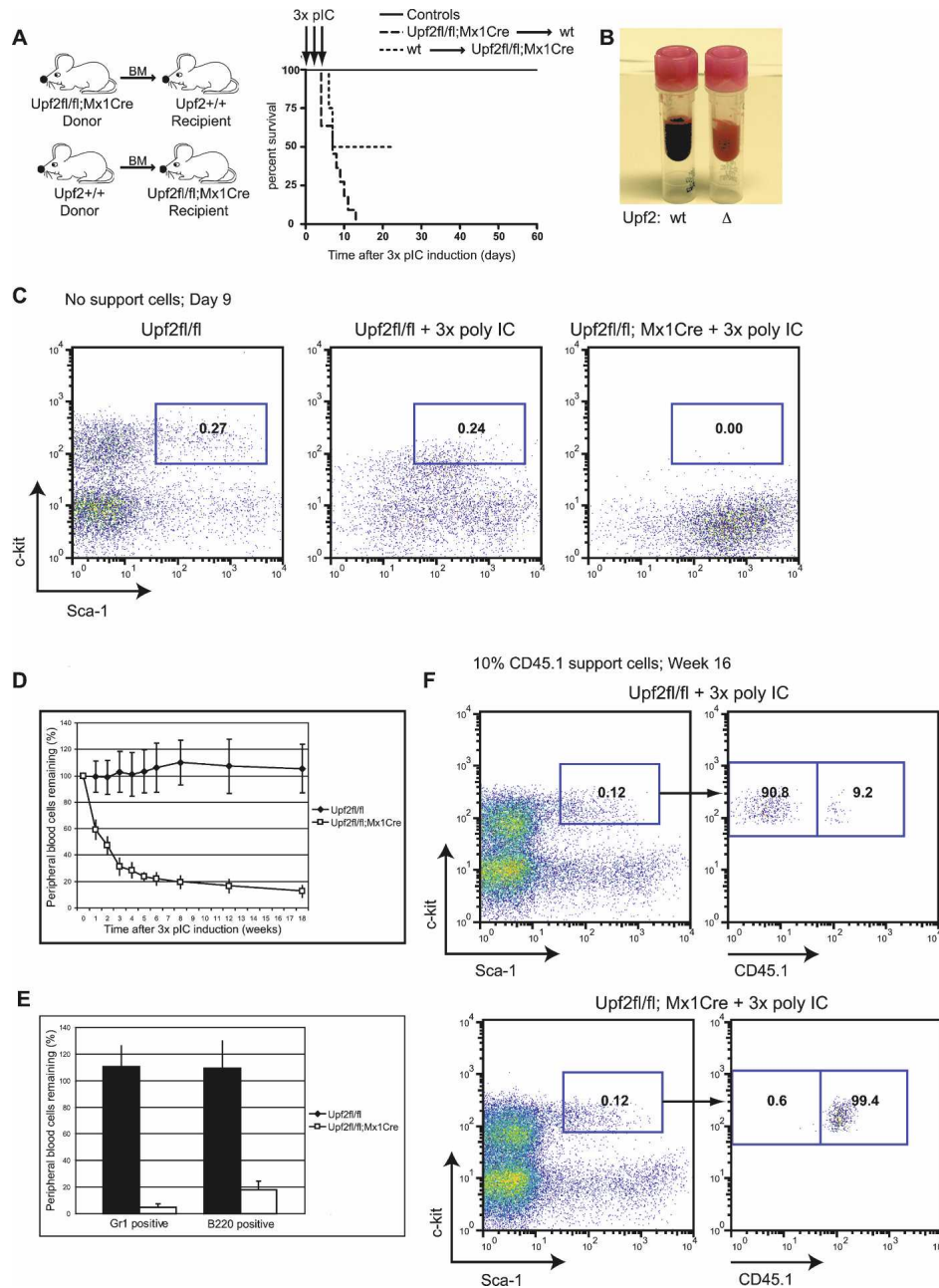


Figure 3. The rapid mortality upon UPF2 deletion is a cell-autonomous feature of BM cells. (A) Lethally irradiated recipient mice were transplanted with 2 million cells as indicated and left for 16 wk before UPF2 deletion. pIC injection led to rapid mortality of all mice transplanted with *Upf2^{fl/fl};Mx1Cre* BM ($N = 11$). (B) PB from control and *Upf2^{fl/fl};Mx1Cre* transplanted animals at day 9 after pIC injection demonstrating severe anemia upon UPF2 deletion. (C) FACS analysis of Lin⁻, 7AAD⁻ subsets from BM chimeras showing the rapid loss of both the c-Kit⁺ HPC and the HSC-containing LSK populations upon UPF2 deletion (day 9 post-pIC). Please note that the interferon response leads to up-regulation of Sca-1 expression in pIC-injected control mice. LSK frequencies are related to total BM. (D) CD45.2 contribution to PB of recipient mice transplanted with a 9:1 mixture of *Upf2^{fl/fl};Mx1Cre* (or control) (CD45.2) and support cells (CD45.1/2). After 5 wk of engraftment, mice were subjected to pIC injections, leading to the selective loss of CD45.2⁺ *Upf2^{fl/fl};Mx1Cre* cells. Data are presented as the mean \pm SD of a cohort of 10 UPF2 ablated and eight control mice. (E) Lineage analysis of PB isolated from the BM chimeras from D at week 12 post-pIC. Data are presented as the mean \pm SD of 10 UPF2 ablated and eight control mice. (F) FACS analysis of Lin⁻, 7AAD⁻ subsets from BM chimeras from D analyzed at week 16 post-pIC. UPF2 deletion leads to a selective loss of CD45.2 *Upf2^{fl/fl};Mx1Cre* (depicted as CD45.1⁻).

UPF2 deletion leads to long-term ablation of hematopoiesis. Analysis of the BM compartment at the experimental endpoint (18 wk after pIC injection) demonstrated

that whereas *Upf2^{fl/fl}* cells contributed massively to all tested lineages including B-cells, granulocytes, progenitors (Supplemental Fig. S2B–D), and LSK cells (Fig. 3F),

the contribution of *Upf2^{fl/fl};Mx1Cre* (CD45.1⁻) was reduced to background levels. These data clearly demonstrate a long-term cell-autonomous requirement of UPF2 for hematopoietic maintenance.

To rule out the possibility that the absence of LSK cells could be due to their exhaustion during the course of the experiment, we analyzed additional BM chimeras transplanted with a similar 1:9 ratio of wild-type (CD45.1) and *Upf2^{fl/fl};Mx1Cre* (CD45.2) BM cells. Sixteen weeks post-transplantation, the animals were injected with three consecutive pIC injections and analyzed at day 18, which is the earliest time point possible if we are to avoid the pIC effects in control mice (data not shown). Consistent with our previous analysis, UPF2 deletion led to the striking and complete replacement of CD45.2 HSCs (LSKFlt3⁻) and c-Kit⁺ progenitor cells with CD45.1 control cells (Fig. 4A). These findings demonstrate an obligate requirement of UPF2 for the maintenance of the HSC and c-Kit⁺ progenitor compartments.

Loss of full-length UPF2 leads to rapid loss of HPCs in vivo and HPC activity in vitro

The rapid loss of c-Kit⁺ cells upon deletion of full-length UPF2 in the BM could be caused by a specific effect on these cells or by down-regulation of the c-Kit receptor itself. We therefore assayed the levels of progenitor populations with low or absent c-Kit expression. Consistent with a general effect on all tested stem and progenitor populations, UPF2 ablation led to a pronounced down-regulation in both common lymphoid progenitors (CLP, as assayed by CD45.2 donor contribution) and B-cell progenitors (Fig. 4B; Supplemental Fig. S3).

We next tested the in vitro performance of myeloid progenitors and LT-HSCs. First, we used a colony-forming assay to demonstrate that UPF2-deleted BM cells were devoid of any myeloid colony-forming potential (Fig. 4C). Second, prospectively isolated HSCs (LSKFlt3⁻) from control and *Upf2^{fl/fl};Mx1Cre* mice were subjected to a 3-d in vitro treatment with interferon (to facilitate UPF2 deletion) during and after which the growth of the cultures was tested. Deletion of UPF2 resulted in a robust cessation of growth and in the appearance of highly dysmorphic *Upf2^{Δ/Δ}* cells (Fig. 4D; Supplemental Fig. S4).

Collectively our phenotypic analysis of the BM deletion of UPF2 demonstrates a unique and absolute requirement for UPF2, and by inference NMD, for the maintenance of all tested stem and progenitor subsets within the BM.

Loss of full-length UPF2 impact on T-cell development, clearance of PTC⁺ Tcrb mRNA, and in vitro stimulation of lymphocytes

Having demonstrated the importance of full-length UPF2 for the maintenance of hematopoiesis within the BM, we next sought to address the phenotypical consequences of deleting UPF2 during development of the

T-cell lineage. To that end we used the T-cell-specific deleter *LckCre*, in which expression of the Cre recombinase has been reported to be activated at the double-negative stage (DN; CD4⁻CD8⁻) during thymic T-cell development (Hennet et al. 1995). In this system, deletion of UPF2 (Fig. 5A) resulted in thymic atrophy with significant reductions in the total numbers of both double-positive (DP; CD4⁺CD8⁺ [threefold]) and single-positive (SP; CD4⁺ [fourfold to fivefold] and CD8⁺ [two-fold to threefold]) T-lymphocytes (Fig. 5B; Supplemental Fig. S5A). Furthermore, the reduction of *Upf2^{fl/fl};LckCre* thymocytes was accompanied by increased numbers of AnnexinV⁺ preapoptotic cells (Fig. 5C).

Programmed DNA rearrangements at the *Tcr* loci occur in immature CD4⁻CD8⁻ DN T-cells and are essential for generating the large functional repertoire of the immune system. Only one in three recombination events is expected to lead to transcripts with full coding potential, and the remaining PTC⁺ *Tcrb* transcripts (encoding the TCR-β protein) are most likely cleared from thymocytes by NMD (Carter et al. 1995; Li and Wilkinson 1998). To test this directly, RNA isolated from either SP T-cells or whole thymus derived from *Upf2^{fl/fl};LckCre* and control mice was subjected to a RT-PCR protocol across the V-D-J recombination junctions of the *Tcrb* transcript (Supplemental Fig. S6). The *Tcrb* PCR products were cloned, and the resultant inserts were subjected to sequencing. Whereas no PTC⁺ fragments were identified in any sequences derived from wild-type mice, 29% and 27% of the sequences derived from SP and total *Upf2^{fl/fl};LckCre* thymocytes, respectively, contained PTCs as a result of nonproductive rearrangements (Fig. 5D). These findings unequivocally demonstrate that the NMD pathway is highly active in T-cells and that its abrogation leads to accumulation of PTC⁺ *Tcrb* transcripts.

We consistently observed CD4⁺ and CD8⁺ SP T-cells in spleen and lymph nodes from *Upf2^{fl/fl};LckCre* mice at ~30% of the wild-type control levels (Supplemental Fig. S5A,B). In contrast to what was found in thymocytes, sequencing of *Tcrb* cDNA did not reveal any appreciable accumulation of PTC⁺ *Tcrb* transcripts in *Upf2*-null peripheral T-cells (one out of 26), suggesting that an aberrantly rearranged *Tcrb* allele is deleterious to resting T-cells (Fig. 5D). We then asked whether the peripheral T-cells without PTC⁺ *Tcrb* mRNAs had indeed lost the expression of full-length UPF2. Surprisingly, we could detect fully recombined peripheral T-cells (Supplemental Fig. 5C,D), as measured by recombination of the *Upf2^Δ* allele in spleen and lymph node from *Upf2^{fl/Δneostop};LckCre* mice (the *Upf2^{Δneostop}* allele is another null allele derived by recombination of the original *Upf2^{neostop}* allele). This implies that whereas resting peripheral T-cells are unable to tolerate PTC⁺ *Tcrb* transcripts, they can survive in the absence of UPF2 and, by inference, NMD.

Having demonstrated that UPF2 is required for proper T-cell development but appears dispensable in peripheral T-cells lacking PTC⁺ *Tcrb* transcripts, we next asked whether acute disruption of UPF2 in mature T-cells had any impact on their ability to be activated in response to exogenous stimuli. We therefore isolated splenocytes

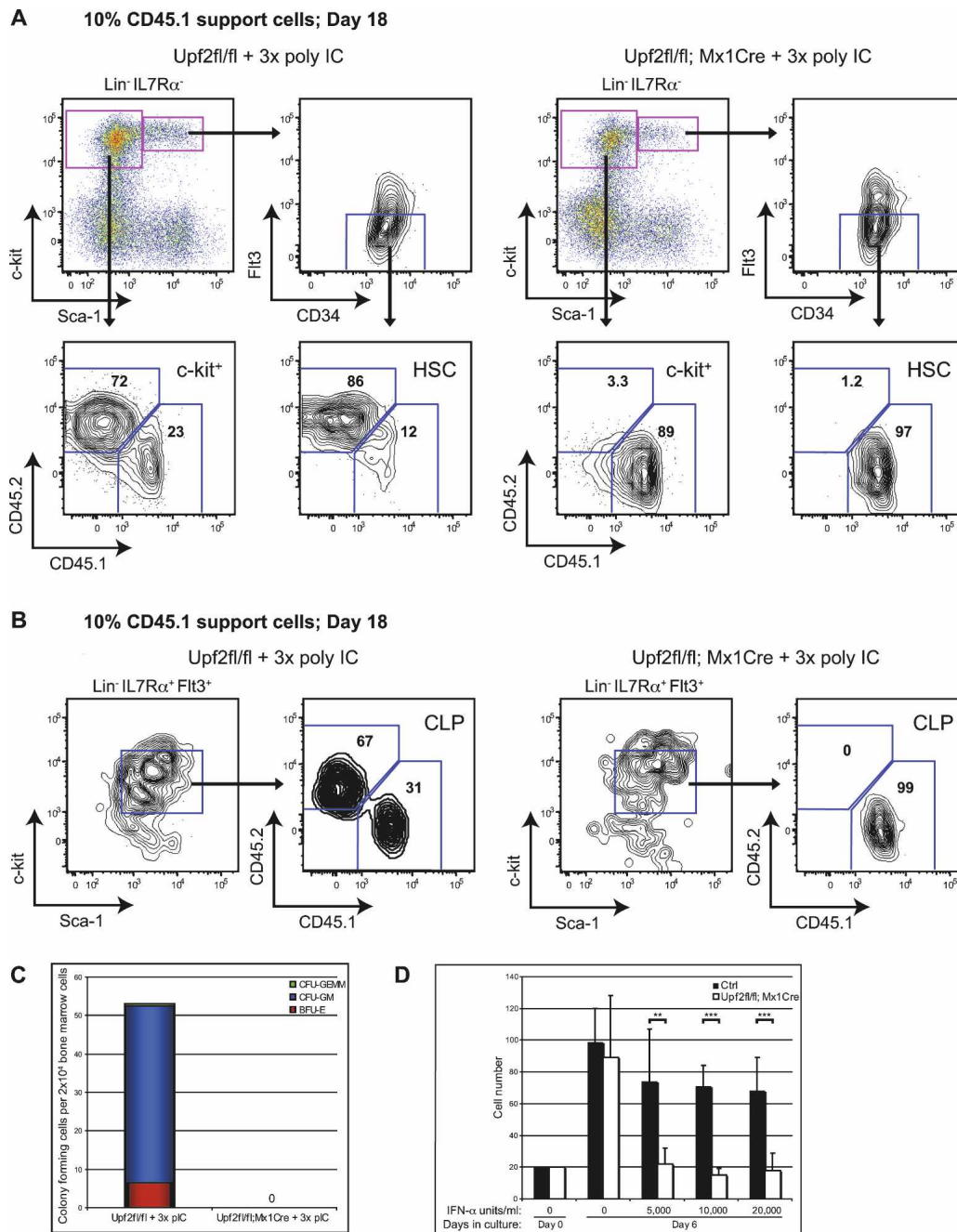


Figure 4. UPF2 deletion leads to fast clearance of the entire HSC and progenitor compartment. (A) FACS analysis of Lin⁻, IL-7Rα⁻ BM subset from lethally irradiated recipients transplanted with a 9:1 mixture of Upf2^{fl/fl};Mx1Cre or control (CD45.1) and support cells (CD45.1). Sixteen-week post-transplant mice were subjected to 3× PIC injections and analyzed at day 18. UPF2 deletion leads to loss of both c-Kit⁺ progenitors and HSCs (LSKFlt3⁻). (B) FACS analysis of Lin⁻IL-7Rα⁺Flt3⁻ BM subset from the mice from A demonstrating the strong selection against Upf2^{Δ/Δ} cells within the CLP fraction. (C) The number of colony-forming units (CFUs) of BM cells derived from PIC-injected (3×, cells plated at day 6) Upf2^{fl/fl} and Upf2^{fl/fl};Mx1Cre mice. Colonies were scored after 12 d in M3434 medium as either multipotent CFU-GEMM (granulocyte/erythrocyte/macrophage/megakaryocyte), committed myeloid CFU-GM (granulocyte/macrophage), or committed erythroid BFU-E (burst-forming unit; erythrocyte). More than 200,000 Upf2^{fl/fl};Mx1Cre BM cells were assayed. (D) FACS-sorted LSKFlt3⁻ cells were grown for 3 d in 96-well plates, in the absence or presence of IFN-αA/D, in medium containing thrombopoietin and stem cell factor. Growth was continued for another 3 d after which the numbers of cells were scored. Each data point is averaged from six individual wells (means of triplicates of sorted cells from two different mice of each genotype ± SD). (***) P < 0.001.

from pIC-treated *Upf2^{fl/fl};Mx1Cre* animals, labeled them with CFSE, and stimulated their proliferation in vitro. Strikingly, deletion of UPF2 led to a profound reduction in the ability of both CD4⁺ and CD8⁺ SP T-cells to enter the cell cycle in response to exogenous stimuli (Fig. 5E). Similar observations were made when mature B-cells were stimulated in vitro, demonstrating the common requirement of resting lymphocytes for functional UPF2 for activation-induced cell cycle entry.

Collectively, these data demonstrate that functional UPF2, and by inference NMD, is required for the development of T-cells, for the clearance of nonfunctional *Tcrb* transcripts, and for the ability of lymphoid cells to re-enter the cell cycle in response to extracellular stimuli. Finally, the increased accumulation of proapoptotic cells in the thymus strongly suggests that UPF2-depleted thymocytes are cleared by apoptosis.

Deletion of UPF2 is tolerated in the myeloid lineage

Given the strong phenotypic consequences of UPF2 deletion in both the HSC/HPC compartments of the BM, it

could not be established whether the observed reductions in cells of the myeloid lineages were secondary to impaired stem and progenitor function or due to UPF2 being directly required for the integrity of myeloid cells. Thus, we next addressed the functional consequences of specifically deleting UPF2 in the myeloid lineage. To that end we crossed the *Upf2^{fl/fl}* allele into the *LysMCre* knock-in strain, which has been reported to express Cre recombinase mainly in the granulocytic/monocytic lineage (Clausen et al. 1999). Surprisingly, FACS analysis of BM cells isolated from *Upf2^{fl/fl};LysMCre* animals did not reveal any reduction in the numbers of either granulocytes or monocytes, despite the significant reduction of full-length UPF2 in the former (Fig. 6A,B).

We next tested the impact of UPF2 deletion during Macrophage Colony-Stimulating Factor (M-CSF)-mediated differentiation of BM cells. This in vitro differentiation protocol resulted in essentially pure populations of BM-derived macrophages (BMMs), which were able to phagocytose irrespectively of their UPF2 status (Fig. 6B,C). Transfection with wild-type and PTC⁺ β -globin reporter constructs demonstrated that *Upf2^{fl/fl};LysMCre* BMMs were indeed devoid of any NMD activity (Fig.

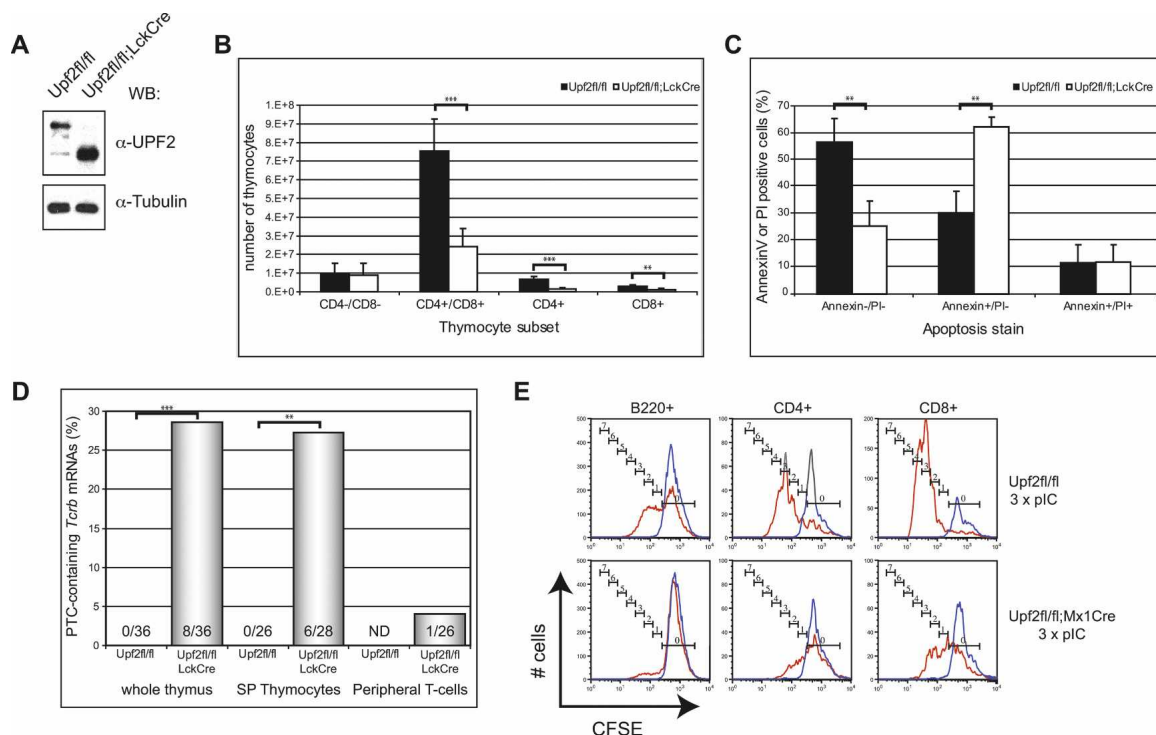


Figure 5. T-cell-specific loss of UPF2. (A) Western blot showing efficient recombination of UPF2 in thymus (*Upf2^{fl/fl};LckCre*). (B) Quantification of thymocyte subsets from *Upf2^{fl/fl};LckCre* mice, based on the expression of CD4 and CD8. (C) UPF2 deletion leads to the accumulation of early apoptotic thymocytes in *Upf2^{fl/fl};LckCre* mice as assayed by the presence of AnnexinV⁺, PI⁺ cells. (D) Accumulation of PTC⁺ *Tcrb* mRNAs in UPF2-deficient thymocytes but not mature peripheral cells (*Upf2^{fl/fl};LckCre*) as assayed by a strategy involving RT-PCR, cloning, and sequencing (Supplemental Fig. S6). Numbers of PTC⁺ sequences (x) and numbers of total clones sequenced (y) are given for each condition (x/y). (E) CFSE-labeled UPF2-deficient splenocytes (*Upf2^{fl/fl};Mx1Cre*) failed to proliferate in response to mitogenic stimuli. Splenocytes (day 6; i.e., 2 d after the last pIC injection) were stimulated in vitro for 72 h with either LPS (B-cells) or CD3 + CD28 antibodies (T-cells); stained with conjugated B220, CD4, or CD8 antibodies; and assayed by FACS analysis. Blue and red lines indicate nonstimulated and stimulated cells, respectively. The numbers of cell divisions, as given by the "Proliferation" feature of FlowJo, are shown. Data in B and C are presented as the mean \pm SD of four UPF2 ablated and 11 control mice. (***) $P < 0.001$; (**) $P < 0.01$.

6D). Finally, when BM cells were pulse-labeled with CFSE at either day 2 or day 4 during a 7-d M-CSF-mediated in vitro differentiation protocol, we did not observe any proliferative disadvantage in *Upf2^{fl/fl};LysMCre* BMMs (Fig. 6E).

Collectively, these findings demonstrate that UPF2, and by inference NMD, is dispensable during terminal differentiation of the myeloid lineage.

Gene expression profiling of *Upf2^{Δ/Δ}* BMMs and thymocytes

Knockdown experiments in transformed human cells suggested that NMD components had extensive effects on the regulation of 4%–10% of the transcriptome (Mendell et al. 2004; Wittmann et al. 2006). To examine the importance of NMD on global transcription under physiological conditions, we subjected BMMs and thymocytes derived from *Upf2^{fl/fl};LysMCre* and *Upf2^{fl/fl};LckCre* mice, respectively, to microarray-based gene expression profiling (Supplemental Tables S2, S3). Both these cell types show complete ablation of NMD as assayed by transfection with reporter constructs or sequencing of *Tcrb* transcripts (Figs. 5D, 6D). *Upf2^{Δ/Δ}* BMMs displayed significant changes (greater than twofold; $P < 0.05$) in the expression of 189 genes (234 probe sets) of which 186 were up-regulated. Similarly, in *Upf2^{Δ/Δ}* thymocytes, we found the expression of 37 genes (42 probe sets) to be significantly changed with only two of these being down-regulated (Fig. 7A). Close to 75% (26 out of 35) of the up-regulated genes in thymocytes were also up-regulated in BMMs, thereby identifying these as core UPF2 targets (Fig. 7C). Most of these transcripts have features of bona fide NMD targets including upstream ORFs (uORFs) and 3' untranslated region (UTR) introns or can generate PTC⁺ isoforms through AS (Fig. 7B,C). The

strong bias toward up-regulation of transcript levels is consistent with the removal of a mRNA degradation component.

Within the conserved UPF2 core set, we noticed three well-described SnoRNA host genes—*Snord22*, *Snhg6*, and *Gas5* (Fig. 7C,D). SnoRNAs belong to the ever-expanding world of small noncoding RNAs and are mainly described as serving as site-specific guides for post-transcriptional modifications of rRNA, but also for other RNAs (Kiss et al. 2006). In addition, six of the eight up-

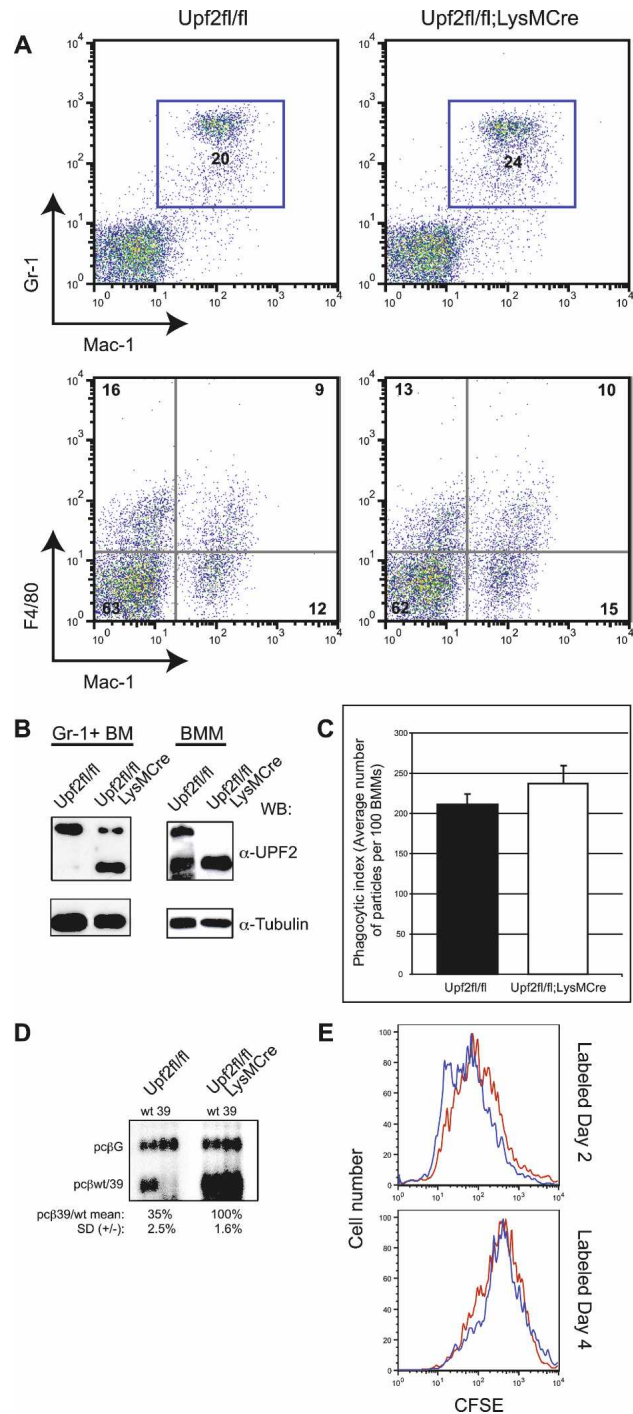


Figure 6. UPF2 deletion is well-tolerated within the myeloid lineage. (A) FACS analysis of BM from control and *Upf2^{fl/fl};LysMCre* mice demonstrates no alterations in the frequencies of either Mac-1⁺Gr-1⁺ granulocytes or Mac-1⁺F4/80⁺ monocytes. (B) Western blot analysis of MACS-purified Gr-1⁺ BM cells and in vitro derived BMMs demonstrate a partial (albeit significant) and complete deletion of UPF2, respectively. (C) The phagocytic index of BMMs, measured by the average number of ingested Zymosan A particles per 100 BMMs, reveals no functional alterations upon deletion of UPF2. Data are presented as the mean ± SD of three mice per genotype. (D) The ability of UPF2-deleted BMMs to perform NMD is totally abolished as assayed by transient transfections of BMMs with NMD-sensitive (pcβ39) and NMD-insensitive (pcβWT) reporter constructs. Band intensities on the Northern blot were normalized to a control (pcβG), and relative resistance toward NMD is shown as the ratio between pcβ39 and pcβWT for a given genotype (see Supplemental Fig. S1 for details). (E) FACS-based CFSE analysis of BMM outgrowth labeled at either day 2 or day 4 during a 7-d differentiation protocol. No significant changes in the loss of CFSE labeling during BMM differentiation was observed between *Upf2^{fl/fl}* (blue lines) and *Upf2^{fl/fl};LysMCre* (red lines).

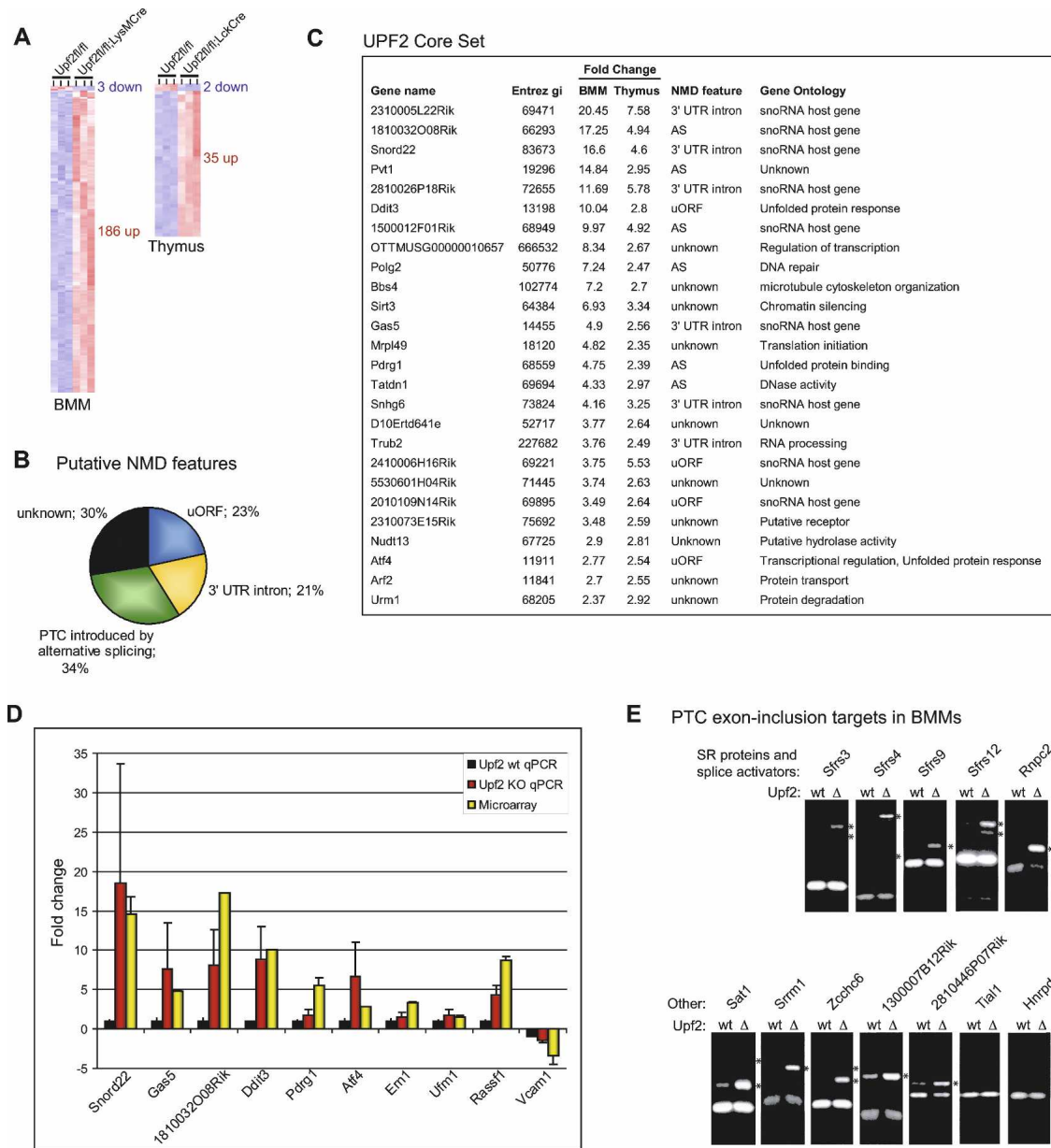


Figure 7. Deletion of UPF2 derepresses snoRNA host genes and interferes with the autoregulatory circuitry regulating the levels of splicing activators. (A) Microarray-based expression analysis of UPF2-depleted BMMs and thymocytes (a detailed list is given in Supplemental Tables S2, S3). Note the strong bias toward up-regulation. The numbers of deregulated genes (greater than twofold; $P < 0.05$) are given to the right of the heat maps. The data were obtained from three independent samples of each genotype (males, pairwise litter-matched). (B) Graphical representation of NMD features for the 60 most up-regulated genes in the BMM data set. (C) The UPF2 core set constitutes genes that are deregulated upon UPF2 deletion in both BMMs and thymocytes. (D) Quantitative RT-PCR validation of a subset of genes in the UPF2 core set. The fold change of the deregulation for individual genes are depicted as determined by quantitative PCR (red) and microarray (yellow). Data are presented as the mean \pm SD of three mice per genotype. (E) Deletion of UPF2 affects the distribution of alternative splice isoforms of mRNAs containing highly or ultraconserved elements (Ni et al. 2007). RT-PCR analysis of RNA isolated from BMMs shows that the PTC⁺ AS transcripts were stabilized for 10 out of 12 tested genes (including five known splicing activators). Splice isoforms that are up-regulated in the absence of UPF2 are indicated by an asterisk.

regulated Riken sequences in our core set were also found to contain SnoRNAs within their introns upon manual inspection. Thus, 35% of the genes in the UPF2 core set serve as SnoRNA host genes, representing a total of 23 different SnoRNA genes (Supplemental Table 4).

These findings demonstrate an unexpected requirement for NMD in down-regulation of several SnoRNA host genes.

The UPF2 core set also contained several genes (*Ddit3*, *Atf4*, *Ufm1*, *Ern1*, *Pdrg1*) involved in endoplasmic re-

ticulum (ER) stress, protein degradation, and the unfolded protein response (UPR). The latter pathway senses unfolded proteins in the ER by various sensors and initiates a program resulting in an increase in ER folding capacity, temporary cessation of protein synthesis, and, if the unfolded protein load is too severe, apoptosis (Ron and Walter 2007). The up-regulation of UPR genes in UPF2-depleted cells could be due to global up-regulation of PTC⁺ transcripts encoding truncated proteins with reduced folding potential leading to the induction of UPR. However, we were unable to detect a significant difference in the UPR of UPF2-depleted BMMs and thymocytes, as assayed by up-regulation of the ER chaperone BiP, increased phosphorylation of the general translation initiation factor eIF2 α , or increased cleavage of Xbp1 pre-mRNA (Supplemental Fig. S7), suggesting that at least in these cells, the UPR pathway is not activated in response to loss of NMD. Possibly the presence of NMD-sensitive UPR transcripts (e.g., *Atf4*) could reflect the existence of an autoregulatory loop, where UPR-mediated inhibition of protein synthesis (and thereby NMD) leads to the selective and sustained stabilization of key UPR transcripts during UPR.

Ablation of NMD results in stabilization of alternatively spliced transcripts containing PTC

AS has been hypothesized as a mechanism to extend the protein-coding diversity without expanding the number of genes. Approximately one-third of all AS transcripts contain a PTC, and NMD has been proposed to safeguard the cell from these potentially deleterious transcripts (Lewis et al. 2003). Previous reports using knockdown of UPF1 have demonstrated that a fraction of PTC⁺ AS transcripts is destabilized by NMD, a mechanism coined AS coupled with NMD (AS-NMD) (Pan et al. 2006; Ni et al. 2007). Interestingly, a group of transcripts with highly conserved PTC⁺ exons were enriched among AS-NMD targets, and splicing activators (e.g., SR proteins) and splicing repressors (e.g., hnRNP proteins) were further overrepresented in this group, suggesting that their levels could be regulated by AS-NMD (Lareau et al. 2007; Ni et al. 2007). To test whether these findings were valid in primary cells, we performed RT-PCR on alternatively spliced mRNAs previously identified as PTC⁺ exon targets (Ni et al. 2007), to determine if the levels of the AS PTC⁺ transcripts were stabilized in *Upf2*^{Δ/Δ} BMMs (Fig. 7E). For 10 out of 12 genes tested we could indeed show that the AS PTC⁺ isoform was up-regulated in NMD ablated cells. Significantly, all five tested splice activators were verified. These findings suggest that NMD plays an important role in vivo in modulating regulated AS events.

Discussion

The NMD pathway has emerged as an important post-transcriptional mechanism that eliminates PTC⁺ mRNAs, thereby prohibiting the synthesis of potential

harmful protein products. Although having been extensively studied at the biochemical level, this study represents the first in-depth analysis of the NMD pathway in the context of a mammalian organism.

Since UPF2, in contrast to several of the other NMD components, has not been associated with non-NMD functions, we chose to target the *Upf2* locus. Similar to the previously characterized *Upf1*-null mice, mice homozygous for our functional *Upf2*-null allele died during early embryogenesis, further substantiating NMD as an essential pathway during fetal development.

We sought to model the importance of NMD in one of the central adult self-renewing organ systems, the BM. Here, induced deletion of UPF2 led to fast removal of all nucleated cells, including c-Kit⁺ progenitor cells, in a cell-autonomous manner, and death of the animals within 5–10 d. We demonstrated that LSK cells are completely depleted from BM in as little as 9 d after initiating UPF2 deletion. More elaborate experiments using mixed BM chimeras (to ensure survival of the transplanted animals) confirmed this finding and allowed us to demonstrate that UPF2 loss is associated with a fast, complete, and lasting extinction of the HSC compartment.

In sharp contrast to the profound effect of UPF2 deletion in hematopoietic stem and progenitor cells, we observed essentially no phenotypic consequences of deleting UPF2 in the myeloid lineage. Moreover, peripheral B- and T-cells were only mildly affected upon acute UPF2 deletion despite the *Upf2* locus being efficiently recombined. These observations are suggestive of a specific function of UPF2 in proliferating cells, a hypothesis consistent with the inability of peripheral *Upf2*^{Δ/Δ} B- and T-cells to enter the cell cycle upon mitogenic stimuli. With respect to the rarely dividing HSCs, we hypothesize that the fast clearance of progenitor cells upon UPF2 deletion either forces HSCs into cycle, leading to their rapid exhaustion, or that these cells become unable to coordinate proliferation/differentiation events and are cleared by apoptosis. Thus our data on the phenotypic description of the hematopoietic system are consistent with an obligate requirement for UPF2, and by inference NMD, for the survival of proliferating, but not terminally differentiated, cells.

NMD has been reported to be of particular importance in lymphoid cells (Li and Wilkinson 1998). During their maturation, B- and T-cells undergo extensive programmed DNA rearrangements involving the genes encoding Ig and TCR, respectively. Although these rearrangement events, which are necessary in order to generate the diverse immune repertoire, are predicted to result in a significant fraction of nonproductively rearranged alleles of these genes, PTC⁺ *Tcrb* transcripts are not accumulating in wild-type T-cells. In contrast, ~28% of *Tcrb* transcripts from NMD-deficient *Upf2*^{Δ/Δ} thymocytes contain PTCs. Strikingly, PTC⁺ *Tcrb* transcripts did not accumulate to any appreciable extent in mature T-cell populations despite the presence of *Upf2*-null, and therefore, NMD ablated T-cells. These observations suggest two interrelated conclusions: First, resting T-cells appear to display a unique requirement for NMD-mediated

ated down-regulation of PTC⁺ *Tcrb* transcripts, as opposed to a general requirement for NMD activity. Secondly, in the absence of NMD, resting T-cells expressing a nonproductively rearranged *Tcrb* allele are selected against during T-cell maturation. Thus, the NMD pathway appears to improve the efficiency of T-cell maturation by facilitating the survival of cells carrying both a nonproductively and a productively rearranged *Tcrb* allele, and we speculate that this could be generalized to other rearranged lymphoid genes. The reason(s) for the apparent toxicity of a PTC⁺ *Tcrb* transcript in resting T-cells is currently not known; however, one possibility could be that truncated TCR-β interferes with the formation of the normal TCR/CD3 complex.

NMD and gene expression

In contrast to the pronounced effects of deleting UPF2 in stem, progenitor, and T-cells, we observed no gross cellular phenotype when UPF2 was deleted in the myeloid lineage using the *LysMCre* deleter strain. This allowed us to use an in vitro differentiation protocol to generate *Upf2*^{Δ/Δ} BMMs, which appeared functionally normal. Comparison of gene expression profiles of wild-type and *Upf2*^{Δ/Δ} cells revealed a marked preference for up-regulation of transcripts in both thymus (95%) and BMMs (98%) upon ablation of NMD. This observation compares well to expression profiles obtained by knockdown experiments in *Drosophila* Schneider cells but contrasts to those from human cells, where comparable levels of up- and down-regulated genes were observed (Mendell et al. 2004; Rehwinkel et al. 2005; Wittmann et al. 2006). We hypothesize that this reflects a fundamental difference between heavily mutated mammalian cancer cell lines and primary cells.

Comparison of the gene sets derived from thymus and BMMs yielded a list of 26 core transcripts, which were commonly up-regulated upon deletion of UPF2 in the two cell types. Nine of these represented SnoRNA host genes that encode SnoRNA genes within their introns. Although some, like *Gas5*, may encode a protein, the exons of these genes are considered to facilitate the presence of the SnoRNA-containing introns and to mediate their appropriate expression. To our knowledge, NMD has not previously been implicated as a regulator of SnoRNA host gene expression in mammalian cells. In *C. elegans*, NMD was reported to down-regulate the expression of several pseudogenes, one of them hosting a single SnoRNA (Mitrovich and Anderson 2005). Here it was proposed that expressed pseudogenes constitute a pool of genes under constant evolution. To allow this extensive probing of the sequence space and to avoid their untimely resurrection, these genes are silenced by NMD, as indeed was shown in *C. elegans*. Such a mechanism may be particularly relevant in mammalian cells given the current estimates of pseudogenes (20,000) within the human genome (Torrents et al. 2003). Due to their poor representation on standard expression microarrays, our data do not reveal any information as to the global importance of NMD in down-regulation of expressed pseu-

dogenes or other ncRNAs. However, we do speculate that the NMD-regulated SnoRNA host genes we identified in the expression profiling analysis of *Upf2*^{Δ/Δ} cells may represent the tip of an iceberg made up of a large number of expressed pseudogenes. Experiments are underway to probe this possibility. Finally, it should be noted that in addition to the NMD target genes reported here, there could also be mRNAs that are regulated through an UPF2-independent NMD pathway (Gehring et al. 2005).

AS has emerged as a major mechanism of regulating gene expression and most protein-encoding genes have one or more AS transcripts (Blencowe 2006). Analysis of EST sequences revealed that ~10% are derived from PTC⁺ AS transcripts (Xing and Lee 2004). It would therefore seem reasonable to argue that ablation of NMD would strongly enhance the levels of AS-NMD transcripts with associated fatal consequences (Lewis et al. 2003). However, controversy exists concerning the magnitude of AS-NMD targets (Pan et al. 2006; Ni et al. 2007).

NMD has been convincingly demonstrated to act in a negative feedback loop regulating the activity of ~20 splicing regulators (Lareau et al. 2007; Ni et al. 2007). Here transcripts containing ultraconserved elements were shown to be involved in regulated AS events in a manner dependent on the inclusion and exclusion of PTC⁺ exons in the transcripts of splicing activators and repressors, respectively. According to this model, an autoregulatory circuit is set up, in which elevated amounts of splicing activators will result in increased levels of an NMD-sensitive transcript (by exon inclusion) leading to reduction of the activator, as exemplified by SFRS3 and SFRS10 (Jumaa and Nielsen 1997; Stoilov et al. 2004), whereas elevated amounts of a splicing repressor will repress the inclusion of a coding exon, again leading to NMD and down-regulation of the repressor (Ni et al. 2007). We tested the NMD dependence on the stabilization of PTC⁺ AS transcripts for a subset of the transcripts regulated by ultraconserved elements in *Upf2*^{Δ/Δ} BMM cells. Indeed, we were able to show that even in vivo 10 out of 12 of the tested PTC⁺ AS transcripts were deregulated in an NMD-dependent manner, including SFRS3 and SFRS10. These findings strongly suggest that disruption of the NMD pathway leads to disruption of the homeostatic control of splicing regulators, which in turn may have profound consequences on a plethora of other regulated splicing events.

Conclusions and future directions

Although we focused on the NMD-related issues in our phenotypic description of the *Upf2*^{Δ/Δ} mice, there is mounting evidence that some NMD components do have non-NMD functions in mammalian cells (Azzalin and Lingner 2006a,b). Recently it was demonstrated that NMD components, including UPF2 (although to a lesser extent), negatively regulate the accumulation of the telomere-encoded Terra RNA at telomeric regions (Azzalin et al. 2007). Interestingly, alleviation of this repression,

led to various chromosomal abnormalities, suggesting that NMD components may play a role in telomeric maintenance as previously reported for SMG-6 (Reichenbach et al. 2003). To what extent this is due to an indirect effect of ablating canonical NMD or truly novel functions for these proteins is currently uncertain. Future studies will address to what extent UPF2 deletion may affect telomere functions *in vivo*.

In summary, we provide compelling evidence for an essential role of UPF2 and, by inference, NMD in the survival and maintenance of hematopoietic stem and progenitor cells. Moreover, we show that *in vivo*, NMD impacts on several layers of gene regulation, including programmed DNA rearrangements at the *Tcrb* locus, processing of non-protein-coding genes and regulation of AS. Similar to the global effects observed by disruption of miRNA biogenesis *in vivo* (Bernstein et al. 2003), our challenge for the future will be to determine the relative contributions of these regulatory mechanisms to the strong phenotype of the *Upf2^{Δ/Δ}* mice, and to determine to what extent they can be ascribed to the deregulation of individual molecular players, to the global impact conferred by the loss of NMD or to any non-NMD functions of UPF2.

Materials and methods

Colony assays

For the analysis of colony-forming potential, 20,000 BM cells were plated in semisolid methylcellulose-based medium (M3434; StemCell Technologies) supplied with erythropoietin, IL-3, IL-6, and stem cell factor. After 12 d in cultures, colonies were scored as CFU-GM, BFU-E, or CFU-GEMM.

BM transplantations

Recipient mice were lethally irradiated by a single radiation dose (900 cGy) and transplanted with 2 million donor BM cells. For the experiments testing the impact of UPF2 truncation solely in hematopoietic cells, we generated BM chimeras by transplanting B6-SJL (CD45.1) recipients with either *Upf2^{fl/fl}* or *Upf2^{fl/fl};Mx1Cre* (both CD45.2) BM cells. Donor contributions in the PB were >90%. Sixteen weeks after transplantation, mice were subjected to pIC injections.

In order to test the effects of UPF2 truncation in nonhematopoietic cells, we generated reverse BM chimeras by transplanting *Upf2^{fl/fl}* or *Upf2^{fl/fl};Mx1Cre* (CD45.2) recipients with wild-type B6-SJL (CD45.1) BM. Donor contributions in the PB were >90%. Sixteen weeks after transplantation, mice were subjected to pIC injections.

For the experiments testing the performance of *Upf2^{fl/fl};Mx1Cre* donor cells over time, lethally irradiated B6-SJL (CD45.1) recipients were transplanted with 9:1 mixtures of donor cells (CD45.2 *Upf2^{fl/fl};Mx1Cre* or *Upf2^{fl/fl}*) and carrier cells (CD45.1/2 or CD45.1 wild-type cells). Donor contributions in the PB were >90%. Five weeks or 16 wk after transplantation, mice were subjected to pIC injections.

All mouse work was approved by the Danish Animal Ethical Committee ("Dyreforsøgstilsynet").

Mitogenic stimulation of lymphoid cells

Spleen cells were harvested and red blood cells were lysed in 1 mL of RBC lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 1 mM

EDTA) for 2 min at room temperature, followed by washing in Lymphocyte medium (RPMI1640 + 10% FCS) supplemented with 20 mM HEPES. Splenocytes (50 × 10⁶/mL) were resuspended in Lymphocyte medium and CarboxyFluorescein Succinimidyl Ester (CFSE; CellTrace, Invitrogen) was added to a final concentration of 5 μM, incubated for 5 min at room temperature, and washed three times with Lymphocyte media supplemented with 20 mM HEPES. For lymphocyte stimulation, splenocytes (10⁵ splenocytes in 200 μL of Lymphocyte Medium) were added to round-bottomed 96-well plates in the presence of either 30 μg/mL LPS (Sigma) or 0.5 μg/mL of anti-CD3 + anti-CD28 antibodies (eBioscience) to stimulate B- and T-cells, respectively. Following 72-h incubation at 37°C, cells were dissociated in 0.5 mM EDTA/PBS for 5 min at 37°C, washed, and resuspended in PBS/5% FCS prior to FACS analysis.

In vitro differentiation and functional assays of BMMs

BM cells were plated at 2 × 10⁶/mL in BMM media (RPMI1640 + Glutamax-I, 10% FCS, supplemented with 10% L929-conditioned media [M-CSF source]) and grown for 7 d. BMM medium was replaced every 2–3 d until analysis. For CFSE labeling, cells were labeled with CFSE either at day 2 or 4 during the *in vitro* differentiation protocol before flow cytometric analysis.

The phagocytotic properties of BMMs were assayed using Alexa-fluor 594-conjugated Zymosan A (*Saccharomyces cerevisiae*) bioparticles (Invitrogen) according to the manufacturer's instructions. Briefly, particles were concentrated on BMMs by centrifugation followed by a 1-h incubation at 37°C. Following extensive washing, BMMs were centrifuged onto microscope slides, fixated, and costained with DAPI and phalloidin-FITC (Sigma).

The ability of *in vitro* differentiated BMMs to perform NMD was assayed by transiently transfecting BMMs using the Nucleofector Kit (Amaxa). Briefly, BMMs (1 × 10⁶ in 100 μL of Nucleofector Solution [Amaxa]) were transfected with 1 μg of pcβwtGAP3 UAC (β-globin internal control) and either 1 μg of pcβwt (NMD-insensitive) or 1 μg of pcβ39 (NMD-sensitive) β-globin reporter plasmids (Lykke-Andersen et al. 2000) in a cuvette using the Amaxa Nucleofector. After culturing for 48 h, RNA was isolated and subjected to Northern analysis.

DNA microarray analysis

RNA isolated from BMMs or whole thymus was labeled according to the one-cycle eukaryotic target-labeling protocol (Affymetrix) and hybridized to Affymetrix Mouse Genome 430 2.0 GeneChips. The .CEL files were obtained using the Affymetrix GCOS version 1.4 software and analyzed using the statistical software R. Affymetrix and *gcRMA* packages were used to correct for optical noise and nonspecific binding and to summarize multiple probes into a single expression value. Default settings for *gcRMA* modeling were used (background correction, quantiles normalization, PM-only modeling, and median polish summarization). Detection calls were determined by MAS5.0 and required 100% present calls within a replicate group to be considered expressed. Replicate groups were compared using "compare samples" from dChip (Li and Wong 2001) requiring a significant fold change >2 (*P* < 0.05, Welch *t*-test). Microarray data were deposited in ArrayExpress (accession no. E-MEXP-1371).

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search Council, and the Copenhagen Hospital Cooperation, Region H. We have no conflicting interests to declare.

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