Enhanced stability of Mcl1, a prosurvival Bcl2 relative, blunts stress-induced apoptosis, causes male sterility, and promotes tumorigenesis

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The B-cell CLL/lymphoma 2 (Bcl2) relative Myeloid cell leukemia sequence 1 (Mcl1) is essential for cell survival during development and for tissue homeostasis throughout life. Unlike Bcl2, Mcl1 turns over rapidly, but the physiological significance of its turnover has been unclear. We have gained insight into the roles of Mcl1 turnover in vivo by analyzing mice harboring a modified allele of *Mcl1* that serendipitously proved to encode an abnormally stabilized form of Mcl1 due to a 13-aa N-terminal extension. Although the mice developed normally and appeared unremarkable, the homozygous males unexpectedly proved infertile due to defective spermatogenesis, which was evoked by enhanced Mcl1 prosurvival activity. Under unstressed conditions, the modified Mcl1 is present at levels comparable to the native protein, but it is markedly stabilized in cells subjected to stresses, such as protein synthesis inhibition or UV irradiation. Strikingly, the modified *Mcl1* allele could genetically complement the loss of *Bcl2*, because introduction of even a single allele significantly ameliorated the severe polycystic kidney disease and consequent runting caused by Bcl2 loss. Significantly, the development of c-*MYC*–induced acute myeloid leukemia was also accelerated in mice harboring that *Mcl1* allele. Our collective findings reveal that, under certain circumstances, the N terminus of Mcl1 regulates its degradation; that some cell types require degradation of Mcl1 to induce apoptosis; and, most importantly, that rapid turnover of Mcl1 can serve as a tumor-suppressive mechanism.

protein turnover | programmed cell death

Apoptosis, an evolutionarily conserved process of programmed cell death for removing excess, damaged, or infected cells, is required for normal development and maintenance of tissue homeostasis throughout life. Whether a cell exposed to developmental cues or cellular stresses lives or dies is largely determined by interactions among members of the Bcl2 protein family (1). The "prosurvival" faction, comprising Bcl2 itself, Bclx$_L$, Bclw, Mcl1, and A1, maintains cell survival by blocking the activation of the second group, the "cell death mediators" Bax and Bak. Apoptosis is initiated when members of the third subfamily, the "BH3-only proteins" (e.g., Bim, Bad, Noxa) are activated by diverse cytotoxic stimuli. They trigger apoptosis by relieving the brake on Bax/Bak activation imposed by the prosurvival Bcl2 proteins and by directly activating Bax/Bak (1). Once activated, Bax and Bak permeabilize the mitochondrial outer membrane and the apoptogenic factors thereby released into the cytosol (particularly cytochrome *c*) unleash the caspase cascade that drives cellular demolition (2).

The normal physiological roles of prosurvival Bcl2 proteins have been established by studies on gene-targeted mice. For example, the absence of Bcl2 causes lymphopenia, premature graying due to loss of melanocytes, and severe polycystic kidney disease that provokes runting and early death (3, 4). Conversely, overexpression of Bcl2 causes tissue hyperplasia and promotes tumor development (5, 6). Thus, for normal development and adult life, the levels and activity of the prosurvival Bcl2 proteins must be tightly regulated.

Mcl1 plays an essential role in maintaining stem/progenitor cell populations, including those within the hematopoietic compartment (7, 8), and its enforced overexpression, like that of Bcl2, promotes cell accumulation and lymphomagenesis (9, 10). Pertinently, the human *MCL1* locus is amplified in ~10% of cancer-derived cell lines (11). Elevation of MCL1 levels by its stabilization might also contribute to tumorigenesis (12, 13). Normally, MCL1 is a short-lived protein with a $t_{1/2}$ of less than 30 min in most cell types studied. Its turnover is regulated by the ubiquitin–proteasome system, and several E3 ubiquitin ligases [HECT, UBA, and WWE domain containing 1 (HUWE1), beta-transducin repeat containing E3 ubiquitin protein ligase (βTRcP), F-box and WD repeat domain containing 7 (FBWX7)] (13–16) and the deubiquitinatinase ubiquitin specific peptidase 9, X-linked (USP9X) (12) have been implicated in controlling MCL1 levels. Moreover, Mcl1 may also undergo ubiquitin-independent proteasomal degradation (17).

The association of loss of the tumor suppressor FBWX7 (13, 16) and overexpression of the candidate oncogene USP9X (12) with tumorigenesis and poor patient prognosis has prompted proposals that stabilization of MCL1 driven by these genetic alterations is critical for the tumorigenesis and resistance of these tumor cells to standard therapeutics. However, because both

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Significance

We obtained evidence that the rapid turnover of Mcl1 has physiological significance by analyzing mice bearing a modified allele of *Mcl1* that proved to encode a stabilized form of Mcl1. In cells under stresses such as protein synthesis inhibition or UV radiation, its life span was much longer than WT Mcl1. Male mice bearing only the modified allele were sterile due to excess early spermatogenesis, and the modified allele ameliorated the polycystic kidney disease arising in mice lacking prosurvival Bcl2. Notably, the *Mcl1* allele accelerated Myc-induced acute myeloid leukemia. Thus, under certain circumstances, the Mcl1 N terminus regulates its degradation, certain cell types require Mcl1 degradation to induce apoptosis, and Mcl1 turnover serves as a tumor-suppressive mechanism.

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FBWX7 and USP9X enzymatically target multiple cellular substrates, their action on other substrates might play greater roles in tumor development.

Our serendipitous identification of mice that harbor an abnormally stabilized form of Mcl1 has allowed us to obtain direct evidence of the physiological significance of Mcl1 turnover in vivo. Although these mice are overtly normal, we show that the stabilized Mcl1 protein enhances prosurvival activity in vivo in certain circumstances. Notably, the male mice are infertile due to abnormally reduced cell death during early spermatogenesis. Strikingly, the altered *Mcl1* allele genetically complements loss of Bcl2, greatly retarding the development of polycystic kidney disease. Most importantly, our studies also establish that abnormal Mcl1 stabilization can promote tumorigenesis in vivo, at least under certain conditions.

Results

Male Mice Harboring a Conditional *Mcl1* Allele Are Unexpectedly Infertile. To generate mice in which Mcl1 can be conditionally deleted, we previously created mice with a floxed (fl) allele of *Mcl1* (18–20). As anticipated, in the absence of Cre recombinase, mice carrying one or both $Mcl1^{fl}$ alleles appeared grossly normal, without any overt signs of pathology in the major organs examined (lung, kidney, liver, colon, and heart). Moreover, the composition of the hematopoietic compartment in $Mcl1^{fl/fl}$ mice was normal (Fig. S1*A*), as was the capacity of bone marrow- or spleen-derived hematopoietic cells to grow and form colonies in vitro when stimulated with cytokines (Tables S1 and S2). Furthermore, the death of diverse cell types, such as thymocytes, exposed to apoptotic stimuli (e.g., dexamethasone) in vitro was indistinguishable from that of the corresponding WT cells (Fig. S1*B*).

Although $Mcl1^{fl/fl}$ females were fertile, we unexpectedly found that homozygous $Mcl1^{fl/fl}$ males were infertile, whereas $Mcl1^{fl/+}$ males sired normal numbers of offspring, whether mated with WT ($Mcl1^{+/+}$), $Mcl1^{fl/+}$, or $Mcl1^{fl/fl}$ females. To determine why $Mcl1^{fl/fl}$ males are infertile, we compared their testes with those of $Mcl1^{fl/+}$ and WT mice. Whereas the $Mcl1^{fl/+}$ and WT testes were indistinguishable, those from $Mcl1^{fl/fl}$ males were severely atrophic (Fig. S2 *A* and *B*). Notably, in $Mcl1^{fl/fl}$ males, the seminiferous tubules were abnormally small and the epididymides lacked mature spermatozoa (Fig. 1*A*), revealing a severe defect in spermatogenesis.

Testicular Atrophy in Male $Mcl1^{fl/fl}$ Mice Reflects Enhanced Mcl1 Prosurvival Activity. The unexpected sterility in $Mcl1^{fl/fl}$ males might indicate that Mcl1 function was altered somehow, perhaps as an unintended consequence of our gene targeting strategy. Because the loss of Bclw (21, 22) or reduction of $Bclx_L$ (23, 24) causes male infertility, we first considered whether attenuated Mcl1 function might account for the infertility of $Mcl1^{fl/fl}$ males. However, this seemed unlikely, because we have found no obvious evidence of impaired Mcl1 function (Fig. S1 and Tables S1 and S2). Moreover, Mcl1 deficiency due to unwarranted recombination of the floxed Mcl1 alleles during meiosis could be ruled out, because no $Mcl1^{fl}$ recombined cells were detectable in testes from $Mcl1^{fl/+}$ or $Mcl1^{fl/fl}$ males (Fig. S3).

Because normal spermatogenesis requires a delicate balance between cell death and survival, insufficient as well as excessive apoptosis in the testis can cause male infertility (25). Specifically, sperm cell development is blocked by loss of the cell death mediator Bax (26), or combined deficiency of the cell death initiating BH3-only proteins Bim and Blk (the mouse ortholog of BIK) (27). To assess whether $Mcl1^{fl/fl}$ males might be infertile due to abnormally enhanced rather than diminished Mcl1 function, we compared the testicular phenotype of adult $Mcl1^{fl/fl}$ males with that of similarly aged $Bax^{-/-}$ males, as well as WT and $Mcl1^{fl/+}$ controls, which were phenotypically indistinguishable (Fig. 1 and Figs. S2 and S3).

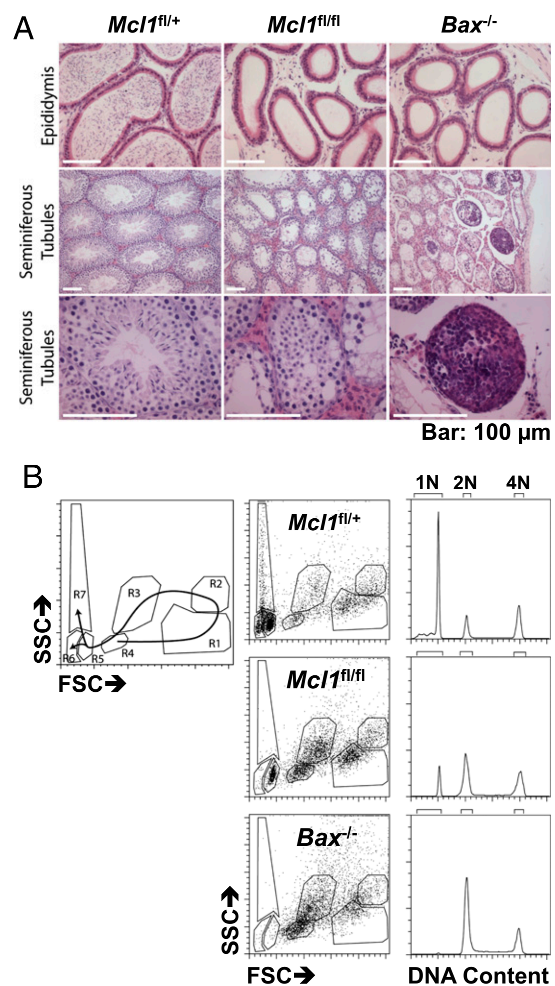

Fig. 1. Male mice homozygous for the $Mcl1^{fl/fl}$ are unexpectedly infertile. (*A*) Absence of mature and elongating spermatids in the testes of adult male $Mcl1^{fl/fl}$ mice. Representative histological sections (H&E stained) show epididymis (*Top*) and seminiferous tubules at medium (*Middle*) or high magnification (*Bottom*) from testes of $Mcl1^{fl/+}$ and $Mcl1^{fl/fl}$ males and, as a control for the complete loss of spermatogenesis, $Bax^{-/-}$ males (*Right*). (Scale bars: 100 μm.) (*B*) Flow cytometric analysis (fluorescence-activated cell sorting) of germ cell development shows that spermatogenesis arrests later in $Mcl1^{fl/fl}$ males than $Bax^{-/-}$ males. (*Left*) Schematic representation of normal germ cell development. The arrow depicts the order of maturation from 2N spermatogonia (R3/4), to 4N spermatocytes (R1/2), to 1N spermatids (R5), to mature sperm (R6/7). (*Center*) Representative forward scatter (FSC)/side scatter (SSC) profiles of germ cells from $Mcl1^{fl/+}$, $Mcl1^{fl/fl}$, and $Bax^{-/-}$ males. (*Right*) Representative DNA content profiles of germ cells from $Mcl1^{fl/+}$, $Mcl1^{fl/fl}$, and $Bax^{-/-}$ males. (*Right* and *Center*) Unlike $Bax^{-/-}$ males, $Mcl1^{fl/fl}$ males can produce immature 1N spermatids, but they fail to mature further.

The seminiferous tubules of $Mcl1^{fl/fl}$ males contained some round spermatids as well as spermatocytes (Fig. 1*A*), and flow cytometric analysis of germ cell development (Fig. 1*B*) revealed a relative accumulation of early stages, particularly 2N spermatogonia (in regions R3/R4), but a marked dearth of 1N spermatids (R5) and essentially no mature sperm (R6/R7). Accordingly, DNA content analysis revealed a far lower proportion of haploid (1N) cells and a much higher proportion of the earlier 2N cells than in the WT or $Mcl1^{fl/+}$ testis (Fig. 1*B* and Fig. S2*D*). Consistent with previous reports (26), sperm cell maturation in Bax-deficient males was arrested even earlier, before differentiation into spermatids. Although some $Bax^{-/-}$ seminiferous tubules were laden with spermatogonia and premeiotic spermatocytes, most were acellular

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(Fig. 1*A*), and flow cytometric analysis failed to identify haploid (1N) cells (Fig. 1*B* and Fig. S2*D*). Accordingly, the epididymis of Bax-deficient males was devoid of sperm, like that of the $Mcl1^{fl/fl}$ mice (Fig. 1*A*). We conclude that in $Mcl1^{fl/fl}$ males, spermatogenesis is arrested later in development than in $Bax^{-/-}$ germ males (Fig. 1 and Fig. S2). Consistent with this, a lower proportion of spermatogonia and early spermatocytes (c-Kit^+ cells) accumulated in the former (Fig. S2*C*). In fact, the spermatogenesis defect in the $Mcl1^{fl/fl}$ males is very similar, if not identical, to that in Bim/Blk double-deficient males (27).

Floxed *Mcl1* Allele Inadvertently Encodes for an Altered Form of Mcl1. The enhanced antiapoptotic activity in $Mcl1^{fl/fl}$ mice inferred from the defective spermatogenesis might suggest that the cellular levels of Mcl1 protein were elevated in these mice. However, blots of lysates from mouse embryonic fibroblasts (MEFs) and thymocytes showed that, in the absence of any stress stimuli, the levels of *Mcl1* mRNA (Fig. S4*A*) and Mcl1 protein (Fig. S5*A*) were comparable in WT and $Mcl1^{fl/fl}$ mice. To our surprise, however, the Mcl1 protein in $Mcl1^{fl/fl}$ cells was discernibly larger than that in WT cells (Fig. S5*A*). This prompted us to reexamine the targeting construct used to generate the floxed *Mcl*1 locus. We found that our placement of the loxP element in the 5′ untranslated region had inadvertently created an initiation codon upstream of, and in frame with, the native start codon, resulting in the translation of an Mcl1 protein with an additional 13 amino acids at its N terminus (Fig. S5*B*). We designate this protein N^+Mcl1 to distinguish it from the native WT protein.

In many cell types, N^+Mcl1 seems to function indistinguishably from WT Mcl1. For example, thymocytes from the $Mcl1^{fl/fl}$ mice were normally sensitive to diverse stress signals (Fig. S1*B*), whereas Mcl1 overexpression in transgenic mice renders these cells refractory to such insults (9, 10). Moreover, $Mcl1^{fl/fl}$ MEFs were normally sensitive to apoptosis induced by overexpression of a panel of BH3-only proteins (Fig. S4*B*). This suggests that, like WT Mcl1, N^+Mcl1 is functionally inactivated by BH3-only proteins, including Noxa, which preferentially binds and degrades Mcl1 (28). Like WT Mcl1, N^+Mcl1 was also degraded by Noxa (Fig. S5*C*).

To verify further that N^+Mcl1 retains full prosurvival function, we reconstituted *Mcl1*-deficient MEFs with either WT or N^+Mcl1. Mcl1 deficiency renders MEFs highly sensitive to the BH3 mimetic compound ABT-737 (29), whereas those reconstituted with WT Mcl1 are fully protected. Notably, N^+Mcl1 conferred comparable high-level protection to ABT-737 (Fig. S5*D*). Thus, N^+Mcl1 is fully functional and these cell survival assays did not distinguish it from WT Mcl1 (Figs. S4 and S5).

N^+Mcl1 Stability Is Enhanced in Response to Certain Cytotoxic Signals. Because Mcl1 is degraded rapidly in response to certain stress stimuli (30, 31), we explored whether turnover of N^+Mcl1 and WT Mcl1 differed in cells subjected to various stresses. Indeed, N^+Mcl1 was degraded more slowly than WT Mcl1 in MEFs following inhibition of protein synthesis or UV irradiation (Fig. 2*A*), and thymocytes yielded similar results upon inhibition of protein synthesis (Fig. S6*A*). Moreover, studies using Mcl1-deficient MEFs reconstituted with WT or N^+Mcl1 (Fig. S6*B*) confirmed that the degradation of N^+Mcl1 is delayed during steady-state turnover (after cycloheximide treatment) or after exposure to UV radiation (Fig. 2*A* and Fig. S6*A*); intriguingly, however, both proteins were comparably degraded in cells overexpressing Noxa (Fig. S5*C*). Consistent with the findings on protein turnover under conditions in which Mcl1 is degraded, such as inhibition of protein synthesis (31) or UV irradiation (30), the cells expressing N^+Mcl1 are more resistant to apoptosis (Fig. 2*B*).

Because our floxed *Mcl1* allele codes for a stabilized form of Mcl1 (N^+Mcl1), we subsequently denote this allele here as $Mcl1^{flN}$ to distinguish it from any other floxed allele encoding WT Mcl1.

Stabilized Mcl1 Can Partially Compensate for the Loss of Bcl2. Because our data suggested that, in certain circumstances, cells expressing N^+Mcl1 are more refractory to apoptosis than those expressing WT Mcl1, we explored whether N^+Mcl1 can compensate for the reduced activity or loss of another prosurvival Bcl2 family member within the whole animal. We first tested whether $Mcl1^{flN}$ can prevent the loss of platelets in mice bearing a hypomorphic allele of *Bclx*, $Bclx^{Plt20}$ (18). However, introduction of either one or two $Mcl1^{flN}$ alleles did not augment platelet numbers in the $Bclx^{Plt20/+}$ heterozygous or $Bclx^{Plt20/Plt20}$ homozygous mice (Fig. S7).

We then investigated whether N^+Mcl1 could alleviate any of the marked degenerative disorders arising in mice lacking Bcl2 (Fig. 3). Remarkably, in contrast to the $Bcl2^{-/-}$ runts, $Bcl2^{-/-}Mcl1^{flN/flN}$ doubly mutant mice grew to normal size (Fig. 3*A*), because the onset of polycystic kidney disease was greatly retarded (Fig. 3*C*). Although $Bcl2^{-/-}Mcl1^{flN/flN}$ mice eventually succumbed

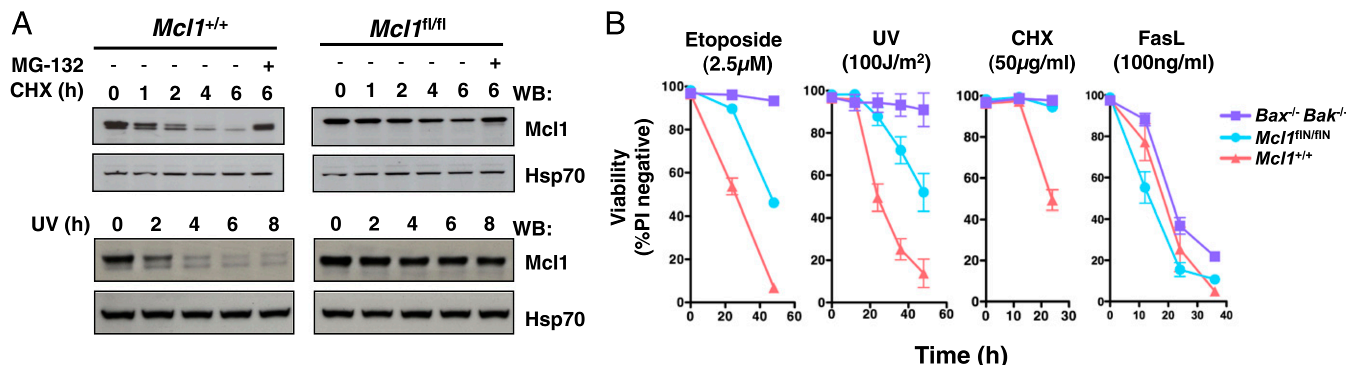

Fig. 2. Floxed *Mcl1* allele encodes a stabilized form of Mcl1, N^+Mcl1, that counters apoptosis more effectively than WT Mcl1 in some settings. (*A*) Floxed *Mcl1* allele encodes an abnormally stabilized form of Mcl1. (*Upper*) Lysates prepared from WT ($Mcl1^{+/+}$) or $Mcl1^{flN/flN}$ MEFs that were incubated for up to 6 h with the protein synthesis inhibitor cycloheximide (CHX; 50 μg/mL), with or without the proteasome inhibitor (10 μM MG132) and the broad-spectrum caspase inhibitor N-(2-Quinolyl)-L-valyl-L-aspartyl-(2,6-difluorophenoxy) methylketone (qVD-OPh) (to block degradation of Mcl1 by caspases). (*Lower*) Lysates prepared from the cells that were UV-irradiated (100 J/m²). The lysates were probed by immunoblotting for Mcl1 and HSP70 (loading control). WB, Western blot. (*B*) Under certain circumstances, N^+Mcl1 inhibits cell death more effectively than WT Mcl1. MEFs derived from WT ($Mcl1^{+/+}$), $Mcl1^{flN/flN}$, or $Bax^{-/-}Bak^{-/-}$ mice were treated with etoposide (2.5 μM), UV irradiation (100 J/m²), CHX (50 μg/mL), or Fas ligand (FasL, CD95L; 100 ng/mL). Cell viability was determined at the indicated times by propidium iodide staining and flow cytometric analysis. Data represent the mean ± SD of two experiments done with a representative cell line of each genotype.

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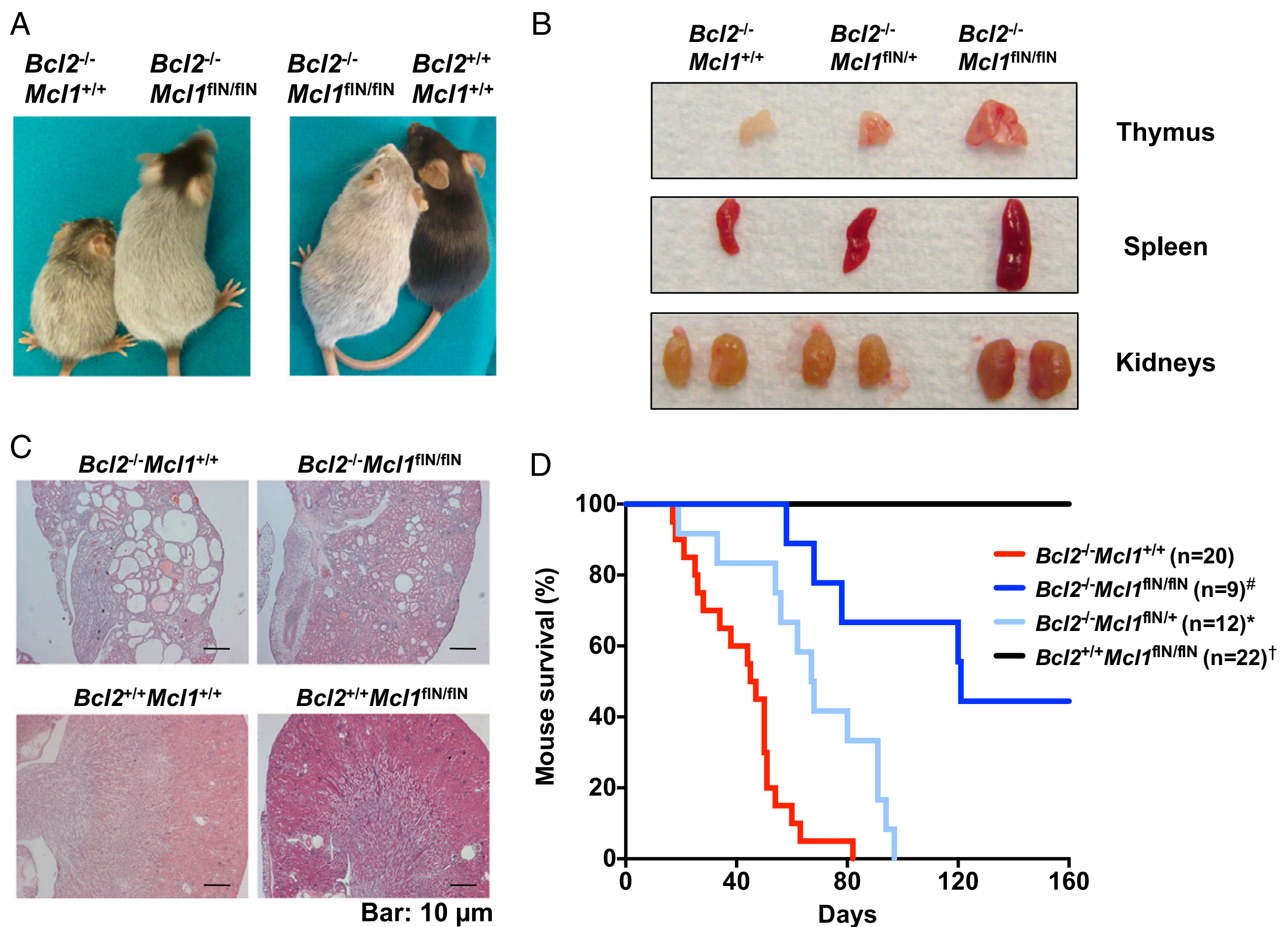

Fig. 3. N^+Mcl1 ameliorates some of the physiological defects caused by Bcl2 deficiency. (*A*) N^+Mcl1 can partially rescue some of the defects caused by Bcl2 deficiency. (*Left*) As previously reported (3, 4), *Bcl2*-deficent mice (*$Bcl2^{-/-}Mcl1^{+/+}$*) were runted and turned prematurely gray. (*Right*) Age-matched (52-d-old) *$Bcl2^{-/-}Mcl1^{flN/flN}$* mice grew to a normal size but still turned prematurely gray like the *$Bcl2^{-/-}$* mice. (*B*) N^+Mcl1 prevents the lymphoid hypoplasia and developmental kidney defects caused by Bcl2 loss. Gross appearance of the thymus, spleen, and kidneys from representative *$Bcl2^{-/-}Mcl1^{+/+}$*, *$Bcl2^{-/-}Mcl1^{flN/+}$*, and *$Bcl2^{-/-}Mcl1^{flN/flN}$* mice (shown in *A*). Note that the kidney from a *$Bcl2^{-/-}Mcl1^{flN/flN}$* mouse was larger and well-aerated (pink, not pale) compared with that isolated from a *$Bcl2^{-/-}Mcl1^{+/+}$* mouse. (*C*) N^+Mcl1 attenuates the polycystic kidney disease caused by Bcl2 loss. Representative low-power, H&E-stained kidney sections are shown from representative mice of the indicated genotypes. Note the significant reduction in cyst formation in the kidney from a *$Bcl2^{-/-}Mcl1^{flN/flN}$* mouse compared with that from a *$Bcl2^{-/-}Mcl1^{+/+}$* mouse. (Scale bars: 10 μm.) (*D*) Mcl1 stabilization extends the life span of *Bcl2*-deficient mice. Kaplan–Meier survival curves of cohorts of mice of the indicated genotypes are shown. Notably, even a single *$Mcl1^{flN}$* allele significantly prolonged the survival of *$Bcl2^{-/-}$* mice. The *P* value determined by log rank analysis of *$Bcl2^{-/-}Mcl1^{+/+}$* mice compared with *$Bcl2^{-/-}Mcl1^{flN/+}$* mice is 0.0032 (*), that compared with *$Bcl2^{-/-}Mcl1^{flN/flN}$* mice is <0.0001 (#), and that compared with *$Bcl2^{+/+}Mcl1^{flN/flN}$* mice is <0.0001 (†).

to the disease (Fig. 3*D*), they survived far longer than the *$Bcl2^{-/-}$* mice (mean survival: ∼120 d vs. ∼40 d). Notably, the introduction of even a single *$Mcl1^{flN}$* allele nearly doubled the life span of *$Bcl2^{-/-}$* mice (from ∼40 to ∼70 d). Introduced *$Mcl1^{flN}$* alleles also ameliorated the lymphopenia elicited by Bcl2 loss (Fig. 3*B*), but the *$Bcl2^{-/-}Mcl1^{flN/flN}$* mice still turned prematurely gray (Fig. 3*A*).

Importantly, the genetic compensation for loss of Bcl2 by *$Mcl1^{flN}$* demonstrates that N^+Mcl1 is a gain-of-function variant of Mcl1. We surmise that the increased stability of N^+Mcl1 over WT Mcl1 raises the apoptotic threshold in the renal epithelial progenitor cells sufficiently to compensate substantially for the loss of Bcl2.

N^+Mcl1 Accelerates Tumorigenesis in Vivo. Because our functional studies identify *$Mcl1^{flN}$* as a hypermorphic allele (Figs. 1–3), we used *$Mcl1^{flN}$* mice to investigate whether abnormal Mcl1 stabilization can promote tumorigenesis, as inferred from studies on oncogenes and tumor suppressors that regulate Mcl1 turnover (12, 13). To investigate whether N^+Mcl1 could enhance tumor formation in vivo, we focused on the development of acute myeloid leukemia (AML), because these hematological malignancies are highly dependent on Mcl1 for their development and sustained growth (20). For these experiments, fetal liver-derived hematopoietic stem cells (WT, *$Mcl1^{flN/+}$*, or *$Mcl1^{flN/flN}$*) were retrovirally transduced with the *MLL-ENL* or c-*MYC* oncogene, transplanted into lethally irradiated (C57BL/6-Ly5.1) mice and the recipients were monitored for the onset of leukemia. Although the expression of N^+Mcl1 had no impact on the rapid induction of AML by the *MLL-ENL* gene (Fig. S8), the development of c-*MYC*–driven AML (32) was markedly accelerated by the presence of two *$Mcl1^{flN}$* alleles or even a single allele (Fig. 4). Thus, stabilization of Mcl1 with concomitant increased antiapoptotic activity can enhance tumorigenesis, at least in certain settings.

Discussion

The physiological importance of Mcl1 is underscored by its critical role in very early embryogenesis (7) and key cell types in the adult (8, 19). Moreover, increasing evidence implicates Mcl1 in the survival of cells from several tumor types and in chemoresistance

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(11, 20). A striking feature of Mcl1 is its rapid turnover, which is far greater than that of Bcl2, $Bclx_L$, or Bclw. It therefore appears likely that control of its turnover has physiological significance, but direct evidence for this has been lacking. Genetic purchase on this notion came when we discovered that a floxed Mcl1 allele that we had generated earlier, designated here as $Mcl1^{flN}$, produces an abnormally stabilized Mcl1 variant due to a 13-residue extension on its N terminus (N^+Mcl1). In cells subjected to certain stresses, such as inhibition of protein synthesis or UV irradiation, N^+Mcl1 is much more stable than WT Mcl1 (Fig. 2 and Figs. S5 and S6) but the modified protein is still readily degraded in response to engagement by its selective endogenous antagonist, the BH3-only protein Noxa.

Our genetic and cellular studies both strongly suggest that $Mcl1^{flN}$ is a hypermorphic *Mcl1* allele. Male $Mcl1^{flN/flN}$ mice proved infertile due to a severe block in spermatogenesis at the spermatid stage (Fig. 1 and Fig. S2). The block is slightly later than that seen in Bax-deficient mice and most closely resembles that in males lacking both of the BH3-only proteins Bim and Blk (27). Tellingly, a very similar block is evoked by transgenic overexpression of Bcl2 or $Bclx_L$ in the testis (33). These results indicate that the spermatogenesis defect in the $Mcl1^{flN/flN}$ males probably reflects abnormally enhanced prosurvival activity in their germ cells. Consistent with this notion, the phenotype differs significantly from the delayed atrophy observed in Bclw-deficient males, which is ascribed to attrition in both the germ and supporting Sertoli cell populations (21, 22). It may seem counterintuitive that atrophy of a tissue can arise from enhanced prosurvival activity. However, copious excess germ cells are normally produced during the first, pioneer wave of spermatogenesis, commencing a few days after birth in mice. Without the substantial attrition of this population, driven by Bax (26) activated by Bim and Blk (27), the germ cell excess is thought to damage the supporting Sertoli cells and thereby preclude adult spermatogenesis (27, 33). Stabilized Mcl1 presumably moderates the early wave of apoptosis in premeiotic germ cells by sequestering Bim and Blk, and by inhibiting activated Bax.

The impact of stabilizing Mcl1 was particularly striking in the absence of Bcl2. Even a single $Mcl1^{flN}$ allele ameliorated the severe polycystic kidney disease evoked by Bcl2 loss and substantially prolonged survival (Fig. 3). The life span of $Mcl1^{flN/flN}$ mice lacking Bcl2 was even longer, and the drop in lymphocytes was precluded, but the premature greying remained.

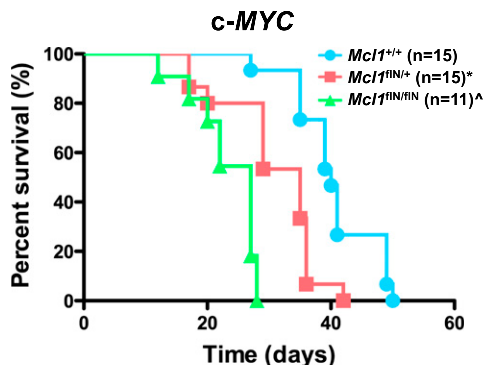

Fig. 4. N^+Mcl1 accelerates development of MYC-driven AML in vivo. Abnormal Mcl1 stabilization promotes c-*MYC*–driven development of AML. Shown is a Kaplan–Meier plot of the survival of lethally irradiated (2 × 5.5 Gy 2 h apart) mice transplanted with embryonic day 13.5 fetal liver cells (a rich source of hematopoietic stem/progenitor cells) derived from WT ($Mcl1^{+/+}$), $Mcl1^{flN/+}$, or $Mcl1^{flN/flN}$ embryos and infected with a c-*MYC*–expressing retrovirus. Note the dose-dependent impact of the $Mcl1^{flN}$ allele on AML-free survival. The *P* value (log rank analysis) of WT ($Mcl1^{+/+}$) mice compared with $Mcl1^{flN/+}$ mice is 0.0014 (*) and that compared with $Mcl1^{flN/flN}$ mice is <0.0001(^).

Most importantly, we established that Mcl1 stabilization can contribute to tumorigenesis, as has been proposed based on studies with proteins that can regulate its stability (12, 13). We showed that the $Mcl1^{flN}$ allele promoted c-*MYC*–driven AML development (Fig. 4). AML driven by the *MLL-ENL* gene, however, was not accelerated (Fig. S8), perhaps because *MLL-ENL* provokes less cytotoxic stress than *MYC*, or because these leukemias initiate in a slightly different cell type.

Despite the striking phenotypes provoked by the $Mcl1^{flN}$ allele, it may seem puzzling that only a few tissues are affected. A partial explanation is that the N-terminal extension only impairs Mcl1 degradation in response to certain stress stimuli. Moreover, whether the Mcl1 stabilization raises the apoptotic threshold sufficiently to have physiological consequences is very likely to differ greatly among cell types, because their expression of particular Bcl2 family proteins varies widely, and also among stress stimuli, because activation of the various BH3-only proteins is highly dependent on the stress stimulus (1). These considerations may well explain why the phenotype in $Mcl1^{flN/flN}$ mice is restricted and why the modified allele rescued some cell types in $Bcl2^{-/-}$ mice (kidney epithelial and lymphoid cells) but not melanocytes (Fig. 3).

A recently recognized but puzzling aspect of Mcl1 regulation is that a proportion of the Mcl-1 protein molecules is processed to remove the N-terminal 33 amino acid residues and the truncated protein enters the mitochondrial matrix (34–36). One study has reported that the matrix form influences mitochondrial fission/fusion and energetics (36). Although our experiments have not directly addressed this pathway, we note that Western blots on MEFs and thymocytes from $Mcl1^{flN/flN}$ mice, whether stressed or not, show only a single prominent Mcl-1 band of the size expected for N^+Mcl1 (Figs. 2 *A* and *C* and 3*A* and Fig. S5). Cleavage of N^+Mcl1 at the identified processing site for the matrix form of Mcl-1 would remove its N-terminal 46 residues, leaving a product that should be readily detectable. Thus, at least in fibroblasts and thymocytes of these mice, little if any of the expected matrix form seems to be generated. A plausible interpretation is that the extended N-terminal sequence in N^+Mcl1 impedes the processing step that produces the matrix form of Mcl1. If so, these mice should provide a useful resource for identifying any significant physiological role of the matrix form. However, we cannot definitively exclude the possibility that the absence of the processed form contributes to some phenomena described here.

Our studies lend strong support to the notion that the control of Mcl1 stability is highly context-specific. The control depends not only on the stress stimuli but on cell type. For example, whereas the response of immortalized $Mcl1^{flN/flN}$ MEFs to DNA damaging agents is impaired (Fig. 2*B*), the response of thymocytes to such agents is unaffected (Fig. S1*B*). At a mechanistic level, precisely how the additional N-terminal amino acids block basal Mcl1 turnover is unclear. The N-terminal region of Mcl-1 may contain a poorly understood degron, because Mcl-1 turnover can be enhanced not only by lengthening the N terminus, as observed here, but by shortening it (34).

Our findings add to the growing evidence that Mcl1 levels and turnover are subject to multiple exquisite controls. This conclusion is pertinent to the current efforts to target prosurvival Bcl2 family members for cancer therapy (1). The most advanced approach is the development of organic compounds that mimic the action of the BH3-only proteins (37). Some of these "BH3 mimetics" have advanced into the clinic, and recent reports validate this approach for targeting BCL2 in lymphoid malignancies (38). However, BH3 mimetics selectively targeting Mcl1 with high affinity have not yet been described, and in any case, systemic inhibition of Mcl1 would raise serious concerns, because Mcl1 sustains the survival of many critical stem and progenitor

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cells (7, 8). Our results should prompt further dissection of the molecular control of Mcl1 stability, because the striking context-dependent differences in its regulation suggest that targeting the machinery that stabilizes Mcl1 in specific tumors (12, 13) may well provide a wider therapeutic window than is possible with a direct Mcl1 antagonist.

Materials and Methods

Mice. The *Mcl1*$^{fl/fl}$ (*Mcl1*$^{flN/flN}$) mice used here were described previously (18–20). They were generated on a C57BL/6 background using C57BL/6-derived ES cells. The *Bax*$^{+/-}$ mice (a kind gift from the late S. Korsmeyer, Washington University School of Medicine, St. Louis) and *Bcl2*$^{+/-}$ mice (kindly provided by D. Loh, Washington University School of Medicine, St. Louis) were generated on a mixed C57BL/6 × 129SV background using 129SV-derived ES cells but were backcrossed onto a C57BL/6 background for >12 generations. *Mcl1*$^{flN/flN}$*Bcl2*$^{-/-}$ mice were generated by serial intercrossing of appropriate parental mice. Mice harboring the *Bclx*^{Plt20} hypomorphic allele have been described (18), and *Mcl1*$^{flN/flN}$*Bclx*$^{Plt20/Plt20}$ mice were generated by serial intercrossing of appropriate parental mice. All mice used were of C57BL/6 origin or had been backcrossed (>10 generations) to this background, and their genotype was determined as previously described (details are available from the authors). All animal experiments followed the guidelines of the Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee.

Analysis of Mcl1 Degradation. Mcl1 turnover was determined by incubating cells with 50 μM cycloheximide, together with proteasomal inhibitor MG132 (10 μM) where indicated. DNA damage was induced by exposure to 100 J/m^2 of UV light or by treatment with 2.5 μM etoposide in the presence of 50 μM qVD-OPh, a broad-spectrum caspase inhibitor, to prevent the caspase-dependent proteolysis of Mcl1 that occurs as a consequence of apoptosis rather than the initiating events on which we are focused. The cells were harvested at the indicated times, the protein concentration was quantified (Bradford reaction), and Western blotting was performed. NOXA was over-expressed in MEFs by infection with retroviruses encoding NOXA or the inert variant NOXA3E, and the transduced (GFP$^+$) cells were selected by fluorescence-activated cell sorting.

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