A critical role for Mnt in Myc-driven T-cell proliferation and oncogenesis

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Mnt (Max's next tango) is a Max-interacting transcriptional repressor that can antagonize both the proproliferative and proapoptotic functions of Myc in vitro. To ascertain the physiologically relevant functions of Mnt and to help define the relationship between Mnt and Myc in vivo, we generated a series of mouse strains in which Mnt was deleted in T cells in the absence of endogenous c-Myc or in the presence of ectopic c-Myc. We found that apoptosis caused by loss of Mnt did not require Myc but that ectopic Myc expression dramatically decreased the survival of both Mnt-deficient T cells in vivo and Mnt-deficient MEFs in vitro. Consequently, Myc-driven proliferative expansion of T cells in vitro and thymoma formation in vivo were prevented by the absence of Mnt. Consistent with T-cell models, mouse embryo fibroblasts (MEFs) lacking Mnt were refractory to oncogenic transformation by Myc. Tumor suppression caused by loss of Mnt was linked to increased apoptosis mediated by reactive oxygen species (ROS). Thus, although theoretically and experimentally a Myc antagonist, the dominant physiological role of Mnt appears to be suppression of apoptosis. Our results redefine the physiological relationship between Mnt and Myc and requirements for Myc-driven oncogenesis.

cancer | antioxidant | buthionine sulfoximine | Ras | Bcl-2

Myc family transcription factors are critical effectors of mitogenesis that promote oncogenesis when their normally tightly controlled expression is dysregulated (1–3). c-Myc (hereafter, Myc) promotes proliferation by coordinating a variety of cellular processes, including those promoting nutrient uptake and metabolism, ribosome biogenesis and translation, mitochondria biogenesis, and cell cycle progression (3–5). The broad program of gene expression and cellular processes governed by Myc can make cancer cells highly dependent on its continued activities, and downregulating or inactivating Myc can suppress tumor growth (2, 6, 7).

Although constitutively expressed Myc can drive oncogenesis, it also sensitizes cells to apoptosis, which can impede tumor formation (8, 9). One factor that controls the balance between Myc-driven apoptosis or proliferation is the amount of Myc expressed (10). Whereas low Myc levels promote productive cell proliferation and oncogenesis with minimal apoptosis, high Myc levels can trigger robust apoptosis. The importance of apoptosis as a limiting factor in Myc-driven tumor formation is illustrated by the acceleration of tumor formation observed when ectopic Myc is coexpressed with antiapoptotic proteins, such as Bcl-2 or Bcl-xL, or when genes encoding proapoptotic proteins, such as p53 or Bax, are inactivated (8, 9). Thus, genetic and/or epigenetic events that suppress Myc-dependent apoptosis in human cancers likely facilitate Myc-driven malignant progression. Moreover, the prevalence of high Myc levels in cancers suggests that tumorigenic cells must undergo changes that allow them to tolerate Myc levels that would otherwise trigger apoptosis. Accordingly, strategies that can restore, promote, or exacerbate the proapoptotic activity of deregulated/overexpressed Myc in tumors may be generally effective for the therapeutic control of cancer (11, 12).

Mnt (Max's next tango) is a transcriptional repressor that shares with Myc a related bHLHZip (basic helix–loop–helix–leucine zipper) motif that mediates dimerization with Max and DNA binding to enhancer box (Ebox) sites (13, 14). Mnt-Max complexes can compete with Myc-Max for binding to Ebox sites, and deletion or knockdown of Mnt leads to misregulation of a number of well-established Myc target genes (15–19). Moreover, results from combined chromatin immunoprecipitation and transcriptome analysis in breast epithelial cells (20) and transcriptome analysis in Mnt-deficient *Drosophila* (21, 22) and human cells (19) suggest that Mnt and Myc bind and coregulate an overlapping set of target genes. Consistent with the notion that Mnt and Myc are functional antagonists, *Mnt* deletion or siRNA knockdown was shown to rescue, at least transiently, the proliferative arrest of cells caused by loss of Myc (16, 17), and deletion of *Drosophila Mnt* partially rescued the viability and cell growth defects caused by deletion of *Drosophila Myc* (21). Conversely, Mnt overexpression suppresses Myc-dependent cell transformation (13). These data support the concept that as a Myc antagonist, Mnt can function to restrict the proproliferative activities of Myc.

The ability of Mnt to antagonize Myc-driven proliferation suggested that *Mnt* deletion, inactivation, or down-regulation might accelerate Myc-driven oncogenesis (16, 23). However, like Myc overexpression, Mnt deficiency strongly sensitizes cells to apoptosis (15, 16, 18, 24). Thus, an alternative possibility is that as a Myc antagonist, Mnt might play an important role in countering the proapoptotic tendencies of Myc that can trigger intrinsic tumor suppression (11). To better define the normal physiological relationship between Myc and Mnt and the role of Mnt in Myc-driven oncogenesis, we developed a set of mouse strains that lack Mnt and Myc in T cells or that lack Mnt and ectopically express Myc in T cells. Our results show that the dominant consequence of *Mnt* deletion in vivo is increased cell death that is exacerbated by elevated Myc and can prevent Myc-driven oncogenesis.

Results

Mnt Promotes Intrinsic Survival of Mature Thymocytes. Mice with conditional *Mnt* deficiency in T cells (MntTcKO) have altered thymocyte maturation and significantly fewer mature CD4/CD8 double-positive (DP) thymocytes and splenic T cells than control mice (18). One possible cause of this defect was reduced proliferation of immature CD4/CD8 double-negative (DN) thymocytes. However, the mean absolute number of immature DN thymocytes was not lower in MntTcKO thymi (control: 2.9×10^6; MntTcKO: 5.1×10^6; and ref. 18). Additionally, DN thymocytes did not show proliferation defects by FACS analyses of DNA content or DNA synthesis in vivo (Fig. S1). Thus, a failure to produce or expand immature precursors was not responsible for fewer mature DP thymocytes.

Another potential explanation for the reduced number of mature thymocytes produced in the absence of Mnt was cell death. Because apoptosing thymocytes are rapidly cleared by phagocytes

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in vivo (25), we analyzed the survival of mature DP thymocytes after 24 h ex vivo. Survival of MntTcKO DP thymocytes was significantly lower than control cells (Fig. 1*A*), and this correlated with significantly fewer thymocytes (Fig. 1*B*) and a lower relative percentage of mature thymocytes (Fig. 1*C*). These findings are consistent with the small size of MntTcKO thymi (Fig. 1*C*).

Unlike MntTcKO thymocytes, mice with Lck-Cre–driven conditional deletion of *Myc* (MycTcKO) (26) did not have a thymocyte-survival defect (Fig. 1*A*). However, MycTcKO thymi had significantly fewer cells and were smaller than control thymi (Fig. 1 *B* and *C*). These results are consistent with a previous report (27), and reiterate that defective proliferation - not defective survival - inhibits production of mature MycTcKO thymocytes. Deletion of both *Myc* and *Mnt* in thymocytes (DTcKO) resulted in a reduction in the number of mature thymocytes produced and extremely small thymi (Fig. 1 *B* and *C*). Thus, DTcKO thymocytes have both survival and proliferation defects and suggest that Mnt-mediated survival and Myc-mediated proliferation function as separate but supportive mechanisms during thymocyte development.

Overexpression of Myc Increases Dependence on Mnt-Mediated Survival. c-Myc promotes thymocyte proliferation and can lead to tumorigenesis when ectopically expressed (27–30). To decipher whether Mnt antagonizes the consequences of Myc overexpression, we used mice in which the *c-Myc* gene was "knocked in" to the *ROSA-26* locus downstream from a LoxP-flanked transcription termination sequence (31) and used Lck-Cre for T-cell–specific Myc expression. T-cell conditional ROSA-Myc (TMyc) mice produced significantly more thymocytes (Fig. 1*B*) and had larger thymi (Fig. 1*C*, *Right*) than control mice but did not have a noticeable impairment in thymocyte development (Fig. 1*C*, *Left*). Consistent with studies showing that low-level, ectopic Myc expression has little effect on cell survival (10), there was only a nonsignificant ($P = 0.07$) trend toward more apoptosis in TMyc thymocytes (Fig. 1*A*). However, the combination of Mnt deficiency and ectopic Myc (MntTcKO+TMyc) caused significantly more apoptosis than either TMyc or MntTcKO thymocytes. This severe apoptosis paralleled a decrease in thymus size and essentially nullified the increased cellularity otherwise caused by ectopic Myc expression. The survival defect of MntTcKO thymocytes, with or without ectopic c-Myc, was not associated with increased p53 (Fig. S2). Moreover, Mnt-deficient thymocytes did not show abnormal kinetics of p53 induction or activation in response to genotoxic insult (Fig. S2). These data suggest that the proproliferative effects of enforced Myc expression might be dependent on a unique pro-survival function of Mnt.

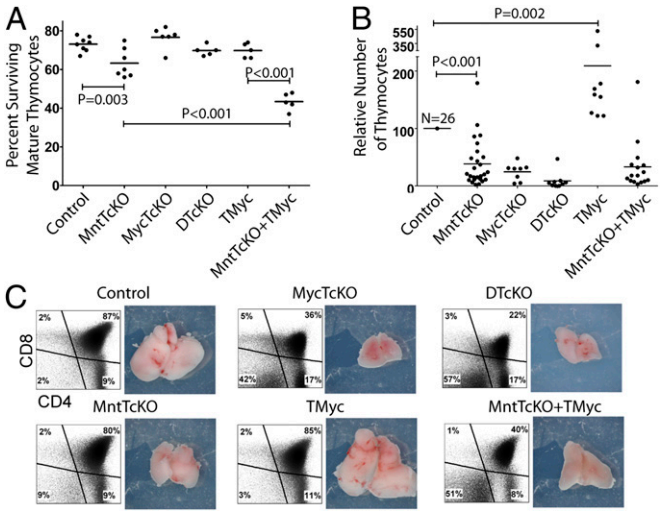

Fig. 1. Control of thymocyte production and survival by Mnt and Myc. (*A*) Single-cell suspensions of thymocytes were cultured ex vivo for 24 h, and the percentage of mature thymocytes ($CD4^+CD8^+$) surviving ($AnnexinV^{NEG}7AAD^{NEG}$) was assessed by FACS. Each dot represents cell survival from one thymus. (*B*) Thymocytes were counted, and the number of cells per thymus was normalized to an age-matched control thymus in the same experiment to avoid age-related changes in thymocyte number. Each dot represents thymus cellularity from one mouse; *n* represents the number of control thymi analyzed. (*C*) The plots are representative FACS plots from thymocytes stained with CD4 (*x* axis) and CD8 (*y* axis) and illustrate the relative dearth of mature thymocytes when Mnt and/or Myc are absent. The images in *C* are representative thymi from each mouse type and illustrate that the number of mature thymocytes correlates with thymus size.

Increased Apoptosis Prevents Expansion of Mitogen-Stimulated, Mnt-Deficient T Cells. Although Mnt-deficient thymocytes were more prone to apoptosis than control cells, Mnt-deficient splenic T cells were not (Fig. 1*A* and Fig. S3 *A* and *B*). One explanation for this difference is the activation state of the cell. Whereas DP thymocytes express cell cycle proteins and exist in a quasiactivated state (32), splenic T cells are mostly quiescent. Only upon antigen encounter do splenic T cells increase glycolysis, nutrient uptake, proliferation, and c-Myc expression (33–35). We reasoned that mitogen-induced, endogenous Myc expression in splenic T cells would trigger apoptosis in MntTcKO T cells, similar to the enhanced apoptosis of unstimulated MntTcKO+TMyc T cells (Fig. S3*A*). To test this, we cultured splenic T cells with the lectin concanavalin (Con)A, which induces Myc expression and is mitogenic at low concentrations but induces apoptosis at high concentrations (36, 37). ConA stimulated the transcription of both *Myc* and *Mnt* genes in control T cells, and *Myc* induction was not affected by *Mnt* deletion (Fig. 2*A*). After 48 h of ConA stimulation, MntTcKO T cells and TMyc T cells showed increased apoptosis relative to control T cells, particularly at the higher (0.5 μg/mL) concentration, and MntTcKO+TMyc cells remained far more apoptotic with or without ConA treatment (Fig. 2*B*). To determine how the mitogen-induced apoptosis in Mnt-deficient cells affected their proliferative capacity in vitro, we exposed splenocytes to a range of ConA concentrations and measured the mitogen-induced increase in live $CD4^+$ T cells after five days (Fig. 2*C*). MntTcKO and MntTcKO+TMyc T cells were much less able to expand in culture than control cells. Neither an alternate mitogenic signal (via CD3 and CD28) nor addition of the T-cell survival factor IL-2 were able to restore expansion of MntTcKO or MntTcKO+TcMyc T cells (Fig. S4 *A* and *B*). In contrast, TMyc T cells expanded better than control cells despite a significantly greater tendency to apoptose (Fig. 2*B*). This result suggests that increasing Myc by either transgene expression or mitogen exposure promotes apoptosis but that enhanced proliferation more than compensates for the increased apoptosis. Taken together, these data indicate that Myc expression (whether ectopic or mitogen induced) promotes proliferation of T cells but also makes these cells more dependent on Mnt for survival.

To exclude that proliferation defects per se were the cause of the reduced expansion of Mnt-deficient T cells, we examined cell cycle entry by measuring BrdU incorporation by $CD4^+$ T cells (both live and apoptosing) 48 h after ConA exposure. We found that BrdU incorporation was unaffected by *Mnt* deletion compared with control cells (Fig. 2*D*). Furthermore, ectopic Myc expression resulted in more BrdU positive T cells for both TMyc and MntTcKO+TMyc T cells. Thus, the failure of Mnt-deficient T cells to expand in culture is not due to an inability to enter the cell cycle, a result supported by previous studies (15–17).

We also examined whether MntTcKO and MntTcKO+TMyc cells were able to survive complete cell divisions by measuring the dilution of the fluorescent dye CFSE in live ($7AAD^{NEG}$) $CD4^+$ T cells 48 h after ConA exposure (Fig. S4*C*). We found that all cell types were able to complete multiple cell divisions, but that there were fewer live, divided MntTcKO and MntTcKO+TMyc cells compared with control and TMyc cells, respectively. This result is consistent with reduced survival of cells that are effectively stimulated by ConA and begin to proliferate. We conclude that

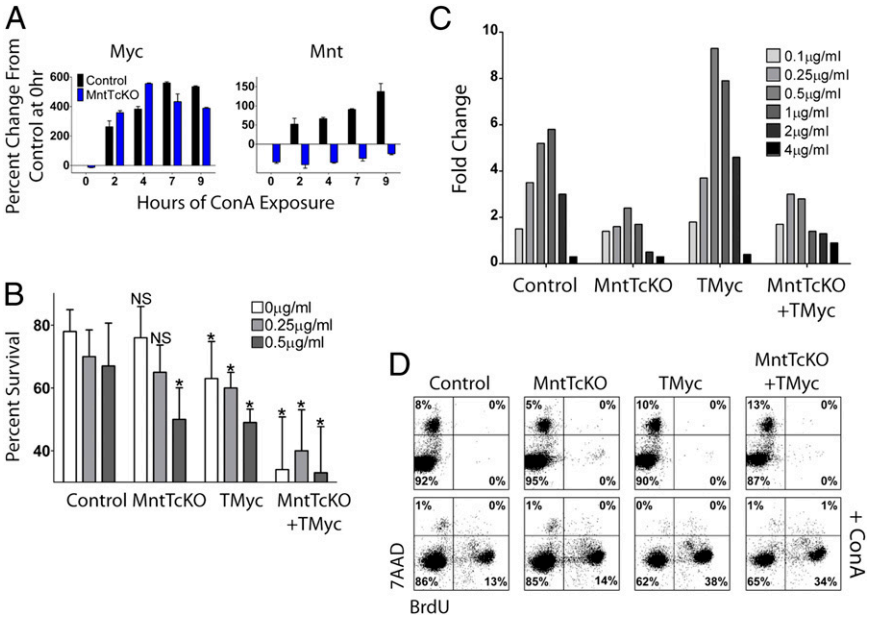

Fig. 2. Mnt is required for survival of ex vivo mitogen-stimulated T cells. (*A*) $CD4^+$ splenic T cells from control and MntTcKO mice were cultured with 1μg/mL ConA and relative amounts of *Myc* and *Mnt* transcripts were assessed by quantitative RT-PCR using *β-actin* as a control. Amount of RNA is given as the percentage change from unstimulated (0hr) control cells. (*B*) Splenocytes were cultured for 48 h with the indicated concentrations of ConA, and the percentage of $CD4^+$ cells surviving ($AnnexinV^{NEG}7AAD^{NEG}$) was determined by FACS. The mean ± SD from four separate experiments is given. Conditions marked with an asterisk (*) have significantly ($P < 0.05$) lower survival than control cells at the same concentration of ConA. NS, not significant. (*C*) Splenocytes were cultured for 5 d with the indicated concentrations of ConA. The number of surviving $CD4^+$ cells for each condition was calculated from the total number of cells generated and the percentage of live $CD4^+$ cells in each culture. Fold expansion from unstimulated cells is plotted. Data are representative of five separate experiments. (*D*) $CD4^+$ T cells were isolated from splenocytes by magnetic bead depletion, and 1×10^6 cells were cultured without or with 1.0 μg/mL ConA and with 10 μM BrdU for 48 h. FACS detection of BrdU incorporation was used to measure DNA synthesis and 7AAD fluorescence was used to measure cell cycle stage.

Mnt-deficient T cells are proliferation-competent and only fail to expand in culture because of reduced survival.

Mnt Deficiency Inhibits Myc-Driven T-Cell Lymphogenesis. Because Myc-driven T-cell expansion in vivo and ex vivo was blocked by loss of Mnt, we considered whether Mnt might be important or required for Myc-driven T-cell lymphoma. Overexpression of wild-type (WT) Myc driven by the *ROSA-26* locus in TMyc mice does not significantly impact mouse survival, and tumors form in these mice only rarely and very late in life. However, mice expressing Myc^{T58A}, an oncogenic version of Myc (38) that accumulates to higher levels than WT Myc (Fig. S5) (39) and is found in some B-cell lymphomas (40), succumb to thymomas with a median survival of 220 d (Fig. 3*A*). To examine the role Mnt plays in Myc-driven tumor formation, we crossed $TMyc^{T58A}$ mice with MntTcKO mice to generate MntTcKO+$TMyc^{T58A}$ mice. As shown in Fig. 3*A*, Mnt deficiency significantly suppressed Myc^{T58A}-driven lethality to a median survival of 355 d ($P < 0.0001$). Furthermore, whereas large thymomas were present in all dead $TMyc^{T58A}$ mice, no thymomas were found in any of the postmortem MntTcKO; $TcMyc^{T58A}$ mice necropsied ($n = 7$) (Fig. 3 *B* and *C*).

Ectopic expression of Myc^{T58A} in cell lines has been associated with moderately reduced apoptosis compared with Myc^{WT}, a feature thought to contribute to its enhanced oncogenic potential (40, 41). We did not observe an increase in survival attributable to the T58A mutation in Mnt-sufficient cells and yet did detect a slight T58A survival effect in Mnt-deficient thymocytes (Fig. 3*D*). However, despite any T58A survival advantage, $TMyc^{T58A}$ thymocytes were still highly dependent on Mnt for survival (Fig. 3*D*). These data support the concept that Myc-driven thymomas depend on the prosurvival activities of Mnt.

Mnt Enhances Survival in Myc-Overexpressing MEFs and Is Required for Myc-Driven MEF Oncogenic Transformation. We found previously that primary MntKO mouse embryo fibroblasts (MEFs) are more susceptible to apoptosis than WT MEFs, rapidly lose Myc expression, and prematurely senesce (15). Addition of $H\text{-}Ras^{G12V}$ or c-Myc plus $H\text{-}Ras^{G12V}$ to primary MntKO MEFs allowed these cells to form transformed foci (ref. 15 and Fig. S6), but in contrast to WT MEFs, MntKO MEFs grew poorly or senesced and could not be readily expanded. Furthermore, whereas primary WT MEFs with ectopic Myc plus the prototypical prosurvival protein Bcl-2, and to a lesser extent Myc alone, produced transformed foci (42), primary MntKO MEFs were refractory to transformation by Bcl-2 plus Myc (Fig. S6*A*) and grew poorly. To overcome these limitations of primary cells and further examine the role of Mnt in Myc-driven apoptosis and oncogenic transformation of MEFs, we used MntKO and WT MEFs made immortal (iMEFs) using a 3T9 protocol (43). MntKO iMEFs, like T cells and primary MEFs, were significantly more sensitive to apoptosis caused by ectopic Myc or Myc^{T58A} than WT iMEFs (Fig. 4*A*), and whereas WT iMEFs with ectopic Myc or Myc^{T58A} formed colonies in soft agar and formed tumors in nude mice, comparable MntKO iMEFs did neither (Fig. 4*B* and Fig. S6 *C* and *D*). Moreover, whereas Bcl-2 protected WT iMEFs from

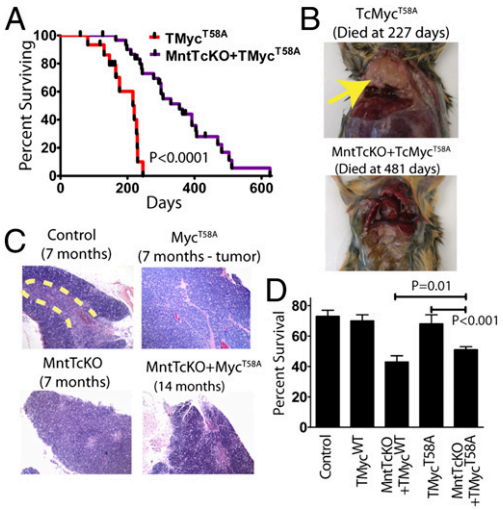

Fig. 3. Myc-mediated tumorigenesis is dependent on Mnt. (*A*) The survival of mice with conditional, biallelic, ectopic expression of Myc^{T58A} in T cells is significantly increased when Mnt is conditionally deleted in T cells ($P < 0.0001$). (*B*) Examples of a thymoma found in $TMyc^{T58A}$ mice and the lack of thymoma formation in MntTcKO+$TMyc^{T58A}$ mice. (*C*) Hematoxylin/eosin (H&E) histology of thymi from the indicated mouse strains and ages. The dashed line demarcates the medulla region. (*D*) Mnt deficiency has a significant effect on survival of $TMyc^{T58A}$ thymocytes. Thymocyte survival was assessed as in Fig. 1. Data are from at least three thymi per mouse type.

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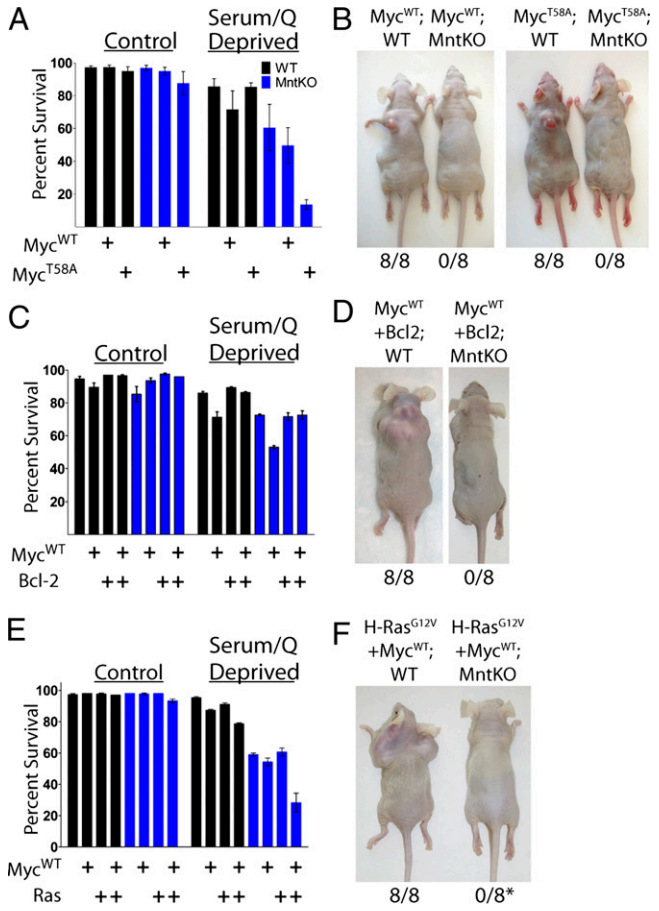

Fig. 4. Mnt is required for MEF survival and tumorigenesis. (*A*, *C*, and *E*) Immortal WT and MntKO MEFs expressing combinations of ectopic Myc^{WT}, Myc^{T58A}, Bcl-2, or Ras^{G12V} were cultured for 24 h in complete media or in media deprived of serum (i.e., 0.1% FBS) and glutamine. The percentage of surviving ($AnnexinV^{NEG}7AAD^{NEG}$) cells was determined by FACS. Data are the means (± SD) of four independent experiments. (*B*, *D*, and *F*) Immortal MEFs expressing Myc^{WT}, Myc^{T58A}, Bcl-2, or Ras^{G12V} were injected s.c. in nude mice at each shoulder per nude mouse and eight mice per cell type to test for tumorigenicity. The frequency of tumor formation observed is given below each photo. Mice injected with WT MEFs expressing Myc^{WT}, Myc^{T58A} Myc+Bcl-2, or Myc+Ras^{G12V} formed tumors of ~2 cm^2 within 3 wk and were killed according to Institutional Animal Care and Use Committee (IACUC) guidelines. Mice injected with MntKO MEFs expressing Myc^{WT}, Myc^{T58A} Myc+Bcl-2, or Myc+Ras^{G12V} failed to form tumors, even after more than 3 mo. This assay was repeated using two independent sets of immortal WT and MntKO MEFs (four injections of each set).

Myc-driven apoptosis and cooperated with Myc in oncogenic transformation as expected (42), Bcl-2 was not effective at suppressing the increased apoptosis caused by loss of Mnt, either in the absence or presence of ectopic Myc (Fig. 4*C*), and MntKO iMEFs were completely refractory to oncogenic transformation by ectopic Myc plus Bcl-2 expression (Fig. 4*D* and Fig. S6 *E* and *F*). Finally, MntKO iMEFs with ectopic H-Ras^{G12V} or Myc plus H-Ras^{G12V} showed increased sensitivity to apoptosis compared with WT iMEFs (Fig. 4*E*) and, unlike WT MEFs, failed to form tumors in nude mice (Fig. 4*F*). However, MntKO MEFs with ectopic H-Ras^{G12V} or $Myc^{(WT\ or\ T58A)}$ plus H-Ras^{G12V} were capable of forming colonies in soft agar, but the colonies were smaller and there were fewer produced (Fig. S6*G*). Thus, although MntKO iMEFs may be partially susceptible to transformation by ectopic H-Ras^{G12V} or Myc plus H-Ras^{G12V} (Fig. S6 *G* and *H* and ref. 15, but also see ref. 16), these cells remained highly prone to apoptosis and resistant to tumorigenesis.

An Increase in Reactive Oxygen Species Is Responsible for Increased Apoptosis in Mnt-Deficient Cells. Proliferating, highly metabolic T cells are susceptible to apoptosis caused by increased production of reactive oxygen species (ROS) (44, 45). To avoid apoptosis and expand in vitro, T cells require either addition of a reducing agent to the media or the presence of non-T cells to naturally lower the redox state of the cell culture (46–48). We found that the presence of a reducing agent [2-mercaptoethanol (2-ME)] or cell-dense, whole-splenocyte cultures could restore a net expansion of MntTcKO and MntTcKO+TMyc T cells (Fig. 5*A*). These results suggested that lowering the redox state in vitro allowed Mnt-deficient T cells to expand by protecting them from ROS-mediated apoptosis and that Mnt-deficient T cells may abnormally accumulate ROS. Consistent with this, we detected increased ROS in MntTcKO and MntTcKO+TMyc T cells immediately ex vivo and after 24 h of culture with or without ConA (Fig. 5*B*).

Unlike T cells, Mnt-deficient MEFs do not have a survival defect under normal culture conditions (Fig. 4 *A* and *B*) but do have increased ROS (Fig. 5*C*). We considered that Mnt-deficient MEFs might be especially dependent on intracellular antioxidant production to survive high amounts of ROS generated by proliferation and/or ectopic Myc expression. In accordance with this explanation, we found that inhibiting the synthesis of glutathione (GSH) (the major intracellular antioxidant) with buthionine sulfoximine (BSO) both increased ROS (Fig. 5*C* and Fig. S7) and potently triggered apoptosis in MntKO and MntKO+Myc MEFs (Fig. 5*D*), despite optimal growth conditions. The BSO-mediated apoptosis was ROS-dependent because the antioxidants 2-ME, GSH, and *N*-acetylcysteine (NAC) decreased ROS and restored survival to MntKO MEFs treated with BSO (Fig. 5*E* and Fig. S7).

MntKO and MntKO+Myc MEFs have normal amounts of GSH (Fig. S8*A*), suggesting that limiting amounts of GSH were not responsible for the BSO sensitivity. Like BSO, inhibition of thioredoxin and cysteine-import antioxidant systems triggered apoptosis in MntKO and MntKO+Myc MEFs (Fig. S8 *B* and *C*). Additionally, the master antioxidant regulator Nrf2 (49, 50) was expressed normally despite ectopic Myc expression and/or Mnt deficiency (Fig. S8*D*). These data suggest that overproduction of ROS, and not a deficiency in antioxidant systems, is responsible for increased apoptosis in Mnt-deficient MEFs.

A major source of ROS production is mitochondrial respiration. We found that a dye that specifically detects superoxide in mitochondria was much more reactive in Mnt-deficient MEFs, thymocytes, and T cells (Fig. 5*F*). A potential cause of aberrant ROS production from mitochondria is altered metabolic activity. T cells provide a useful setting for assessing the relationship between ROS and metabolism because mitogen stimulation is known to induce a dramatic increase in T-cell energy consumption (51), an increase in ROS (Fig. 5*B*), and a dependence on Mnt for survival (Fig. 2*B*).Therefore, we assessed rates of extracellular acidification (EAR) and oxygen consumption (OCR) for T cells in the presence or absence of mitogen stimulation (i.e., ConA+IL-2). As expected, T cells produced a greater EAR and OCR when stimulated (Fig. 5 *G* and *H*). However, although the EAR was indistinguishable between stimulated control and MntTcKO cells (Fig. 5*G*), we observed a lower rate of OCR in stimulated MntTcKO cells (Fig. 5*H*). This result suggests that inefficient oxygen consumption in the absence of Mnt may be responsible for the toxic production of ROS in proliferating cells.

Discussion

Deregulated expression of Myc family oncoproteins can drive robust proliferation and oncogenesis but also sensitize cells to apoptosis, which has long been considered an exploitable weakness of Myc-dependent tumors. Although Mnt overexpression has been found capable of antagonizing the proproliferative activity of Myc (15, 18, 52), Mnt also antagonizes apoptosis (reviewed in refs. 23 and 53 and data described here). Using mouse models with altered

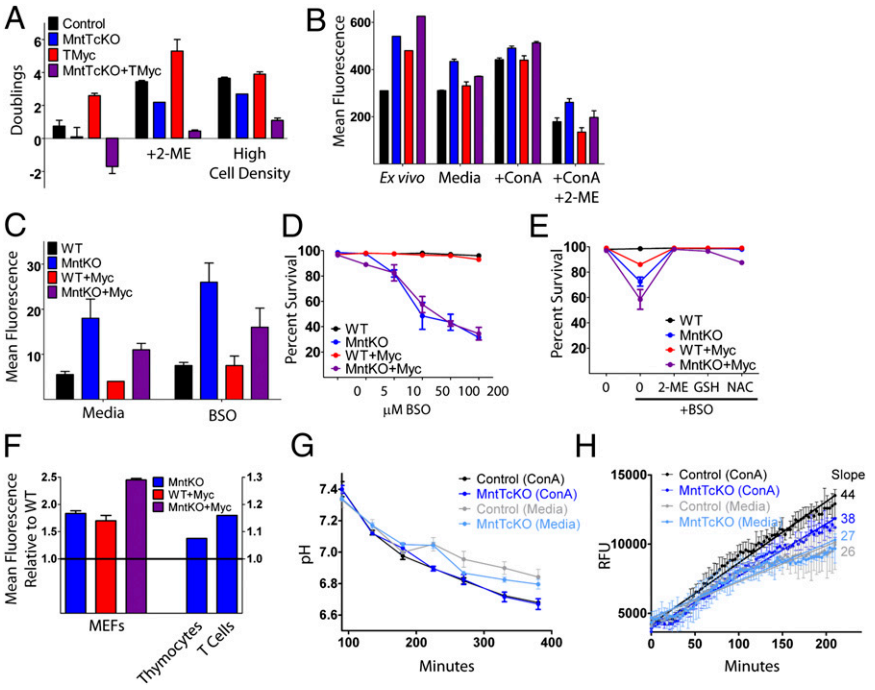

Fig. 5. The survival defect in Mnt-deficient T cells and MEFs is ROS-dependent. (*A*) Splenocytes were cultured at low cell density (0.5×10^6 cells/well) with or without 50 μM 2-ME or at high cell density (2×10^6 cells/well) in the presence of 1 μg/mL ConA. The number of live $CD4^+$ cells was determined before and after 5 d of culture, and the number of doublings is given on the *y* axis. (*B*) The relative amount of intracellular ROS was determined by the mean fluorescence intensity of CM-H_2DCFDA–stained, live $CD4^+$ T cells before and after 24 h of culture with media alone or 1 μg/mL ConA. (*C*) The relative amount of intracellular ROS was determined by the mean fluorescence intensity of CM-H_2DCFDA–stained subconfluent immortal MEFs after 24 h of culture with or without 100 μg/mL BSO. (*D*) The percentage of surviving ($7AAD^{NEG}$) immortal MEFs was determined by FACS after 24 h of culture with a range of BSO concentrations. (*E*) MEFs were treated for 28 h with media only, 50 μM 2-ME, 5 mM GSH, or 5 mM NAC and exposed to 100 μM BSO for the final 24 h of culture. The percentage of surviving ($7AAD^{NEG}$) MEFs was determined by FACS. Error bars represent SD of two replicates. (*F*) Immortal MEFs (left *y* axis), thymocytes and $CD4^+$ T cells (right *y* axis) were stained with the superoxide-specific dye MitoSox, and fluorescence was determined by FACS. Data are given as the fold change in mean fluorescence intensity from WT/control cells (represented by a horizontal black line). Each graph is representative of at least three experiments with nearly identical results. (*G*) Sorted $CD4^+$ T cells were cultured for 3 d with 1 μg/mL ConA and 10 ng/mL IL-2 and then resuspended in fresh media either with or without 1 μg/mL ConA and 10 ng/mL IL-2. The rate at which cells changed the pH of fresh media (*G*) or consumed oxygen (*H*) was determined over time. Error bars represent SD of two replicates. RFU, relative fluorescence units.

expression of Myc and Mnt in T cells, our data reveal the importance of the prosurvival activity of Mnt in Myc-driven proliferation and T-cell lymphoma. We found that an increase in either ectopic or endogenous Myc expression dramatically increased the reliance on Mnt for T-cell survival. The survival defect caused by *Mnt* deletion in vivo worsened as the amount of Myc increased: from no Myc (DTcKO), to endogenous Myc (MntTcKO), to ectopic Myc expression (MntTcKO+TMyc or MntTcKO+$TMyc^{T58A}$) (Figs. 1*A* and 3*C*). MEFs expressing ectopic Myc are also more dependent on Mnt for survival, and it is noteworthy that Myc expression was invariably lower in MntKO than WT MEFs (Fig. S6 *D*, *F*, and *H*), an effect we attribute to selection against MntKO MEFs expressing high Myc (15). Thus, although the prosurvival function of Mnt can be overwhelmed by extremely high Myc expression, our data indicate that cells have difficulty tolerating even small increases in Myc when Mnt is absent. Taken together with data showing that only relatively high amounts of Myc trigger apoptosis (10), our data suggest that loss of Mnt dramatically lowers the threshold at which Myc induces apoptosis such that Myc levels that might normally promote proliferation and oncogenesis instead trigger apoptosis and intrinsic tumor suppression.

Control of ROS by Mnt and Myc. Mnt-deficient cells have elevated ROS and were extremely sensitive to cell death induced by inhibition of GSH, thioredoxin reductase, or the cystine/glutamate antiporter (Fig. 5 *D* and *E* and Fig. S8 *B* and *C*). Because ROS are a natural consequence of oxygen consumption, a greater OCR creates a relative increase in ROS. However, ROS are also reliably induced by artificial inhibition of the electron transport chain (54, 55) or by moderate hypoxia (56), underscoring that toxic ROS may be the product of aberrant oxidative metabolism, not simply more oxidative metabolism. The diminished OCR and increased mitochondrial ROS in Mnt-deficient cells, despite the presence of active antioxidant systems, suggests that Mnt normally limits toxic ROS production by supporting efficient oxidative metabolism. Myc is a known inducer of various metabolic pathways, including mitochondrial oxidative phosphorylation (4, 57–59), that can increase ROS (60). Thus, by facilitating efficient use of oxygen, especially during Myc-driven metabolism, Mnt may allow both normal and cancer cells to maintain the hypermetabolism required for proliferation.

In contrast to their toxic effects, ROS can act as critical signaling molecules that promote cell proliferation and deregulated growth of cancer cells (61). Importantly, Mnt-deficient cells can enter the cell cycle more rapidly than normal cells and can eventually become tumorigenic (15–18). Therefore, although the dominant physiologic outcome of *Mnt* deletion in T cells is increased apoptosis that is linked to increased ROS, increased mitogenesis may occur but be masked by a propensity of mitogen-stimulated and proliferating cells to die and be eliminated. Additionally, although ROS can act to promote proliferation, it can also cause oxidative damage to DNA and lead to the accumulation of oncogenic mutations. Therefore, as has been proposed for deregulated Myc (62), elevated ROS in Mnt-deficient cells could lead to mutations that overcome their propensity to apoptose and expose proproliferative tendencies (15, 17, 18) that lead to malignancy (18, 20).

Mnt as a Critical and Nonredundant Prosurvival Protein. We and others have observed that Mnt still controls survival when Myc is absent in cells (16, 17) (Fig. 1). One hypothesis in line with these observations is that the presence or absence of Myc does not directly impinge on ROS regulation or survival pathways controlled by Mnt, but aberrantly high levels of ectopic Myc may antagonize the function of Mnt more indirectly. For example, we showed previously that high levels of Myc have the ability to compete with Mnt for interaction with Max (17), which would, in theory, act to block Mnt function. In support of this idea, high amounts of Myc expressed in mouse cells or human tumors trigger an apoptotic response akin to that caused by *Mnt* deletion, whereas slight increases in Myc expression are proproliferative but not proapoptotic (10). Thus, although Myc and Mnt may have some overlapping target genes that they regulate in an antagonistic manner (15–17, 20), our results suggest that Mnt and Myc also have separate and cooperative activities in the control of survival and proliferation.

Finally, the finding that immortal MntKO MEFs are susceptible to apoptosis suggests that apoptotic sensitivity is maintained in the absence of a fully functional p53 pathway (63). Furthermore, combining ectopic Myc with antiapoptotic Bcl-2 or oncogenic Ras failed to oncogenically transform Mnt-deficient MEFs or reverse their apoptotic sensitivity (Fig. 4 *C–F*). Taken together, our results suggest that Mnt plays a unique and previously unappreciated role in preventing Myc-induced apoptosis and supporting oncogenesis.

Materials and Methods

Detailed descriptions of the reagents and protocols used in this study can be found in the *SI Materials and Methods*. Flow cytometry methods were used to analyze cell number, survival, proliferative capacity, and ROS. Oncogene introduction by retroviral infection was used to induce transformation and tumorigenesis in nude mice.

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