The mSin3A Chromatin-Modifying Complex Is Essential for Embryogenesis and T-Cell Development†

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The corepressor mSin3A is the core component of a chromatin-modifying complex that is recruited by multiple gene-specific transcriptional repressors. In order to understand the role of mSin3A during development, we generated constitutive germ line as well as conditional *msin3A* deletions. *msin3A* deletion in the developing mouse embryo results in lethality at the postimplantation stage, demonstrating that it is an essential gene. Blastocysts derived from preimplantation *msin3A* null embryos and mouse embryo fibroblasts (MEFs) lacking *msin3A* display a significant reduction in cell division. *msin3A* null MEFs also show mislocalization of the heterochromatin protein, HP1 α , without alterations in global histone acetylation. Heterozygous *msin3A*^{+/-} mice with a systemic twofold decrease in mSin3A protein develop splenomegaly as well as kidney disease indicative of a disruption of lymphocyte homeostasis. Conditional deletion of *msin3A* from developing T cells results in reduced thymic cellularity and a fivefold decrease in the number of cytotoxic (CD8) T cells, while helper (CD4) T cells are unaffected. We show that CD8 development is dependent on mSin3A at a step downstream of T-cell receptor signaling and that loss of mSin3A specifically decreases survival of double-positive and CD8 T cells. Thus, *msin3A* is a pleiotropic gene which, in addition to its role in cell cycle progression, is required for the development and homeostasis of cells in the lymphoid lineage.

mSin3A functions as a platform that mediates the association of histone deacetylase (HDAC) enzymes and other chromatin-modifying activities with a large number of DNA-binding transcriptional repressors (for reviews, see references 4 and 29). In order to interact with a wide range of repressors, Sin3 proteins from yeast to mammals have evolved and maintained four imperfect repeats known as paired amphipathic helix (PAH) domains (6, 49, 63), each of which appear to possess innate specificity for recruiting a defined repertoire of transcriptional repressors or corepressors. Recent structural studies have begun to define the basis for specific interactions between the second PAH domain of mSin3A (PAH2) and the transcriptional repressors Mad1 and HBP1 (8, 11, 19, 57, 58). The association of Sin3 with sequence-specific transcription factors serves to bring the complex in the vicinity of the transcription factor target genes, thereby permitting the complex to modulate transcriptional output by altering chromatin structure.

Sin3 is thought to predominantly influence chromatin structure by mediating histone deacetylation through its recruitment of class I HDACs (HDAC1 and HDAC2) (3, 21, 22, 34, 41). Another Sin3 complex protein, SDS3, acts as a bridging protein between the HDACs and the Sin3 HDAC interaction

domain (HID), a highly conserved region lying between PAH3 and PAH4 (2, 13, 15, 16, 36). The RbAp46 and RbAp48 proteins also associate with HDACs in the Sin3 complex and appear to target HDAC activity to histone tails. Other components of the complex may serve to couple Sin3 to specific repression systems. For example, SAP30 is involved in repression by antagonist-bound nuclear hormone receptors (33) and mediates binding to RbAp-HDAC1 (73), the Rb docking protein RBP1 (35), and the negative growth regulator p33ING1b (31).

While HDAC activity is clearly central to Sin3 repressive function, other proteins with the potential to modify chromatin have also been reported to be present in Sin3 complexes. These include histone methyltransferases (42, 68) as well as an O-linked *N*-acetylglucosamine transferase (69), both of which could in principle contribute additional repressive modifications to deacetylated histone tails. Furthermore, components of the Swi/Snf nucleosome remodeling complex have been detected in a subset of mSin3 complexes (31, 44, 54). Because a number of Sin3 complex components are apparently present at substoichiometric levels (54, 69, 72, 73), the composition of intracellular Sin3 complexes is likely to be heterogeneous, suggesting that specialized subsets of complexes may be directed towards specific repression functions.

In mice two distinct Sin3 genes, *msin3A* and *msin3B*, encode the mSin3A and mSin3B paralogs, respectively (6). Mammalian Sin3A and Sin3B proteins are approximately 57% identical throughout the length of their polypeptide chains, with the highest degree of homology localized in the PAH and HID regions (6). Both proteins have been found to be widely expressed (frequently within the same cells and tissues) and to bind many of the same transcriptional repressors and complex

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components (references 6, 8, and 57 and unpublished data). Therefore Sin3A and Sin3B have been generally assumed to perform redundant functions.

Biological roles of Sin3 complexes. Given that Sin3 complexes are recruited by a wide range of transcription factors, it is not surprising that Sin3 functions have been linked to a large number of cellular processes. For example, in *Saccharomyces cerevisiae* Sin3 has been shown to play roles in the unfolded protein response (51), rRNA processing (39), and the response to DNA damage (26, 52). In *Drosophila melanogaster*, small interfering RNA knockdown of Sin3 compromises G₂ progression without causing global changes in chromatin acetylation (46). Expression array analysis of *Drosophila* Sin3 knockdown cells indicated that dSin3 regulates cell cycle genes as well as many genes involved in mitochondrial genesis and function (47). Sin3 in mammalian cells has been implicated in the maintenance of pericentric heterochromatin and binding by HP1 α (67). Furthermore, specific cellular functions related to proliferation arrest and differentiation mediated by regulators such as Rb and p130 (35, 48), p53 (31, 40, 74), and the Mad protein family (5, 27, 49, 50) have been linked to their ability to interact with Sin3 complexes.

Reports of new Sin3-interacting transcription factors and Sin3/HDAC complex components appear regularly (e.g., references 16, 58, 70, and 71), suggesting that the range of cellular functions involving Sin3 is likely to be even larger than those mentioned above. Nonetheless, it is apparent that many of the functions of Sin3 appear to converge on regulation of gene expression coupled to proliferation arrest and differentiation. In order to understand in more detail the role of Sin3 *in vivo*, we have generated mice with a constitutive or conditional disruption of the *msin3A* gene. We have employed the constitutive deletion to analyze the general effects of *msin3A* loss of function on murine development. We have found that *msin3A* is an essential gene and that heterozygous mice develop splenomegaly and glomerulopathy due to alterations in lymphoid homeostasis. Furthermore, because T-lymphoid cell development represents a compartment in which chromatin modification and remodeling (18, 30) play crucial roles, we have generated a conditional *msin3A* deletion in T cells and have found specific effects on T-cell proliferation, differentiation, and homeostasis.

MATERIALS AND METHODS

Targeting construct, colony generation, and genotyping. A genomic DNA fragment containing the *msin3A* gene was isolated from a murine 129/Sv genomic library (a kind gift from P. Soriano, Fred Hutchinson Cancer Research Center [FHCRC]) using the *msin3A* cDNA (6) as a probe. The resulting clones were confirmed by sequencing. The mSin3ALoxP targeting vector was constructed as follows: a 2.76-kb EcoRI fragment, containing exon 3 of the *msin3A* gene, was used to form the long arm of the targeting vector. An upstream LoxP site and additional EcoRI and HindIII restriction sites were inserted as a double-stranded oligonucleotide into a unique XbaI site. The short arm (3' end) of the vector was generated by PCR and cloned into the HindIII site of the plasmid. The final targeting vector, mSin3ALoxP, was sequenced in full to ensure the absence of point mutations. We screened clones, initially by PCR and then by Southern blotting analysis, using EcoRI and the region outlined in Fig. 1C as a probe to identify correctly targeted recombinants. The Neo^c cassette was removed by transient expression of Cre followed by PCR and Southern analysis. Correctly targeted embryonic stem (ES) cell clones were injected into blastocysts to generate chimeric mice, which were subsequently bred onto a C57BL/6J strain to produce germ line-targeted (*msin3A*^{L/+}) animals. *msin3A*^{L/+} mice were initially intercrossed with a Mox2-Cre germ line deleter strain (61) and then back-

crossed to produce *msin3A*^{+/-} heterozygotes. Mice were routinely genotyped by multiplex PCR. Primer sequences used were as follows: SinFor, GATGTTTGC ACATAAGTGTGG; SinRev, TTCAGCTTAACAATAGGAGAC; SinMut, AC AATTATGTTAATTGAGAC. A PCR with all three primers was used to distinguish wild-type alleles (497-bp) and alleles with exon 3 deleted (583-bp). Wild-type (493-bp) and LoxP-targeted (545-bp) alleles can be distinguished using only SinFor and SinRev primers. PCR conditions were 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s.

Cellular analysis. Mouse embryo fibroblasts (MEFs) were prepared from wild-type, *msin3A*^{L/+}, and *msin3A*^{L/L} embryonic day 14.5 (E14.5) embryos essentially as described previously (75), genotyped by PCR, and routinely cultured in Dulbecco's modified Eagle's medium plus 10% fetal calf serum, 50 mM β -mercaptoethanol, penicillin, and streptomycin. The deleted allele was reconstituted by infecting early-passage MEFs with an murine stem cell virus retrovirus expressing both Cre and green fluorescent protein (GFP) using standard protocols. Infected cells were isolated by sorting for GFP-positive populations using a Vantage cell sorter (Becton Dickinson). To perform growth curve analysis of Cre/GFP MEFs, 15,000 cells were plated into a 24-well plate, 3 days after viral infection, and were harvested and counted using a hemocytometer on the indicated days. Immortalized MEFs were derived as follows: supernatants containing a large T antigen retrovirus were prepared from Psi 2 packaging cells infected with pZIPNeo SV(X)1 vector (7). The supernatant was then used to infect wild-type and *msin3A*^{L/L} MEFs by standard protocols followed by G418 selection. Cell cycle analysis of both primary and immortalized MEFs was performed by harvesting cells, 7 days after Cre/GFP infection and staining with Hoechst 33342 (Molecular Probes), followed by analysis using a FACSCalibur (Becton Dickinson) and CellQuest software.

Protein analysis. Lysates for Western blotting were prepared from equivalent cell numbers as follows: cells were washed twice with phosphate-buffered saline (PBS) before addition of hot 2 $\times$ protein loading buffer (4% sodium dodecyl sulfate [SDS], 10% [vol/vol] glycerol, 1% [vol/vol] β -mercaptoethanol) and boiling for 5 min, followed by sonication. Cell extracts were resolved using SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and then probed using standard protocols. All antibody incubations were performed in PBS containing 0.05% Tween 20 (vol/vol) and 2% bovine serum albumin (wt/vol). Standard horseradish peroxidase-conjugated secondary antibodies were used in conjunction with enhanced chemiluminescence reagents for Western analysis unless stated otherwise. Quantitative Western analysis was performed as above, but with Alexa Fluor 680-conjugated anti-rabbit (Molecular Probes) and IRDye 800-conjugated anti-mouse secondary (Rockland) antibodies. Blots probed in this fashion were analyzed using the LI-COR Odyssey linear detection system. Immunofluorescence was performed on MEFs grown in eight-well chamber slides, fixed in 2% paraformaldehyde, permeabilized with 0.5% Triton X-100, and then probed with the indicated antibodies in PBS, 5% normal goat serum, and 0.05% Tween 20. The following antibodies were used in this study: the PIE7 monoclonal was generated using the purified PAH2 domain of mSin3A (S. M. Cowley and R. N. Eisenman, FHCRC); anti-mSin3A (sc-994), anti-HDAC2 (sc-7899), anti-slp76 (sc-9062), anti-LAT (sc-7948), and anti-LCK (sc-13) rabbit polyclonals (Santa Cruz Biotechnology); anti-mSin3B monoclonal (sc-13145; Santa Cruz Biotechnology); antitubulin and antiactin monoclonal (Sigma); anti- β -tubulin (a gift from Don Ayer, Huntsman Institute, Salt Lake City, UT); anti-Runx3 (a gift from Yoram Groner, Weizmann Institute, Rehovot, Israel); and anti-HP1 α (Upstate Biotechnology).

Isolation and cytometric flow analysis of lymphoid cells. Cells were isolated from bone marrow, spleen, and thymus as described previously (25). Briefly, harvested cells were resuspended in Hanks' balanced salt solution (Gibco) plus 3% fetal calf serum (HyClone), and erythrocytes were then depleted by ammonium chloride lysis. Staining for surface markers using flow cytometry was performed as described previously (25), and 10,000 gated events were collected utilizing a FACSCalibur machine (Becton Dickinson). Data were then analyzed using CellQuest software (Becton Dickinson). Cells were stained with the following antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-CD25 (Pharmingen), anti-CD8 (Caltag), phycoerythrin (PE)-conjugated CD4 (Caltag), anti-CD44 (Pharmingen), biotinylated anti-CD3 ϵ (Pharmingen), and anti-heat-stable antigen.

T-cell proliferation assays. T cells were cultured in RPMI 1640 medium (Gibco-BRL) supplemented with 10% fetal calf serum (HyClone), 2 mM L-glutamine (Gibco), 100 mM nonessential amino acids (Gibco), 100 U/ml penicillin, 100 U/ml streptomycin (Gibco). Cells were stimulated to divide by addition of anti-CD3 and anti-CD28 antibodies. For [³H]thymidine incorporation assays, cells were grown for 48 h following stimulation and then pulsed with [³H]thymidine for 24 h prior to harvesting onto printed glass fiber filtermats using an LKB-Wallac Cell Harvester. Incorporation of [³H]thymidine was measured using

FIG. 1. (A) The *msin3A* gene present on mouse chromosome 9 consists of 22 exons, with exon 2 containing the initiator ATG and first 63 amino acids. The *msin3A* locus is flanked by the mannosyltransferase gene, *man2c1*, and the tyrosine phosphatase gene, *ptpn9*. (B) Targeting strategy and mutant alleles of *msin3A*. The Neo^r cassette and exon 2, the region to be deleted, were flanked by LoxP sites (triangles). The endogenous EcoRI sites plus one synthetic site, engineered into the 5' LoxP element, used for Southern analysis are also depicted. The probe used for Southern blot analysis is shown. (C) Southern analysis of ES cell clones (digested with EcoRI; left panel) showing targeting of the *msin3A* locus (*msin3A^{Neo}*), removal of the Neo^r cassette by transient expression of Cre (*msin3A^L*), and the allele with exon 2 deleted (*msin3A⁻*). (Right panel) Genomic PCR analysis was also used to confirm the presence of the wild-type (1.6-kb) or deleted (0.6-kb) alleles.

an LKBWallac 1205 Betaplate liquid scintillation counter. For cell division experiments, cells were labeled with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) according to the manufacturer's instructions. Cells were harvested after 48 and 96 h poststimulation and then analyzed using a FACSCalibur machine (Becton Dickinson).

RESULTS

Given the widespread expression pattern and putative transcriptional roles of mSin3A, it seemed possible that constitutive *msin3A* gene disruption would result in early lethality. Therefore, we generated a conditional *msin3A* allele that can be inactivated using standard Cre recombinase technology. The *msin3A* gene, present on mouse chromosome 9, consists of 22 exons spanning a region of 52 kb (Fig. 1A). We constructed a vector for targeting ES cells in which loxP recombination sites were introduced 5' and 3' to the first coding exon (Fig. 1B, exon 2). Correctly targeted ES cells (*msin3A^{Neo/+}*) were determined by both PCR and Southern blotting (Fig. 1C). Following the removal the Neo^r cassette by transient expression of Cre, the resulting ES cell clones, *msin3A^{L/+}* (L for loxP), were injected into blastocysts to generate chimeric animals that were crossed onto a C57BL/6 line to generate germ line *msin3A^{L/+}* mice. *msin3A^{L/+}* mice were further crossed with a germ line

deleter strain, Mox2-Cre (MORE) (61) to generate MORE-*Sin3A^{L/+}* animals, which were subsequently backcrossed to create germ line heterozygous *msin3A^{+/-}* animals with a constitutive deletion.

msin3A^{+/-} pups appeared phenotypically normal and developed into fertile adults. Intercrosses between *msin3A^{+/-}* mice generated both wild-type (*msin3A^{+/+}*) and heterozygous (*msin3A^{+/-}*) pups but failed to yield any homozygous null animals (*msin3A^{-/-}*) (Fig. 2A). The number of *msin3A^{+/-}* pups isolated was less than would be predicted from a normal Mendelian ratio (Fig. 2A), suggesting that there may be haploinsufficiency associated with the loss of one *msin3A* allele. Because the lack of viable *msin3A^{-/-}* pups suggested an embryonic lethal phenotype, we examined embryos generated from the identical cross at different time points during development. None of the embryos genotyped at E10.5 were *msin3A^{-/-}*. However, we did observe a number of empty decidua which were much reduced in size, suggesting that the absent *msin3A^{-/-}* embryos had been reabsorbed at a point following implantation. A second, independently generated, *msin3A* null mouse line also results in the lethality of homozygous null embryos at the peri-implantation stage (J. H. Dannenberg et al., submitted for publication). Consistent with hy-

A

Viable pups or embryos derived from <i>msin3A</i> ^{+/-} × <i>msin3A</i> ^{+/-} cross			
+/+	+/-	-/-	
111	165	0	viable pups
11	15	0	10.5 days p.c
8	16	5	3.5 days p.c

B

FIG. 2. Loss of *msin3A* causes early embryonic lethality. (A) The number and genotypes of pups and embryos generated from *msin3A*^{+/-} heterozygous intercrosses are shown. (B) Embryos harvested at E3.5 from *msin3A*^{+/-} heterozygous intercrosses were cultured in vitro on gelatinized tissue culture plates for 7 days. Representative examples of outgrowth cultures from wild-type, heterozygous, and nullizygous embryos are shown. ICM, inner cell mass.

pothesis that *msin3A* null embryos are reabsorbed at implantation, we were able to isolate E3.5 embryos with all three genotypes (Fig. 2A and B), which were cultured ex vivo by plating them onto gelatinized tissue culture plates. Although *msin3A*^{-/-} embryos appeared phenotypically normal in appearance at the point of isolation, cells derived from the inner cell mass of nullizygous blastocysts produced much smaller colonies, suggesting that they fail to proliferate significantly in comparison to heterozygous and wild-type littermate controls (Fig. 2B). Taken together, our data suggest that mSin3A function is essential for embryonic development beyond the implantation stage.

Reduced proliferative potential in MEFs with *msin3A* deleted. *msin3A*^{-/-} embryos die too early in their development to isolate MEFs. However, we were able to generate MEFs from both wild-type and *msin3A*^{L/L} targeted embryos, in which a deleted allele can be generated by infection with a Cre-expressing retrovirus, resulting in a reduction in expression of full-length mSin3A protein (Fig. 3A). Residual mSin3A observed in L/L cells is likely due to a subpopulation of cells that escaped infection. A comparison of Cre-infected cells revealed that loss of mSin3A causes a reduction in proliferative potential (Fig. 3B). The population doubling time of cultured Cre-infected *msin3A*^{L/L} cells, beginning 3 days after infection, was increased by approximately twofold relative to Cre-infected

msin3A^{+/+} cells. However, despite a reduction in the number of divisions, the cell cycle profile of CreGFP-*msin3A*^{L/L} cells remained similar to wild type, with only a ~7% increase in G₂/M-phase cells (Fig. 3C). To circumvent the growth limitations of primary cells, we attempted to derive an *msin3A*^{L/L} cell line by immortalizing targeted and wild-type MEFs, prior to Cre infection, with large T antigen. However, Cre-mediated deletion of mSin3A in immortalized MEFs resulted in profound apoptosis, as measured by the occurrence of a sub-G₁ peak (Fig. 3D). The occurrence of the sub-G₁ peak is concurrent with a decrease in the G₂/M population, suggesting that post-S-phase cells are undergoing apoptosis, presumably in a p53-independent manner.

As a potential mechanism for these defects in cell-cycle progression, we analyzed the level and patterns of acetylated histones for changes caused by the loss of mSin3A-associated HDAC activity. The relative amounts of euchromatic markers, such as acetylated Lys9 on histone 3 (H3K9Ac) and Lys12 on histone 4 (H4K12Ac), did not change significantly in bulk histone extracts from primary MEFs with reduced mSin3A (data not shown). Although bulk histone acetylation was unaltered, it was possible that the distribution pattern of acetylated histones in the interphase nuclei of CreGFP-*msin3A*^{L/L} cells might be affected. mSDS3, a core component of the mSin3A complex, was previously demonstrated to be required for the maintenance of pericentric heterochromatin in its hypoacetylated state (13). However, the distribution and amount of pericentric heterochromatin, identified by areas of intense 4',6'-diamidino-2-phenylindole (DAPI) staining, did not appear to be altered and were found to lack H3K9Ac and H4K12Ac in both wild-type and *msin3A*^{L/L} cells infected with Cre (see Fig. S1 in the supplemental material). In contrast, the localization of HP1α, which in wild-type MEFs displays punctate staining predominantly colocalized in pericentric heterochromatin, was frequently dispersed throughout the entire nucleus in CreGFP-*msin3A*^{L/L} cells (Fig. 3E). No significant change in HP1α protein level was detected (Fig. S1 in the supplemental material). In conclusion, loss of mSin3A in MEFs, while causing little evident change in histone acetylation, leads to a reduction in proliferative potential, increased apoptosis in immortalized cells, and HP1α mislocalization.

A subset of *msin3A* heterozygous mice develop splenomegaly and membranous glomerulopathy. Pathological analysis of mature *msin3A*^{+/-} animals (over 5 months of age) revealed that a quarter of *msin3A*^{+/-} mice (7 of 28) had spleens of greater than three times the normal size (i.e., >0.3 g), compared to 0 out of 10 wild-type littermates. Indeed, two heterozygous *msin3A*^{+/-} animals had spleens with a mass of over 0.7 g, equivalent to seven times the average wild-type mass (Fig. 4A). Hematoxylin and eosin (H&E)-stained sections of normal and enlarged spleens revealed that the areas of white pulp, which contain the majority of splenic lymphoid cells, were increased in size in *msin3A* heterozygotes (Fig. 4B), indicating an increased lymphoid population. Consistent with this increased size, heterozygous mice with splenomegaly (represented by Het2) had four times the number of splenocytes compared to unaffected heterozygotes (represented by Het1) and wild-type controls (Fig. 4C). In contrast, the cellularity of thymus and bone marrow was largely unchanged. To account for this increase in splenic cellularity, we examined the lym-

FIG. 3. MEFs lacking mSin3A have a reduced proliferative potential. (A) Western blot analysis of mSin3A levels in GFP-positive MEF extracts 3 days after infection with a Cre/GFP-expressing virus. HDAC2 and actin levels were used to normalize protein extracts. (B) Growth curves of GFP-positive MEFs, 3 days after Cre/GFP infection, were determined by counting cells with a hemocytometer at the time points indicated. (C) Flow cytometric analysis of DNA content was used to determine the cell cycle profiles of wild-type, *msin3A*^{L/+}, and *msin3A*^{L/L} MEFs 7 days following Cre/GFP infection. The percentages of cells in each phase of the cell cycle are shown. (D) Wild-type, *msin3A*^{L/+}, and *msin3A*^{L/L} cells, prior to Cre infection, were immortalized by infection with large T antigen. These immortalized cells were then analyzed as for panel C, 7 days after Cre/GFP infection. (E) Cre/GFP-infected wild-type and *msin3A*^{L/L} MEFs were analyzed by immunofluorescence. Specific antiserum was used to determine the localization of HP1α in GFP-positive cells; DAPI was used to stain DNA.

phoid cell populations in the spleen. The percentages of B (B220 positive) and T cells (CD3 ϵ positive) within the spleens of Het2 mice were comparable to wild-type controls (Fig. 4E). However, the total number of B cells and CD4 T cells were increased fourfold compared to controls, consistent with the overall increase in splenic cellularity (Fig. 4F). In contrast, CD8-positive T cells were present at wild-type levels in the enlarged spleens, indicating that the relative proportion of CD8 cells was sharply reduced. The CD4/CD8 ratio for the Het2 example was about eight- and sixfold greater than for wild-type and Het1 animals, respectively (Fig. 4G). On average, we observed an approximately fourfold increase in the ratio of CD4 versus CD8 cells in Het2 animals compared to Het1 and wild-type mice (Fig. 4H). Younger *msin3A*^{+/-} mice (less than 12 weeks of age) showed no indication of splenic hypertrophy or altered CD4/CD8 ratio, suggesting a delayed or gradual onset. Quantitative Western blot analysis of T cells from wild-type, Het1, and Het2 animals revealed only a twofold reduction in mSin3A levels in Het2 mice compared to controls (Fig. 4D), perhaps explaining the gradual occurrence. Alternatively, the partial penetrant phenotype could be related to stochastic differences in the differentiation of cytokine-secreting T cells in *msin3A*^{+/-} mice (see Discussion).

Further histological analysis indicated increased infiltrates of lymphocytes in many tissues, including lung, liver, and kidney of Het2 animals versus normal littermate controls (data not shown). Moreover, examination of two out of three aged Het2 mice revealed severe membranous glomerulopathy (Fig. 4I), a kidney disease in humans and other species characterized by proteinuria and formation of immune deposits in the lamina rara of the glomerular basement membrane (for review, see reference 28). The Het2 mice examined had protein in their renal tubules (pink substance) and extensive protein deposits in their glomeruli basement membrane. Together, these studies suggest that mSin3A has a role in modulating lymphocyte homeostasis and that constitutive loss of one allele is sufficient to result in the expansion of CD4 and B lymphocytes and severe immune complex-mediated kidney disease.

Conditional deletion of *msin3A* in T cells results in reduced thymic cellularity and proliferative potential of mature T cells. To further explore the possibility that loss of mSin3A might influence T-cell development, we generated a T-cell-specific *msin3A* deletion. *msin3A*-targeted mice (*mSin3A*^{L/L}) were bred to animals expressing Cre under the control of the *lck* proximal promoter, which expresses Cre specifically in T cells throughout thymopoiesis (37). A comparison of thymocyte DNA derived from *lckCre-msin3A*^{L/+} and *lckCre-msin3A*^{L/L} animals with DNA from targeted mice lacking Cre demonstrated a robust deletion of exon 2 in the presence, but not absence, of the recombinase (Fig. 5A). Characterization of thymocyte protein extracts from these same cells revealed a reduction in mSin3A levels compared to littermates lacking *lckCre* (Fig. 5B and see below). Other components of the mSin3 complex, including mSDS3 and HDAC2, were found to be expressed at comparable levels in all four genotypes (Fig. 5B). Thymocytes from a number of *lckCre-mSin3A*^{L/L} mice expressed a truncated form of mSin3A as detected by an antibody to PAH2 (amino acids 304 to 386) (Fig. 5B, third row). Because this smaller protein is not recognized by an N-terminal antibody, we conclude that it lacks the N-terminal region

and probably arises as a result of selection for an internal translational initiation event, since it is not detected in heterozygous deleted (*lckCre-mSin3A*^{L/+}) T cells. In general, we observed a dose-dependent correlation between loss of all forms of mSin3A protein and increasing severity of the phenotypes which, taken together with the apparent selection, suggests that the truncated form of the protein does not function as a dominant negative. In addition, Western blot analysis of thymocyte protein extracts with an antibody raised to the C terminus of mSin3A failed to detect any other truncated forms of the protein (data not shown).

Phenotypic analysis of *lckCre-mSin3A*^{L/L} T cells revealed a threefold reduction in total thymocyte cellularity in comparison to *lckCre-mSin3A*^{L/+} and control cells lacking *lckCre*, indicative of an impairment in thymopoiesis (Fig. 5C). The progression of T cells during development can be monitored through the expression of the cell surface markers CD4, CD8, and the T cell receptor (TCR), which help delineate the four major subsets of thymocytes. Immature TCR⁻ CD4⁻ CD8⁻ (double-negative [DN]) cells proliferate and differentiate to become TCR⁺ CD4⁺ CD8⁺ (double-positive [DP]) cells before finally maturing into CD4⁺ or CD8⁺ single-positive (SP) naïve T cells. A comparison of CD4 and CD8 expression revealed that *lckCre-mSin3A*^{L/L} mice had a threefold increase in the percentage of DN cells compared to controls, indicative of a partial block or delay in thymopoiesis and thus explaining the reduced thymic cellularity (Fig. 5D, top row; an increase from 4% to 12%). A delay in thymopoiesis at the DN stage, prior to proliferative expansion, results in a nearly normal number of DN T cells, while DP, CD4SP, and CD8SP T-cell populations, in contrast, are all greatly reduced (Fig. 5E, compare *lckCre-msin3A*^{L/L} cells to *lckCre-msin3A*^{L/+} and the no-*lckCre* controls).

We next examined more specifically where loss of mSin3A function affected development of DN cells. Immature DN T cells can be further subdivided into four populations (DN1 to -4) based on their expression of CD25 (interleukin-2 [IL-2] receptor) and CD44 (Fig. 5C, second row). Maturation from the DN3 (CD25⁺, CD44⁻) to DN4 (CD25⁻, CD44⁻) stage of development, subsequent expansion, and allelic exclusion of the TCR β chain are dependent upon successful in-frame rearrangement of the TCR β -chain and formation of the pre-TCR complex, collectively referred to as the "β-checkpoint." Homozygous deleted *msin3A* T cells showed increased numbers of DN3 cells, suggesting that a reduction in mSin3A levels results in a dose-dependent delay or partial block at the β-checkpoint. However, the formation of gamma delta T cells, which express a variant TCR, was unaffected by loss of mSin3A (Fig. 5C, third row).

The reduction of thymic cellularity in *lckCre-mSin3A*^{L/L} mice might be explained by an impairment in maturation (e.g., the β-checkpoint) or by a more generalized cell cycle defect, as suggested by the reduction in proliferative potential observed in cultured embryos and MEFs (Fig. 2B and 3B). To distinguish between these two possibilities, we assessed the ability of T cells from *lckCre-msin3A*^{L/L} and control mice to proliferate ex vivo. T cells derived from the spleens of *lckCre-msin3A*^{L/+}, *lckCre-msin3A*^{L/L}, and non-Cre-expressing mice were stimulated to proliferate in culture by the addition of anti-CD3 and anti-CD28 antibodies, which mimic TCR activation. Prolifer-

FIG. 5. Characterization of thymocytes lacking mSin3A. (A) Genomic PCR analysis of thymocyte DNA derived from *msin3A*^{L/+} and *msin3A*^{L/L} thymocytes with and without *lckCre*. The position of wild-type (WT), targeted (LoxP), and deleted (Δ exon 2) alleles are shown (upper panel). (B) Western blot analysis of protein extracts derived from thymocytes with the indicated genotype. Antiserum which recognizes the PAH2 domain (amino acids 304 to 380) was used to detect mSin3A protein. The white arrowhead indicates the position of the truncated mSin3A protein. (C) The number of thymocytes harvested from individual animals with the indicated genotype was determined using a hemocytometer. The average cellularity was calculated using four mice of each genotype from three independent experiments. (D) Single cell populations of thymocytes harvested from mice with the indicated genotypes were stained with FITC-conjugated anti-CD25, anti-CD8, anti-gamma delta TCR, PE-conjugated anti-CD44, anti-CD4, and biotinylated-anti-CD3e, followed by streptavidin tricolor. A representative flow cytometric histogram from 8-week-old mice is shown. (E) The average cellularity of each thymocyte subset was determined, using flow cytometric analysis and total thymic cellularity, from three independent experiments. (F) Splenocytes derived from the genotypes indicated were stimulated to proliferate ex vivo by addition of anti-CD3 and anti-CD28 antibodies. The cells were allowed to divide for 48 h and then pulsed with 3 [H]thymidine for 24 h prior to harvesting. A representative histogram of the average 3 [H]thymidine incorporated, normalized for the number of starting T cells, is shown. (G) Splenocytes were labeled with CFSE for 5 min at room temperature. The cells were then stimulated to proliferate as for panel B and then analyzed at 48 and 96 h by flow cytometry to assess division.

FIG. 6. Loss of *msin3A* inhibits the development of CD8SP T cells. (A) Thymocytes and splenocytes derived from mice with the indicated genotype were stained with anti-CD4-FITC, anti-CD4-PE, and anti-CD3e antibodies. A representative flow cytometric histogram is shown. (B) The average ratio of CD4SP versus CD8SP cells in the spleen was determined from mice with the indicated genotypes. (C) Splenocytes expressing a transgenic T-cell receptor (OT-1) were harvested from 6-week-old mice with the indicated genotypes and then stained with FITC-conjugated anti-CD8, PE-conjugated anti-CD4, and biotinylated anti-CD3e, followed by streptavidin tricolor. A representative histogram, with two mice of each genotype, is shown. (D) The LI-COR Odyssey quantitative Western blotting system was used to analyze the relative protein levels of mSin3A, mSin3B, and tubulin in the thymocytes indicated. The amounts of mSin3A and mSin3B were normalized to tubulin and then expressed as a percentage relative to wild-type (+) cells.

ation was measured by the incorporation [³H]thymidine over a 48-hour period following antibody stimulation (Fig. 5F). Incorporation of [³H]thymidine was markedly reduced in *lckCre-mSin3A*^{L/L} mice compared to *lckCre-mSin3A*^{L/+} and no-Cre controls. No proliferation of T cells derived from any of the mice occurred in the absence of stimulation (Fig. 5F, first four columns). Cell division was also assessed from the same splenocytes by labeling with the fluorescent dye CFSE prior to stimulation. Analysis of stimulated CD4SP cells revealed significant division of both *mSin3A*^{L/L} and *lckCre-mSin3A*^{L/+} cells after 2 days, which increased over a 4-day period (Fig. 5G). The cells from *lckCre-mSin3A*^{L/L} mice, in contrast, which had a reduced thymic cellularity and mSin3A levels compared to control cells, did not divide at all, even 4 days poststimulation.

Thus, we conclude that *lckCre-mSin3A*^{L/L} mice have a reduced number of T cells, due to a partial block at the β -checkpoint stage of thymopoiesis and a reduction in proliferative potential of mature T cells, suggesting that loss of mSin3A may cause a generalized defect in cell cycle progression similar to that observed in cultured embryos and MEFs (Fig. 2 and 3).

Loss of mSin3A impairs the development of CD8SP T cells. Closer examination of mature CD3^{hi} thymocytes (CD3^{hi}, up-

per row) revealed an additional effect on T-cell development whereby loss of *msin3A* resulted in a marked decrease in the percentage and total number of CD8SP (cytotoxic) T cells in *lckCre-mSin3A*^{L/L} mice compared to controls (Fig. 5E and Fig. 6A, upper row). Consequently, the ratio of CD4SP to CD8SP cells is increased from 2.2 and 1.9 in *LckCre*-negative control animals to 7.2 in *lckCre-mSin3A*^{L/L} mice lacking *msin3A*. Analysis of spleens from the same animals revealed a similar reduction in the percentage of CD8SP cells (Fig. 6A, bottom row). On average, we observed an increase in the ratio of CD4SP to CD8SP cells in the spleen from 1.4 in non-Cre-expressing animals to 2 and 5.3 in *lckCre-mSin3A*^{L/+} and *lckCre-mSin3A*^{L/L} mice, respectively. This loss of CD8SP cells is reminiscent of the phenotype we observed in *msin3A*^{+/-} mice with splenomegaly (Fig. 4E). However, the size and cellularity of spleens from *lckCre-mSin3A*^{L/L} mice was not significantly different from control mice, in contrast to *msin3A*^{+/-} Het2 mice (see Discussion).

The maturation of CD8SP cells from their DP precursors is dependent upon many competing factors, chief among these being the interaction of the TCR, on thymocytes, with complexes of peptides and the major histocompatibility complex (MHC) I (for CD8SP) or II (for CD4SP) molecules expressed

on thymic epithelial cells, a process called positive selection (see reference 53 for a review). The level of CD3 ϵ and TCR β expression, which indicates the formation of a competent TCR complex, was comparable in control and thymocytes with *msin3A* deleted (Fig. 5D, bottom row, and data not shown). This suggests that mSin3A modulates lymphocyte development and proliferation by controlling signaling pathways that function downstream of the TCR. To test this hypothesis more comprehensively, we bred *lckCre-mSin3A^{L/L}* mice to OT-1 transgenic animals, which constitutively express an MHC class I-restricted TCR (24) and hence are artificially skewed towards the development of CD8 single-positive thymocytes (CD8SP) during positive selection. We reasoned that if mSin3A functioned downstream of the TCR, its loss should readily inhibit the increased accumulation of CD8SP cells on the OT-1 background. As previously published, expression of the OT-1 transgene on the wild-type (*msin3A^{+/+}*) *lckCre⁺* background increases the proportion of peripheral CD8SP cells (Fig. 6C, *lckCre-msin3A^{+/+}; OT-1* mice A and B; 16% and 19% CD8SP, respectively). However, this increased population of CD8SP is significantly reduced in T cells lacking mSin3A (Fig. 6C, compare *lckCre-msin3A^{+/+}; OT-1* and *lckCre-mSin3A^{L/L}; OT-1* mice; a reduction from 16% and 19% to 2% and 3% CD8SP cells, respectively). Interestingly, of the two heterozygous deleted mice (*lckCre-mSin3A^{L/+}; OT-1*) used in this instance, the phenotype of T cells from the first (A) more closely resembled cells derived from a homozygous deleted genotype (only 7% CD8SP), while the second (B) was indistinguishable from wild type (23% CD8SP) (Fig. 6C, compare *lckCre-mSin3A^{L/+}; OT-1* A and B). Quantitative Western blotting (Fig. 6D) revealed that thymocytes from heterozygote A, displaying the strong reduction in CD8SP cells, had only 28% the amount of mSin3A compared to wild-type cells. By contrast, heterozygote B, which had wild-type levels of CD8SP cells, had 88% of wild-type mSin3A. The two *lckCre-mSin3A^{L/L}; OT-1* mice used for this experiment (A and B) had 16% and 8% the amount of wild-type mSin3A, respectively (Fig. 6D) and, consequently, the lowest levels of CD8SP cells. Thus, it appears that successful development of CD8SP cells is sensitive to the levels of mSin3A protein. We also observed that mSin3B protein levels were increased, compared to wild-type controls, from 1.2- to 2.3-fold in mice with reduced levels of mSin3A (Fig. 6D). However, there was no correlation between increased mSin3B levels and the proportion of CD8SP cells.

Thus, we conclude that mSin3A is required for the normal development of CD8SP T cells, in a dose-dependent manner.

Thymocytes lacking mSin3A have an intact T-cell signaling pathway but increased levels of apoptosis. To investigate potential mechanisms for the reduction of DP and CD8SP cells in T cells with *msin3A* deleted, we examined whether the expression of essential components of pre-TCR and TCR-dependent signaling pathways are altered by loss of mSin3A. To start, we analyzed expression of the adaptor proteins LAT and SLP76, the Syk-family PTK ZAP-70, and the Src-family protein tyrosine kinase p56^{LCK} (Fig. 7A), all of which are essential for normal T-cell development. A decrease in ZAP-70 (3a) or an increase in p56^{LCK} protein levels (23), for instance, might reduce the number of CD8SP. However, we found that the expression of these proteins was unaffected by the loss of

msin3A, compared to controls, when *msin3A* was deleted (Fig. 7B).

We next examined the expression of several nuclear proteins that likely function downstream of these signaling molecules. The transcriptional repressors Runx-1 and Runx-3 are required for the silencing of the CD4 locus and the development of CD8SP cells (62, 66). But again, we found that Runx-1 and Runx-3 protein levels in cells with *msin3A* deleted were also largely unchanged compared to controls (Fig. 7B and data not shown). Finally, since mSin3A is the central component of an HDAC-containing complex, we also wanted to assess whether acetylation of core histones was affected in *lckCre-mSin3A^{L/L}* cells. Western blot analysis showed that levels of H3K9Ac and H4K12Ac were unaltered in bulk histones by loss of mSin3A (Fig. 7B and data not shown). These results suggest that loss of mSin3A is not likely affecting T-cell development and proliferation by directly inhibiting the expression of membrane-proximal signaling molecules known to be important for T-cell development, or the nuclear proteins Runx1 and Runx3, which are important for the development of CD8 lineage cells.

We had previously shown that loss of mSin3A in immortalized MEFs results in an increase in sub-G₁ apoptotic cells (Fig. 3). We therefore asked whether the impairment of DP and CD8SP cells in *msin3A*-deficient thymocytes may be due in part to increased selective apoptosis during development. Consistent with this hypothesis, we observed an almost twofold increase in apoptosis of thymocytes with *msin3A* deleted, compared to heterozygous deleted and no-Cre controls (Fig. 7C; 4% to 7% annexin V-positive cells). We next analyzed individual thymocyte populations for apoptosis following 24 h in culture. We found that loss of mSin3A caused an increase in apoptosis in DP and CD8SP thymocytes compared to controls (Fig. 7D) while, in contrast, DN and CD4SP cell populations lacking *msin3A* showed no significant change in the percentage of apoptotic cells. Taken together with our previous results in mSin3A-deficient MEFs, these results suggest that the decrease in CD8SP cells we observed in *lckCre-mSin3A^{L/L}* mice is caused by a reduction in cell viability.

DISCUSSION

***msin3A* is an essential gene.** Sin3 orthologs, possessing highly conserved PAH and HID regions, are present in all eukaryotes from yeast to humans. Deletion of Sin3 in fission yeast (12) and fruit flies (43, 45) results in lethality. In this study, we demonstrate that *msin3A* is also an essential gene for mammalian development (Fig. 2). Because the highly related mSin3A paralog, mSin3B, is widely expressed (e.g., Fig. 6 and 7), our results suggest that these two genes are not functionally redundant. This is surprising, given that no clear differences between mSin3A and mSin3B in terms of binding and complex formation have been reported. The up-regulation of mSin3B in T cells with reduced mSin3A suggests that compensation does occur, albeit at a level insufficient to prevent phenotypic deficiencies (Fig. 6 and 7). It is likely that the presence of this close relative masks otherwise more prominent phenotypes, such as those observed when other components of the Sin3/HDAC complex have been deleted. Mouse knockout studies for mSDS3 (13) and HDAC1 (32) reveal similarities and a few discrepancies with our mSin3A knockout data. Loss of any one

FIG. 7. Thymocytes lacking mSin3A have an intact T-cell signaling pathway but increased levels of apoptosis. (A) Schematic diagram showing relative position of signaling molecules and nuclear factors in T-cell receptor-dependent signaling pathway. (B) Western blot analysis was used to determine the relative expression levels of the indicated proteins from the thymocyte protein extracts specified. (C) Thymocytes derived from mice with the indicated genotype were analyzed for apoptosis by staining with annexin V-FITC and 7-aminoactinomycin (7-AAD) followed by flow cytometric analysis. A representative histogram is shown. (D) Thymocytes of the indicated genotype were isolated and then cultured ex vivo in Dulbecco's modified Eagle's medium for a 24-h period before being analyzed for apoptosis. Apoptosis of individual thymocyte populations was analyzed by costaining with anti-CD4 and anti-CD8 antisera followed by annexin V. Each assay was performed in triplicate. A representative histogram is shown.

of the three genes results in early embryonic lethality at the postimplantation stage. Moreover, an increase in the ratio of viable wild-type to heterozygous pups in the knockouts suggests that haploinsufficiency can also be detrimental. The apparent cause of lethality in all three instances is a marked reduction in proliferative potential.

In three independent systems, cultured blastocysts (Fig. 2), MEFs (Fig. 3), and mature T cells (Fig. 5), we observed that a reduction in mSin3A correlated with decreased cell division. However, while the doubling time of these cells was increased, flow cytometric analysis revealed only a subtle change in cell cycle profile, suggesting that loss of mSin3A may affect changes

in both G₁ and G₂/M phases (Fig. 3). Acute deletion of *msin3A* in MEFs (CreGFP-*Sin3A*^{L/L}) produces a modest increase in the levels of CDK inhibitors p21 and p27 compared to control cells (see Fig. S1A in the supplemental material), possibly accounting for a delay in G₁ phase similar to that observed in cells with HDAC1 deleted (32). On the other hand, cells lacking mSDS3 develop a G₂/M arrest due to defective chromosome segregation caused by aberrant acetylation of pericentric heterochromatin (13). Similar findings were also obtained in *Schizosaccharomyces pombe* cells lacking the Sin3 ortholog, Pst1p (55). However, our examination, using immunofluorescence, of the pericentric heterochromatin in cells with *msin3A*

deleted indicated that it was still present in a hypoacetylated state (see Fig. S1B in the supplemental material). There was also no evidence of aneuploidy, equivalent to that observed in mSDS3-disrupted cells, in CreGFP-*Sin3A*^{L/L} MEFs (Fig. 3C and D). A strong chromatin phenotype such as this might only be expected to only occur upon deletion of both *msin3* genes, or by inactivation of a protein which mediates function of both mSin3 proteins, such as mSDS3. We did, however, detect a reduced number of distinct HP1 α foci in CreGFP-*Sin3A*^{L/L} MEFs compared to control cells (Fig. 3), consistent with previous reports (67). HP1 α is thought to be targeted to heterochromatin via numerous transient interactions (for review, see reference 38). Perhaps the best characterized of these involves binding to trimethylated lysine 9 on histone 3 (H3-K9). In this regard, it is interesting that mice null for the Sin3A-associated H3-K9 methyltransferase ESET (68) die at a peri-implantation stage (14), similar to *msin3A*^{-/-} animals. HP1 α localization can also be modified by prolonged exposure to the HDAC inhibitor trichostatin A, which causes histone hyperacetylation without a global change in histone methylation (59). These findings suggest that one of the targets of trichostatin A might be the mSin3/HDAC complex. However, loss of mSin3A—unlike HDAC1, but similar to mSDS3—does not cause a change in bulk histone acetylation (Fig. 7 and data not shown) (12, 55). It therefore seems unlikely that loss of mSin3A would cause a global change in gene expression. Indeed, only 3% of genes (399/13,137) were found to have altered expression, the majority with less than a twofold change, when microarray studies were performed in *Drosophila* cells with reduced dSin3 (47).

We propose that loss of *msin3A* causes multiple effects, including mislocalization of the heterochromatin-associated protein HP1 α and induction of cyclin-dependent kinase inhibitors, which result in a reduction of cellular proliferation. While we did not observe a global increase in histone acetylation, loss of mSin3A may lead to a more subtle disruption of localized regions of heterochromatin that are perhaps reflected in the observed alterations of HP1 α binding. Such changes may ultimately have a deleterious effect on regulated gene expression during proliferation and development. The embryonic lethality we observe peri-implantation is thus likely due to a failure in proliferation once maternal stores of mSin3A have been depleted. Our conclusions are in general agreement with a second, independently generated *msin3A* null mouse which dies in early development and whose MEFs exhibit reduced proliferation and altered HP1 α localization but not hypoacetylation of pericentric heterochromatin or altered H3K9 trimethylation (Dannenberg et al., submitted).

Systemic reduction in mSin3A alters peripheral lymphoid homeostasis and causes membranous glomerulopathy. Constitutive *msin3A*^{+/-} mice with splenomegaly have an expanded total number of CD4 T cells and B cells. Thymocytes derived from these mice have a twofold reduction in mSin3A protein compared to wild-type and unaffected heterozygote animals (Fig. 4D). Thus, the affected *msin3A*^{+/-} mice appear unable to compensate for the loss of one *msin3A* allele. However, we cannot be certain whether this is sufficient to generate the disruption in lymphoid homeostasis. In all likelihood, the mixed 129/Sv and C57BL/6J genetic background of the *msin3A*^{+/-} mice also plays a role in modifying the severity and

penetrance of the phenotype. We are in the process of backcrossing the *msin3A*^{+/-} mice to generate pure 129/Sv and C57BL/6J lines to further explore these phenomena. In contrast, T-cell-specific disruption of *msin3A* in lckCre-*msin3A*^{L/L} animals does not result in splenomegaly, we presume this is either because the lymphoproliferative phenotype observed in the constitutive deletion is not T-cell autonomous or because the near-complete deletion of *sin3A* during T-cell development results in strong selection for mature T cells that can compensate for reduced mSin3A. Nonetheless, we observed a reduced population of CD8SP T cells in both systems (Fig. 4F and 6A), leading us to conclude that a reduction in mSin3A protein levels curtails cytotoxic T-cell development.

Flow cytometric and terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling analyses of enlarged spleens from *msin3A*^{+/-} mice reveal normal levels of FasL and significant levels of apoptotic cells (data not shown), suggesting that the splenomegaly phenotype we observe is not due to a defect in the Fas pathway, as occurs in lpr or gld mice, which lack Fas or FasL (1, 60, 64). Rather, the splenomegaly phenotype of *msin3A*^{+/-} mice, combined with the impairment in both pre-TCR signaling and TCR-induced proliferation, is reminiscent of mice deficient in the LAT adaptor (56). Despite impaired development of DP cells and impaired T-cell proliferation in response to TCR stimulation, LAT-deficient mice later develop a lymphoproliferative disorder characterized by an expansion of CD4 cells with a T helper 2 IL-4-secreting cytokine profile. Since both LAT and Sin3A are expressed in B and T cells, the lymphoproliferative phenotypes in LAT or *sin3A*^{+/-} mice may reflect defects in both B and T cells (as well as additional cell types), while T-cell-specific disruption of Sin3 in lckCre-*sin3A*^{L/L} animals does not result in splenomegaly, presumably because the lymphoproliferative phenotype is not T-cell intrinsic. Mice heterozygous for *sin3A* also develop membranous glomerulopathy, which is also consistent with the expansion of Th2 cytokine-secreting CD4 T cells, which would stimulate B cells and plasma cells to produce excessive antibodies that deposit in the kidney glomeruli.

Regulation of T-cell differentiation by chromatin-modifying factors. A growing number of transcriptional regulators and chromatin remodeling factors have been implicated in T-cell development. The Swi/Snf complex proteins Brg1 and BAF57 act in a reciprocal manner to silence and activate the CD4 and CD8 loci, respectively, in immature cells (9, 10, 17). The transcriptional regulator Ikaro, which associates with mSin3A (30), also plays a role in activating the CD8 locus (20; see reference 18 for review). Conversely, Mi-2 β , a component of the NuRD complex, is required to initiate CD4 expression during the DN-to-DP transition (65), while the repressors Runx-1 and -3 are required to silence it in immature T cells and mature CD8SP cells, respectively (62, 66). Underscoring the important role played by transcriptional regulation, we have found that selective deletion of *msin3A* in developing thymocytes causes a reduction in the total number and proportion of cytotoxic T cells (Fig. 6A). This novel function of mSin3A is likely to occur downstream of the TCR-dependent signaling pathway, because deletion of *msin3A* was able to block the accumulation of CD8SP cells even in the presence of a transgenic MHC class I-restricted TCR (Fig. 6C). By taking advantage of the variability in mSin3A expression levels in

lckCre-Sin3A^{L/+} thymocytes, we were able to demonstrate that small decreases in mSin3A protein did not effect CD8SP development, while larger reductions produced a concomitant decrease in the level of CD8SP cells (Fig. 6C).

Thymocytes from a number of *lckCre-msin3A^{L/L}* mice expressed a truncated form of mSin3A which lacks a wild-type N terminus (Fig. 5B; determined by Western blotting using antisera raised to different portions of the mSin3A molecule). This protein is probably generated through an internal initiation event, since the first coding exon (exon 2) has been deleted. T cells undergo a proliferative expansion as they progress from the DN to DP stage and are therefore prone to clonal expansion. Thus, a small percentage of cells expressing a truncated form of mSin3A may be selected. Since mSin3A is required for cellular proliferation, it seems likely that this truncated form of mSin3A is more likely to be a hypomorphic form of the protein rather than dominant negative. In general, much less of the truncated mSin3A protein was detected than the wild type, and loss of all forms of the protein correlated with a stronger phenotype.

Our analysis of protein extracts from purified populations of wild-type DN, DP, CD4SP, and CD8SP cells revealed that they all express equivalent amounts of mSin3A (data not shown). Therefore, it is the selective recruitment of mSin3A by other finely regulated transcription factors, and not regulation of mSin3A protein levels per se, that is likely to be important. Only in the context of *lckCre-msin3A^{L/L}* mice, where mSin3A levels become limiting, do we observe a reduction in the number of CD8SP cells. We believe that mSin3A function is necessary for the viability CD8SP cells, since a reduction in the level of mSin3A protein causes an increase in the percentage of apoptotic cells and thus a concomitant decrease in CD8SP cell numbers. Work is in progress to try and discover the mechanism by which CD8SP cells with *msin3A* deleted are more susceptible to apoptosis while CD4SP cells are not.

In summary, we have demonstrated an essential role for mSin3A in embryogenesis and the development of T cells. The impairment of thymic DP and CD8 T-cell development in *lckCre-msin3A^{L/L}* mice, and also the alteration in splenic lymphoid homeostasis in *msin3A^{+/-}* mice, suggests that *msin3A* is a pleiotropic gene required not only for cell cycle progression but also for the proper development and homeostasis of lymphoid cells.

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ADDENDUM

Dannenberg et al. (12a) have also found that targeted deletion of *msin3A* produces early embryonic lethality and inhibits proliferation and survival of fibroblasts in culture.

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