Survivin Loss in Thymocytes Triggers p53-mediated Growth Arrest and p53-independent Cell Death

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Abstract

Because survivin-null embryos die at an early embryonic stage, the role of survivin in thymocyte development is unknown. We have investigated the role by deleting the *survivin* gene only in the T lineage and show here that loss of survivin blocks the transition from CD4$^-$ CD8$^-$ double negative (DN) thymocytes to CD4$^+$ CD8$^+$ double positive cells. Although the pre–T cell receptor signaling pathway is intact in survivin-deficient thymocytes, the cells cannot respond to its signals. In response to proliferative stimuli, cycling survivin-deficient DN cells exhibit cell cycle arrest, a spindle formation defect, and increased cell death. Strikingly, loss of survivin activates the tumor suppressor p53. However, the developmental defects caused by survivin deficiency cannot be rescued by p53 inactivation or introduction of Bcl-2. These lines of evidence indicate that developing thymocytes depend on the cytoprotective function of survivin and that this function is tightly coupled to cell proliferation but independent of p53 and Bcl-2. Thus, survivin plays a critical role in early thymocyte development.

Key words: pre–T cell • cell death • development • thymus • mitosis

Introduction

T lymphocyte development in thymus is a complex process involving distinct stages of proliferation and cell death. These stages are defined by the expression of specific cell surface markers, commencing with CD4$^-$ CD8$^-$ double negative (DN) cells, which proliferate and differentiate into CD4$^+$ CD8$^+$ double positive (DP) cells. DP thymocytes then undergo a culling process involving induced cell death that results in populations of terminally differentiated CD4$^+$ or CD8$^+$ single positive (SP) cells. The SP thymocytes are released from the thymus to become mature peripheral T cells. DN thymocytes are not only classified by their lack of expression of CD4 and CD8, but also by their differential expression of CD44 (Pgp-1) and CD25 (IL-2 receptor α chain): DN1 (CD44$^+$ CD25$^-$), DN2 (CD44$^+$ CD25$^+$), DN3 (CD44$^-$ CD25$^+$), and DN4 (CD44$^-$ CD25$^-$; reference 1). The DN3 stage is then subdivided according to CD44/CD25 expression or cell size: DN3E (CD44$^-$ CD25^{high} small) and DN3L (CD44$^-$ CD25^{low} large). Productive rearrangement of the TCRβ locus takes place during the DN3 to DN4 transition and leads to pre-TCR expression. Only cells expressing a functional pre-TCR undergo exponential expansion, a process referred to as β-selection (2–4).

Survival signals in early thymocyte progenitors are mediated mainly by IL-7 and stem cell factor. IL-7 prevents CD25$^+$ pre–T cells from undergoing cell death by up-regulating expression of the antiapoptotic gene Bcl-2 (5–7). Once a functional pre-TCR is formed at the DN3 stage, however, the pre-TCR takes over and provides survival signals allowing pre–T cells to advance to the DP stage. It remains unclear precisely which downstream signaling molecules are mobilized by the pre-TCR to control survival. Bcl-2 does not

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Abbreviations used in this paper: 7AAD, 7-amino actinomycin D; DN, double negative; DP, double positive; ERK, extracellular signal–regulated kinase; ES, embryonic stem; HSA, heat stable antigen; Lin, lineage marker; SP, single positive.

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appear to play as important a role as in the early progenitor stage because Bcl-2 expression decreases through the DN3L to DN4 transition (8) and is rarely detected in DN4 cells (9, 10). Several lines of evidence also suggest that a checkpoint dependent on the tumor suppressor p53 exists in pre–T cells to inhibit premature differentiation of thymocytes before expression of the pre–TCR complex. Induction of p53 deficiency in CD3γ-deficient (11), RAG-deficient (12), or SCID mice (13–15), all of which lack a functional pre-TCR, rescues the block in DN thymocyte development. Therefore, it has been proposed that p53 may act as a sensor for β-selection. Only thymocytes that express a functional pre-TCR succeed in inactivating the p53-induced cell death pathway and go on to the DP stage.

The cycling and division of almost all cell types are tightly regulated by the controlled activation and inactivation of precise checkpoint mechanisms. Many types of cellular stresses trigger p53, which in turn activates both the G1/S and G2/M cell cycle checkpoints (16, 17). However, damage to the mitotic spindle triggers the p53-independent spindle checkpoint, halting the cell cycle at the metaphase-anaphase transition (18). Cells with wild-type p53 and its target gene p21 then undergo a form of G_1 arrest characterized by resistance to antimicrotubule agents (19, 20). In contrast, p53-deficient cells do not arrest in G_1 and instead endoreduplicate their DNA, resulting in cell death due to aberrant mitosis (21, 22).

Survivin is a member of the inhibitor of apoptosis family (23) but its direct role in apoptosis is still controversial. For example, survivin displays no ability to suppress caspase-3 activity in vitro and does not bind procaspase-9 (24). Survivin is not highly expressed in most adult tissues, but this protein becomes prominent in fast-growing cells such as transformed cell lines and human cancers (23). Accordingly, survivin expression is regulated in a cell cycle–dependent fashion (25, 26). Immunohistochemical analysis has shown that survivin associates with mitotic spindle microtubules, centromeres, and intracellular mid-bodies (27, 28). Recent studies have also shown that survivin is a chromosomal passenger protein that regulates chromosome segregation by interacting with other passenger proteins, such as inner centromere protein and Aurora-B kinase (29). Inactivation of mammalian survivin or its orthologues in lower organisms results in cytokinesis abnormalities (30–32), particularly spindle defects (33).

Because survivin-null embryos die at an early embryonic stage (28, 34), conventional knockout mice cannot provide any information on the role of survivin in organogenesis. To address whether survivin requirement is tissue specific, we have generated conditional knockout mice in which the *survivin* gene is deleted only in the T lineage. This is also an ideal tool to investigate whether survivin directly regulates apoptosis. We found that these animals had few peripheral T cells or SP or DP thymocytes because thymocyte development was impaired at the DN3 to DN4 transition. Mislocalization of proteins crucial for spindle formation was evident in mutant thymocytes. Ex vivo analysis revealed that survivin-deficient thymocytes at the DN3 to DN4 transition underwent cell cycle arrest and cell death. Although loss of survivin induced p53 and p21, neither p53 loss nor introduction of a Bcl-2 transgene could restore the development of survivin-deficient DN3 thymocytes. These results demonstrate that survivin plays an essential role in early thymocyte development and that the cytoprotective function of survivin cannot be replaced by overexpression of Bcl-2 or loss of p53.

Materials and Methods

Generation of Lck-Cre;survivin$^{flox/flox}$ Mice. Three independent overlapping genomic *survivin* clones were isolated from a 129/Sv library and used to construct a targeting vector (see Fig. 1) that was electroporated into E14K embryonic stem (ES) cells (129/Ola). Homologous recombinants were used to generate chimeric mice and survivin$^{flox/flox}$ mice after the removal of the *neo* cassette by transient expression of Flpe recombinase in vitro (35). Germline transmission was confirmed by Southern blot analysis of tail DNA as previously described (36). The primer sequences for genomic DNA PCR are available upon request.

Histological Analysis. Thymi were fixed overnight at 4°C in freshly prepared 4% paraformaldehyde/PBS and processed for histology. Serial sections were stained with hematoxylin and eosin using standard protocols.

Flow Cytometric Analysis and Cell Sorting. Anti-CD4, CD8, CD25, CD44, B220, CD11c, NK1.1, TCR$\alpha\beta$, TER-119, Mac-1, Gr-1, and heat stable antigen (HSA) mAbs were from BD Biosciences. These mAbs were directly coupled to FITC, PE, allophycocyanin, or biotin. Surface marker expression by thymocytes and peripheral T cells was analyzed using a flow cytometer (FACSCalibur™; Becton Dickinson) and CELLQuest™ software according to standard protocols. Intracellular staining was performed using the Cell Fixation/Permeabilization Kit (BD Biosciences).

Cell sorting was performed by FACS Vantage™ (Becton Dickinson). For DN thymocytes, CD4$^+$ CD8$^+$ cells and B220$^+$ cells were depleted using a mixture of CD4/CD8/B220 magnetic beads (Dynal). After two rounds of depletion, the cells were preincubated with Fc-Block (BD Biosciences) and stained with mAbs. DN3E cells were taken as HSA$^+$, lineage marker (Lin)$^-$ (CD4, CD8, B220, CD11c, NK1.1, TCR$\gamma\delta$, TER-119, Mac-1, Gr-1), and small in size. DN3L cells were HSA$^+$, Lin$^-$, and large in size. DN2 cells were Lin$^-$ CD25$^+$ CD44$^+$, whereas DN4 cells were Lin$^-$ CD25$^-$ CD44$^-$.

Cell Cycle Analysis. Cell cycle analysis of thymocytes was performed using the BrdU Flow Kit (BD Biosciences) as previously described (37). Mice were intraperitoneally injected with 1 mg BrdU (Sigma-Aldrich) 2 h before sample preparation. Cells were prepared, stained with cell surface markers followed by anti-BrdU Abs and 7-amino actinomycin D (7AAD; BD Biosciences), and analyzed by flow cytometry. At least 3,000 cells of each DN population were collected.

Western Blot Analysis. DN thymocytes were stimulated with anti–CD3ϵ-biotin (BD Biosciences) and avidin (Sigma-Aldrich) as previously described (38). Protein lysates were subjected to Western blotting using Abs against phospho–extracellular signal–regulated kinase (ERK)1/2, ERK1/2 (both from New England Biolabs, Inc.), or actin (Sigma-Aldrich) according to standard protocols. Anti-p53 (CM5; Novocastra; reference 39) and anti-survivin Ab (27) were used as previously described.

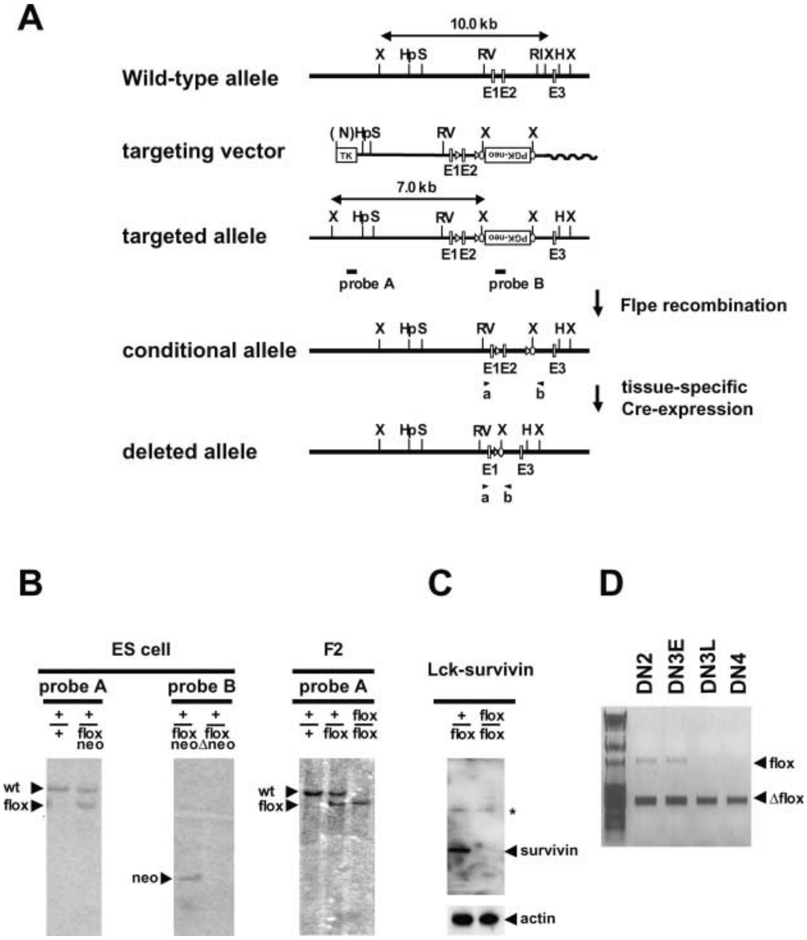

Figure 1. Tissue-specific targeted disruption of the *survivin* locus. (A) A portion of the murine wild-type *survivin* locus (top) showing exons 1–3 (open boxes) and a 10-kb XbaI fragment. The targeting vector was designed to generate floxed exon2 (loxP; triangles), flank the PGK-*neo* cassette with FRT sequences (ovals), and introduce a new XbaI site (X). The targeted allele contains a diagnostic 7.0-kb XbaI fragment. To generate the conditional allele, the *neo* cassette was removed by transient expression of Flpe recombinase. Cre-mediated recombination resulted in the deleted allele. The positions of the 5′ flanking probe (A) and *neo* probe (B) used for genotyping are indicated. (B) Southern blot analyses to identify survivin$^{flox/+}$ ES cells containing *neo* (left), ES cells with *neo* removed (middle), and F2 pups (right). Genomic DNA was digested with XbaI and hybridized with probe A or B. (C) Western blot of survivin protein in Lck-survivin$^{flox/+}$ and Lck-survivin$^{flox/flox}$ DN thymocytes. Wild-type survivin protein (arrowhead) and a nonspecific band (asterisk) are indicated. Actin, loading control. (D) Detection of deleted survivin allele in DN thymocyte subpopulations. The floxed (flox) and deleted (Δflox) survivin alleles were amplified by PCR using primers a and b shown in A. Genomic DNA samples were prepared from the indicated DN subsets.

RT-PCR Analysis. Total RNA was extracted from thymocytes using TRIzol (Life Technologies). cDNA was generated with the SuperScript First-Strand Synthesis System (Invitrogen). The primer sequences for RT-PCR are available upon request.

Immunofluorescence Microscopy. Cells were fixed in cold methanol or in freshly prepared 4% paraformaldehyde/PBS and permeabilized in 0.2% Triton X-100/PBS. Alternatively, cells were preextracted with 0.2% Triton X-100 in PHEM buffer (80 mM Pipes, 20 mM Hepes, 1 mM EGTA, and 2 mM $MgCl_2$, pH 6.8). Samples were simultaneously fixed and extracted with 0.5% Triton X-100, 3.7% formalin, and 0.25% glutaraldehyde in PHEM buffer. Slides were blocked in 0.5% BSA/0.02% glycine/PBS and labeled with primary and secondary antibodies. Hoechst 33258 (Molecular Probes) was used to stain DNA. Images were obtained using an Olympus 1X-70 inverted microscope and Deltavision Deconvolution Microscopy software (Applied Precision). The following primary antibodies were used: mouse anti–bovine α-tubulin (Molecular Probes), mouse–anti IAK/Aurora-A kinase and mouse anti–AIM/Aurora-B kinase (Transduction), rabbit anti–pericentrin (Covance), and mouse anti–phospho-histone H3 (Upstate Biotechnology). The following secondary antibodies were used: Oregon green 488 goat anti–rabbit IgG, Oregon green 488 goat anti–mouse IgG, Alexa Fluor 594 goat anti–mouse IgG, and Texas red goat anti–rabbit IgG (Molecular Probes).

Apoptosis Assays. Evaluation of thymocyte apoptosis in vitro was performed using the Apoptosis Detection Kit (R&D Systems) as previously described (36).

Results

Generation of Thymocyte-specific Survivin-deficient Mice. We constructed a *survivin*-targeting vector in which the *neo* cassette was flanked by FRT sequences and *survivin* exon 2 was flanked by loxP sequences (Fig. 1 A). Therefore, Cre-mediated removal of exon 2 resulted in an early frameshift and translation termination. The conditional targeting vector was used to generate two independent ES cell lines carrying a floxed *survivin* exon 2 and a flrted *neo* gene (Fig. 1 B). Transient transfection of these clones with Flpe recombinase (35, 40) resulted in successful recombination that removed *neo* (Fig. 1 B). Recombined ES clones were used for blastocyst injection followed by standard breeding steps to produce animals homozygous for the floxed survivin allele (survivin$^{flox/flox}$ mice). Survivin$^{flox/flox}$ mice were born at the expected Mendelian ratio and displayed no abnormalities (not depicted), indicating that genetic manipulation of the *survivin* gene did not interfere with survivin function. Survivin$^{flox/flox}$ mice were crossed with Lck-Cre transgenic mice (41) to generate thymocyte-specific survivin-deficient mice (Lck-survivin$^{flox/flox}$). The lack of survivin protein in DN thymocytes was demonstrated by Western blot analysis of survivin protein levels (Fig. 1 C). In addition, genomic DNA prepared from sorted DN cells was analyzed by PCR for the detection of the floxed or deleted (Δfloxed) survivin

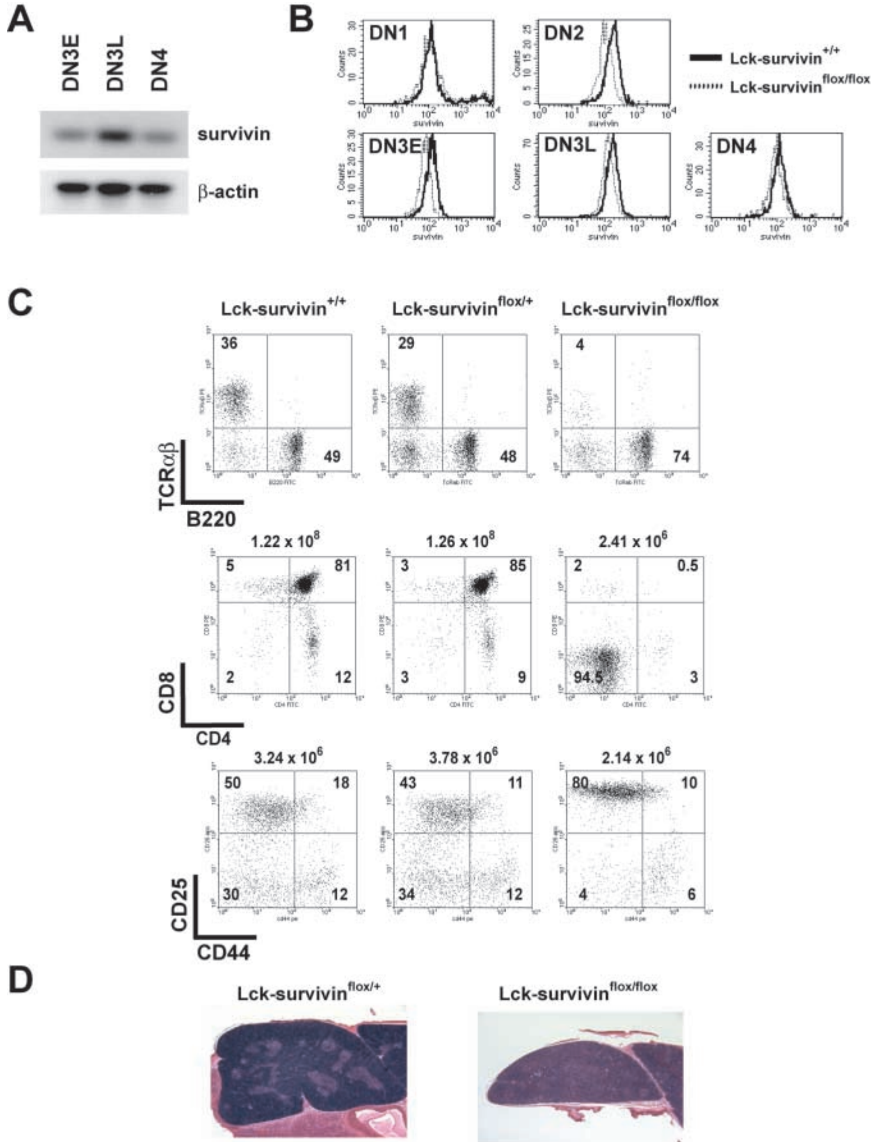

Figure 2. Flow cytometric and histological analyses of peripheral T cells and thymi of Lck-Cre;survivin$^{flox/flox}$ mice. (A) Survivin mRNA expression in DN cells. Levels of survivin mRNA were analyzed in the indicated DN subsets by RT-PCR. RNA was prepared from DN cells sorted from wild-type thymocytes. (B) Survivin protein expression in DN cells. Surface staining of Lck-survivin$^{+/+}$ (thick line) and Lck-survivin$^{flox/flox}$ (dotted line) thymocytes with anti-CD25, anti-CD44, and anti-Lin Abs was followed by intracellular staining with anti-survivin Ab. Lin^{+} cells in the indicated thymocyte subpopulations were electrically gated out. (C) Impaired development of survivin-deficient peripheral T cells and DP thymocytes. Peripheral blood cells from Lck-survivin$^{+/+}$, Lck-survivin$^{flox/+}$, and Lck-survivin$^{flox/flox}$ mice were stained with anti-TCRαβ and anti-B220 (top). Thymocytes were stained with anti-CD4 and anti-CD8 (middle), or anti-CD25 and anti-CD44 (bottom), and the Lin^{-} population was examined. (D) Reduced size and cellularity of the thymus in Lck-survivin$^{flox/flox}$ mice. Hematoxylin and eosin staining of transverse sections of thymus from Lck-survivin$^{flox/+}$ (left) and Lck-survivin$^{flox/flox}$ (right) mice. The mutant thymus is much smaller and lacks the typical cortex medulla structure.

allele. The floxed allele was deleted in the majority of DN2 and DN3E cells and almost all DN3L and DN4 cells in the mutants (Fig. 1 D).

Defects in Thymocyte Development in Lck-survivin$^{flox/flox}$ Mice. We first compared the level of survivin expression in DN subpopulations in mutant and control mice. RT-PCR analysis showed that survivin mRNA was maximally expressed at the DN3L stage in control cells (Fig. 2 A). Flow cytometric analysis demonstrated that compared with Lck-survivin$^{+/+}$ cells, intracellular survivin expression was decreased in Lck-survivin$^{flox/flox}$ cells starting at the DN2 stage (Fig. 2 B). This time frame is consistent with Lck proximal promoter activity (42).

Next, we determined numbers of peripheral T cells and thymocyte subpopulation proportions in Lck-survivin$^{flox/+}$ and Lck-survivin$^{flox/flox}$ mice at 6–8 wk of age. Flow cytometric analysis revealed that the number of peripheral TCRαβ^{+} cells was dramatically reduced in Lck-survivin$^{flox/flox}$ mice (Fig. 2 C, top). The average number of thymocytes recovered from Lck-survivin$^{flox/flox}$ mice was $2.41 \pm 0.5 \times 10^6$ ($n = 6$), ~2.0% of the number of thymocytes in Lck-survivin$^{+/+}$ mice ($1.22 \pm 0.6 \times 10^8$; $n = 6$). Moreover, ~95% of Lck-survivin$^{flox/flox}$ thymocytes were DN cells (Fig. 2 C, middle), suggesting that the loss of survivin affected either the DN to DP transition or the production of DN cells. To distinguish between these possibilities, we stained DN cells from control and mutant mice with the early developmental markers CD25 and CD44 (Fig. 2 C, bottom). DN3 (CD25^{+} CD44^{-}) cells represented ~80% of Lck-survivin$^{flox/flox}$ DN cells compared with <50% of DN cells in Lck-survivin$^{+/+}$ or Lck-survivin$^{flox/+}$ mice. DN4 (CD25^{-} CD44^{-}) cells were reduced to 4% in Lck-survivin$^{flox/flox}$ mice compared with 30 and 34% in Lck-survivin$^{+/+}$ and Lck-survivin$^{flox/flox}$ mice, respectively.

Histological findings confirmed the block in the DN3 to DN4 transition in the absence of survivin. Thymi in Lck-

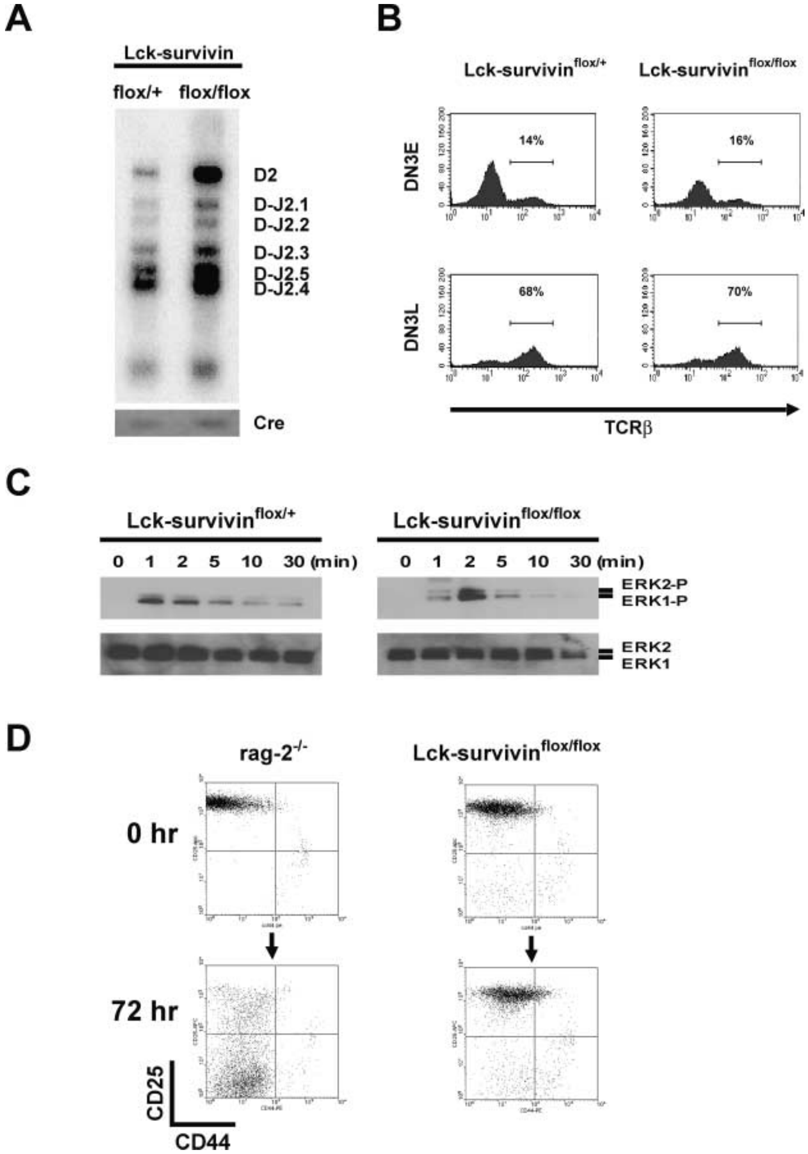

Figure 3. Normal TCRβ gene rearrangement and pre-TCR signaling in Lck-Cre;survivin$^{flox/flox}$ thymocytes. (A) Normal TCRβ D-J rearrangement. TCRβ Dβ2-Jβ2 recombination in Lck-survivin$^{flox/+}$ and Lck-survivin$^{flox/flox}$ DN thymocytes was examined by PCR followed by Southern blotting with ^{32}P-labeled oligonucleotide probes as previously described (reference 59). The *Cre* gene was amplified as an internal control for PCR. (B) Normal TCRβ protein expression. DN3E and DNL3 thymocytes from Lck-survivin$^{flox/+}$ and Lck-survivin$^{flox/flox}$ mice were surface stained with anti-CD25, anti-CD44, and anti-Lin followed by intracellular staining with TCRβ and flow cytometric analysis. (C) Normal MAPK activation. Lck-survivin$^{flox/+}$ and Lck-survivin$^{flox/flox}$ DN thymocytes were treated with anti–CD3ε-biotin followed by cross-linking with avidin for the indicated times. Protein lysates were subjected to Western blot analysis using anti–phospho-ERK Abs (top). To control for loading, the blot was stripped and reprobed with anti–ERK Ab (bottom). (D) Impaired in vivo response of survivin-deficient thymocytes to anti-CD3ε. RAG-2$^{-/-}$ (left) and Lck-survivin$^{flox/flox}$ (right) mice were intraperitoneally injected with 100 mg anti-CD3ε. After 72 h, thymocytes were prepared and stained with anti-CD25, anti-CD44, and anti-Lin Abs, and then subjected to flow cytometry. Unlike RAG-2$^{-/-}$ DN3 cells, Lck-survivin$^{flox/flox}$ DN3 cells failed to advance to DN4. Data shown are representative of three independent experiments.

survivin$^{flox/+}$ mice had a typical structure and contained a cortex (Fig. 2 D, left, dark purple area) with immature T cells and a medulla (Fig. 2 D, left, light purple area) with smaller DP cells. In contrast, thymi of Lck-survivin$^{flox/flox}$ mice were much smaller and lacked cortex medulla compartmentalization (Fig. 2 D, right). The vast majority of thymic cells in the mutants were large and stained light purple, which are characteristics of immature thymocytes. These results indicate that in the absence of survivin, DN thymocytes fail to expand and progress to the DP stage.

Normal TCRβ Gene Rearrangement and In Vitro Pre-TCR Signaling in Survivin$^{flox/flox}$ DN Thymocytes. The accumulation of DN3 thymocytes in Lck-survivin$^{flox/flox}$ mice was strikingly similar to that in RAG-1 (43), RAG-2 (44), TCRβ (45), and Lck (46) knockout mice. Therefore, we analyzed the rearrangement of the TCRβ locus, a requirement for the DN3 to DN4 transition. Analysis of DN thymocyte DNA by PCR and Southern blotting showed that somatic recombination of Dβ2-Jβ2 gene segments was not altered in survivin-deficient thymocytes (Fig. 3 A). Furthermore, flow cytometry revealed no differences in intracellular TCRβ protein between DN3E and DN3L cells of control and mutant mice (Fig. 3 B).

The pre-TCR signaling pathway necessary for the DN3 to DN4 transition can be activated in vitro by ligating CD3ε on the surface of DN cells (47). This activation can be assessed by determining the extent of phosphorylation of the ERKs, downstream targets of pre-TCR signaling (38). DN thymocytes from Lck-survivin$^{flox/flox}$ and Lck-survivin$^{flox/+}$ mice were activated using anti-CD3ε Ab followed by detection of ERK activation via anti–phospho-ERK Abs. As shown in Fig. 3 C, ERK1 and ERK2 were phosphorylated equally and with comparable kinetics in control and mutant DN cells. Intraperitoneal injection of anti-CD3ε Ab can mimic pre-TCR signaling in vivo and induce DN cells to proliferate and differentiate into DP

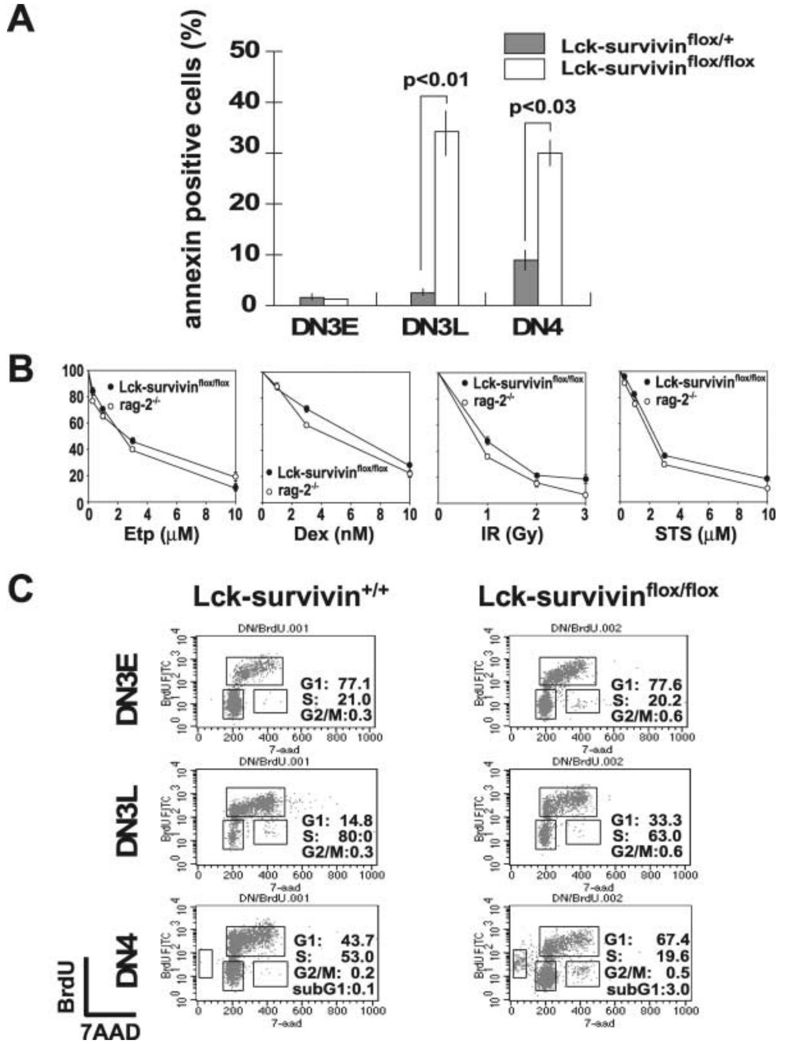

Figure 4. Effect of survivin loss on cell death in vivo and in vitro and on thymocyte proliferation. (A) Increased cell death of proliferating survivin-deficient DN cells. DN thymocytes were prepared ex vivo and stained with anti-CD25, anti-CD44, and anti-Lin Ab followed by annexin. The average number of annexin$^+$ cells in each DN subpopulation was assessed by flow cytometry. Results shown are mean ± SD. Annexin$^+$ cells were significantly increased in Lck-survivin$^{flox/flox}$ DN3L and DN4 populations (ANOVA; $n = 3$). (B) Normal susceptibility of resting survivin-deficient DN cells to various apoptotic stimuli. Lck-survivin$^{flox/flox}$ and RAG-2$^{-/-}$ DN3E cells were treated with etoposide (Etp), dexamethasone (Dex), γ-irradiation (IR), and staurosporine (STS) at the indicated doses. Cell death was evaluated as described in Materials and Methods. Cell viability was normalized to account for spontaneous cell death. Triplicate samples of each treatment in three independent experiments were assayed. Results shown are mean ± SD. (C) Impaired proliferation in vivo in the absence of survivin. Lck-survivin$^{flox/+}$ and Lck-survivin$^{flox/flox}$ mice were injected with 1 mg BrdU and DN thymocytes were purified and stained with anti-CD25, anti-CD44, anti-BrdU, and 7AAD. The percentages of cells in the G1, S, and G2/M phases were measured by flow cytometry for the indicated DN subsets. Results shown are representative of three independent experiments.

cells in RAG-2$^{-/-}$ mice in the absence of pre-TCR signaling (48). Therefore, we treated Lck-survivin$^{flox/flox}$ and RAG-2$^{-/-}$ mice with anti-CD3ε Ab and isolated DN thymocytes 72 h after injection. Although anti-CD3ε Ab induced the differentiation and proliferation of DN3 cells in RAG-2$^{-/-}$ mice, it failed to do so in Lck-survivin$^{flox/flox}$ mice (Fig. 3 D). These results indicate that the loss of survivin does not affect TCRβ rearrangement, intracellular TCRβ expression, or the pre-TCR signaling pathway itself. However, the DN3 to DN4 transition cannot be rescued by a surrogate pre-TCR signal in the absence of survivin, suggesting that survivin plays a critical role in highly proliferative cells.

Impaired Proliferation and Increased Cell Death of Lck-survivin$^{flox/flox}$ DN Thymocytes. Because the pre-TCR signaling required for DN cell proliferation was normal in Lck-survivin$^{flox/flox}$ thymocytes, we investigated whether the loss of DN4 cells in the mutant mice was due to impaired proliferation or increased apoptosis or both. Annexin V staining for cell viability in vivo showed that 37% of DN3L and 32% of DN4 cells were annexin$^+$ in Lck-survivin$^{flox/flox}$ mice (Fig. 4 A). In contrast, only 5–7% cells were annexin$^+$ in control mice, indicating that loss of survivin induces apoptosis of DN3L and DN4 thymocytes.

To test whether loss of survivin impaired cellular responses to external apoptotic stimuli, we subjected purified DN cells from Lck-survivin$^{flox/flox}$ and RAG- 2$^{-/-}$ mice to treatment with etoposide (Fig. 4 B, Etp), dexamethasone (Dex), γ-irradiation (IR), or staurosporine (STS). About 90% of thymocytes in both mutant strains are DN3E cells. When apoptosis was evaluated at 16 h after treatment, no significant differences in the numbers of apoptotic cells were observed under any conditions tested (Fig. 4 B). These results indicate that survivin is not essential in DN3E cells for the execution of apoptosis in response to various external stimuli in vitro. However, a failure in survivin function triggers the death of proliferating cells in vivo.

To examine the effect of survivin loss on the cell cycle, DN thymocytes were pulse labeled in vivo with BrdU and stained with anti-BrdU Ab and 7AAD in vitro (Fig. 4 C). The cell cycle profile of DN3E cells was comparable in Lck-survivin$^{+/+}$ and Lck-survivin$^{flox/flox}$ mice (Lck-survivin$^{+/+}$: G1, 77.1%; S, 21.0%; G2/M, 0.3% vs. Lck-survivin$^{flox/flox}$: G1, 77.6%; S, 20.2%; G2/M, 0.6%). However,

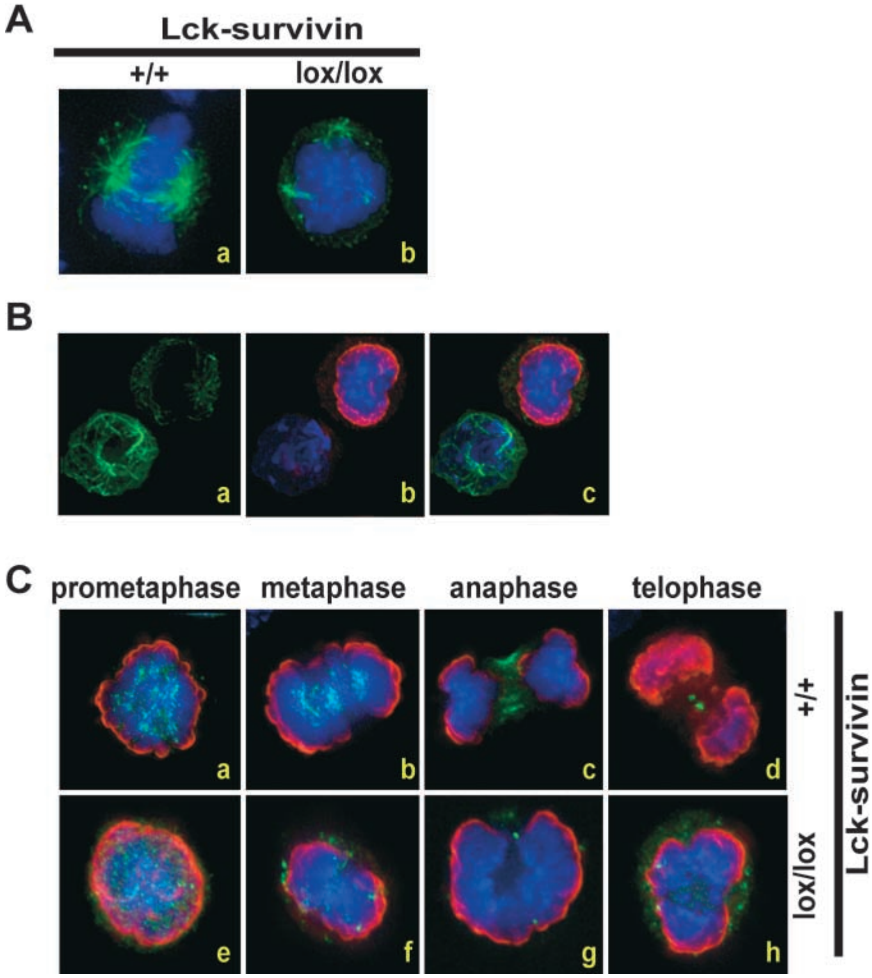

Figure 5. Defects in cytokinesis of Lck-survivin$^{flox/flox}$ DN thymocytes. (A) Impaired spindle formation. Purified DN3L and DN4 cells from Lck-survivin$^{+/+}$ (a) and Lck-survivin$^{flox/flox}$ (b) mice were stained with anti–α-tubulin (green). DNA was visualized with Hoechst 33258 (blue) staining. Data shown are representative of at least three independent preparations per genotype. (B) Severe impairment of spindle assembly in mitotic cells. Spindle assembly in cells from Lck-survivin$^{flox/flox}$ mice was compared at interphase (bottom left) and mitosis (top right). Cells were stained with anti–α-tubulin (green), anti–phospho-histone H3 (p-H3) Ab (red), and Hoechst (blue). a, α-tubulin; b, anti–p-H3 and Hoechst; c, merge. (C) Impaired cytokinesis and localization of Aurora-B kinase. Mitotic DN3L and DN4 cells from Lck-survivin$^{+/+}$ (a–d) and Lck-survivin$^{flox/flox}$ (e–h) mice were stained with anti–p-H3 Ab (red), anti–Aurora-B kinase Ab (green), and Hoechst (blue). a and e, prometaphase; b and f, metaphase; c and g, anaphase; d and h, telophase/cytokinesis.

in Lck-survivin$^{flox/flox}$ DN3L cells, the G1 population was increased to 33.3% (Lck-survivin$^{+/+}$: 14.8%) and the S population was decreased to 63% (Lck-survivin$^{+/+}$: 80%). In Lck-survivin$^{flox/flox}$ DN4 cells, the G1 population was increased still further to 67.4% (Lck-survivin$^{+/+}$: 43.7%), whereas the S population dropped to 19.6% (Lck-survivin$^{+/+}$: 53.0%). In addition, sub-G1 cells represented 3.0% of DN4 thymocytes in Lck-survivin$^{flox/flox}$ mice but only 0.1% of control DN4 cells. Thus, survivin is required for the proliferation and survival of DN3L and DN4 thymocytes, and a lack of survivin leads to cell cycle arrest and cell death at these stages.

Defects in Cytokinesis in Lck-survivin$^{flox/flox}$ DN Thymocytes. Embryos of mice with a null mutation of the *survivin* gene show a cytokinesis defect and die early during embryogenesis (28, 34, and unpublished data). Overexpression of dominant negative survivin or antisense inactivation of survivin also lead to cytokinetic defects (25, 27) and anti-survivin Ab injection results in spindle defects (49). We examined spindle formation in DN3L and DN4 cells from Lck-survivin$^{flox/flox}$ and Lck-survivin$^{+/+}$ mice by staining thymocytes with α-tubulin. Lck-survivin$^{+/+}$ (and Lck-survivin$^{flox/+}$) cells had well-organized and symmetrical spindles (Fig. 5 A, a), whereas Lck-survivin$^{flox/flox}$ cells showed shorter and thicker spindles (Fig. 5 A, b). The number of spindle fibers was reduced in many cases, suggesting a defect in the assembly of spindle microtubules. Significantly, we found that microtubule assembly defects were enhanced in mitotic mutant cells compared with interphase mutant cells (Fig. 5 B). Thus, the organization and assembly of mitotic spindles is severely impaired in the absence of survivin.

Because the intracellular localization of lpl1/Aurora kinase in *Caenorhabditis elegans* is affected by the loss of the *survivin* orthologue *bir-1* (32), we examined the localization of murine Aurora-B kinase (Aurora-B) at each mitotic stage in DN cells of control and survivin-deficient mice. In wild-type cells, Aurora-B localized at the centromeres until metaphase (Fig. 5 C, b), transferred to the central spindle during anaphase (Fig. 5 C, c), and finally accumulated at the spindle midbody during telophase and cytokinesis (Fig. 5 C, d). However, in survivin-deficient cells, Aurora-B could not localize at either the midzone or midbody at either anaphase or telophase (Fig. 5 C, e–h). Indeed, no typical telophase cells could be found among survivin-deficient DN thymocytes.

p53 Induction in Lck-survivin$^{flox/flox}$ DN Thymocytes. Increased cell death and cell cycle arrest are hallmarks of p53

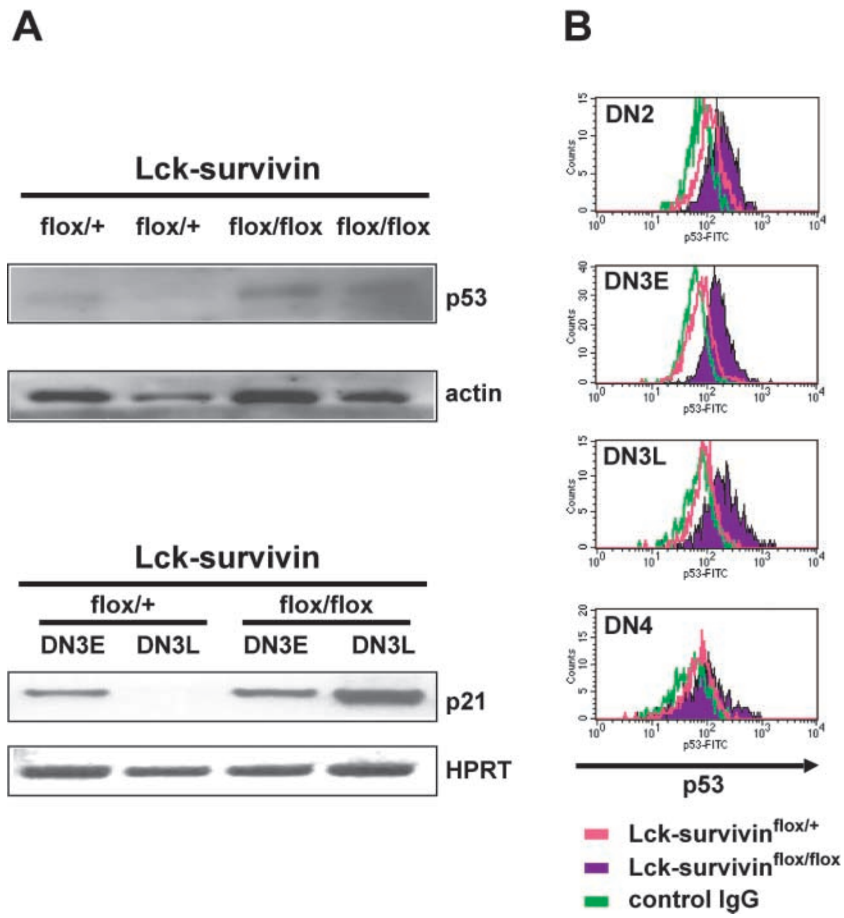

Figure 6. Induction of p53 and p21 in Lck-survivin$^{flox/flox}$ DN thymocytes. (A) p53 and p21 induction in DN thymocytes. Top: Protein samples prepared from Lck-survivin$^{flox/+}$ and Lck-survivin$^{flox/flox}$ DN cells were subjected to Western blot analysis using anti-p53 Ab followed by reprobing with anti-actin as a loading control. Bottom: Levels of p21 and HPRT (loading control) mRNA in control and mutant DN cells were determined by RT-PCR. (B) p53 induction in DN subpopulations. Lck-survivin$^{flox/+}$ and Lck-survivin$^{flox/flox}$ thymocytes were surface stained with anti-CD25, anti-CD44, and anti-Lin followed by intracellular staining with anti-p53 or control IgG. p53 protein in each DN subpopulation was assessed by flow cytometry.

function. To test whether loss of survivin induced p53, we investigated the status of p53 protein in Lck-survivin$^{flox/flox}$ DN cells. We found that p53 protein expression was indeed induced in Lck-survivin$^{flox/flox}$ DN cells but not in Lck-survivin$^{flox/+}$ DN cells (Fig. 6 A, top). Flow cytometric analysis of intracellular p53 protein confirmed that p53 was expressed in survivin-deficient DN2 to DN4 cells (Fig. 6 B). In addition, RT-PCR analysis showed that expression of the p53 target gene p21 was induced in Lck-survivin$^{lox/lox}$ DN cells (Fig. 6 A, bottom). These results suggested that p53 might be responsible for the increased apoptosis and arrest observed in Lck-survivin$^{flox/flox}$ mice.

Gain of Bcl-2 or Loss of p53 Expression Does Not Rescue DN Thymocyte Developmental Defects Induced by Survivin Loss. We determined whether Bcl-2 could rescue survivin deficiency by crossing Lck-survivin$^{flox/flox}$ mice to Eμ-Bcl-2 transgenic mice to generate Lck-survivin$^{flox/flox}$;Bcl-2 animals. The total number of thymocytes in Lck-survivin$^{flox/flox}$;Bcl-2 mice ($2.2 \pm 0.7 \times 10^6$; $n = 4$) was comparable to that in Lck-survivin$^{flox/flox}$ mice ($2.4 \pm 0.5 \times 10^6$; $n = 4$; Fig. 7 A). The number of DN cells was also unaltered in the presence of the Bcl-2 transgene (Lck-survivin$^{flox/flox}$ vs. Lck-survivin$^{flox/flox}$;Bcl-2; $2.3 \pm 0.5 \times 10^6$ vs. $2.1 \pm 0.6 \times 10^6$; $n = 4$), suggesting that Bcl-2 overexpression cannot overcome cell death induced by an absence of survivin.

To test whether survivin deficiency had a direct impact on p53-mediated apoptosis, we generated compound conditional survivin mutants by crossing Lck-survivin$^{flox/flox}$ mice to p53$^{-/-}$ mice to generate Lck-survivin$^{flox/flox}$;p53$^{-/-}$ animals. In mice lacking both survivin and p53, the total number of thymocytes was decreased (0.33 ± 0.10^6; $n = 3$) compared with Lck-survivin$^{flox/flox}$;p53$^{+/-}$ controls ($1.23 \pm 0.12 \times 10^6$; $n = 3$). The percentage of DN thymocytes in the double mutants was decreased by 10% at most ($n = 3$), whereas the percentages of DP and SP thymocytes were slightly (not significantly) increased (Fig. 7 B), indicating that loss of p53 cannot restore DN thymocyte development in the absence of survivin.

Loss of p53 or p21 Expression Releases Cell Cycle Arrest but Accelerates Apoptosis Induced by Survivin Loss. Next, we examined cell cycle status and apoptosis in survivin-deficient thymocytes also lacking either p53 or p21. In both cases, the G1 subpopulation of DN3L thymocytes was restored to the control level (Lck-survivin$^{flox/+}$ vs. Lck-survivin$^{flox/flox}$ vs. Lck-survivin$^{flox/flox}$;p53$^{-/-}$ vs. Lck-survivin$^{flox/flox}$;p21$^{-/-}$; 35.3 vs. 51.1 vs. 35.6 vs. 37.0%; Fig. 7 C, middle). Thus, cell cycle arrest induced by loss of survivin is mediated by the p53 pathway and involves p21 induction. The G1 subpopulation of DN4 thymocytes was reduced by >80% regardless of genetic background, suggesting profound cell damage resulted in these cells. These results suggest that loss

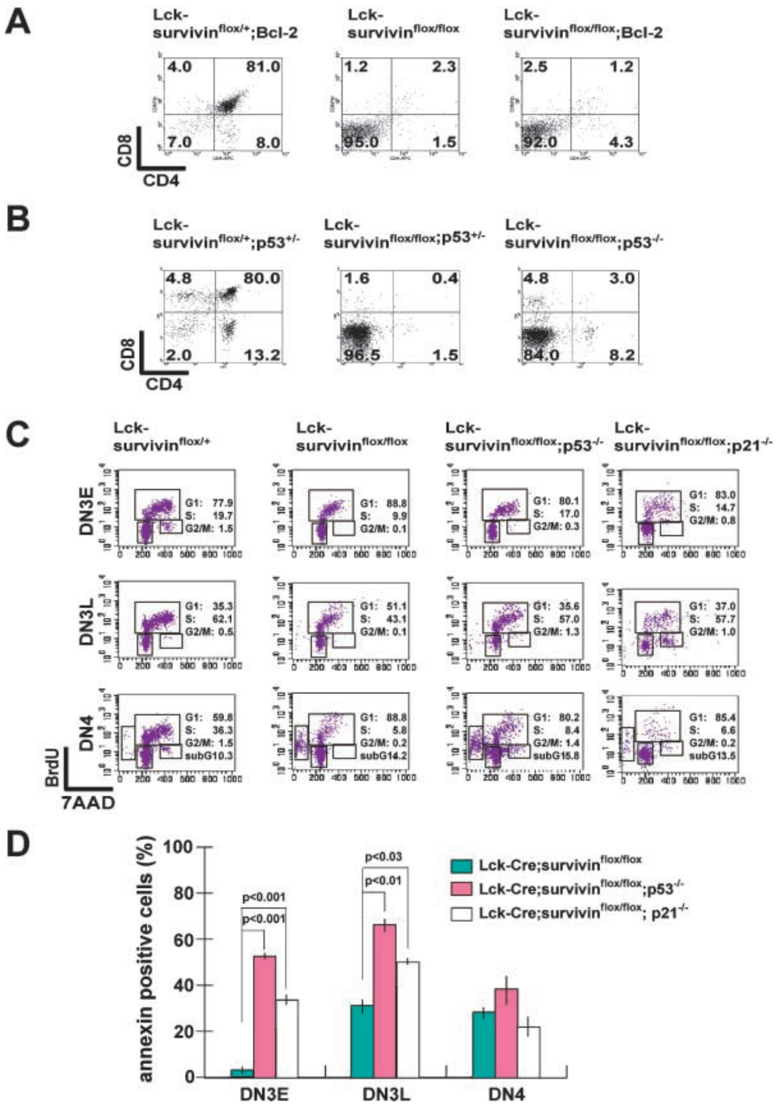

Figure 7. Effects of Bcl-2 gain or p53 loss on phenotypes of survivin-deficient thymocytes. (A) Gain of Bcl-2 does not restore DP cells in the absence of survivin. Thymocytes prepared from Lck-survivin$^{flox/+}$;Bcl-2, Lck-survivin$^{flox/flox}$, and Lck-survivin$^{flox/flox}$;Bcl-2 mice were stained with anti-CD4 and anti-CD8 followed by flow cytometric analysis. One experiment representative of four independent trials is shown. (B) Loss of p53 does not rescue DN thymocyte development in the absence of survivin. CD4/CD8 expression was determined as in A for thymocytes from Lck-survivin$^{flox/+}$;p53$^{-/-}$, Lck-survivin$^{flox/flox}$;p53$^{+/-}$, and Lck-survivin$^{flox/flox}$;p53$^{-/-}$ mice. (C) Decreased G1 arrest of survivin-deficient DN3L cells in the p53$^{-/-}$ and p21$^{-/-}$ genetic backgrounds. Cell cycle profiles of DN cells prepared from Lck-survivin$^{flox/+}$, Lck-survivin$^{flox/flox}$, Lck-survivin$^{flox/flox}$;p53$^{-/-}$, and Lck-survivin$^{flox/flox}$;p21$^{-/-}$ mice were determined as in Fig. 4 B. (D) Increased cell death of survivin-deficient DN cells in the p53$^{-/-}$ and p21 genetic backgrounds. The percentages of annexin$^+$ cells in the indicated DN subpopulations were determined as in Fig. 4 A. Results shown are mean ± SD. Annexin$^+$ cells were significantly increased in p53$^{-/-}$ and p21$^{-/-}$ genetic backgrounds (ANOVA; n = 3).

of survivin, as well as triggering p53-mediated growth arrest, precipitates aberrant mitosis that inexorably causes the death of affected cells regardless of their p53 or p21 status. Indeed, annexin staining showed that the viability of DN3E and DN3L cells was significantly decreased when both survivin and either p53 or p21 were missing (Fig. 7 D). Thus, the loss of the cytoprotective function of survivin appears to trigger p53 induction and cell cycle arrest, but the death of survivin-deficient cells involves a mechanism that is independent of p53.

Discussion

In this study, we have defined the role of survivin in thymocyte development and have investigated the biochemistry underlying its effects. The conditional deletion of the *survivin* gene in thymocytes revealed that survivin has vital functions in both cell death and proliferation in vivo. But we could not find any evidence that survivin directly regulates apoptotic pathway.

Control of cell survival and proliferation is fundamental to normal T cell development, and signaling via the pre-TCR contributes to the regulation of both these processes during early thymocyte maturation (4, 50). Whether the pre-TCR also mediates anti-apoptosis has not been well clarified. Is survivin required for these pre-TCR–mediated signals? Our data show that the DN3 to DN4 transition is severely impaired in Lck-survivin$^{flox/flox}$ mice. Profound reductions in DP and SP cell numbers occur that lead to a decrease in total thymic cellularity (Fig. 2, C and D). Thus, the proliferation and survival of DN3L and DN4 cells, which are known to depend on pre-TCR signaling, are also dependent on survivin function. Once survivin is inactivated, DN3L and DN4 cells lose their ability to expand and eventually die. In our study, survivin was up-regulated during the DN3E to DN3L transition (Fig. 2, A and B), and loss of survivin led to a drastic increase in the number of cells undergoing cell cycle arrest and cell death (Fig. 4). This phenotype is reminiscent of that of mice missing any component (TCRβ, pTα, CD3 molecules) of the pre-

TCR. However, survivin-deficient DN cells retain normal expression of rearranged TCRβ and pre-TCR signaling components (Fig. 3, A–C). Although anti-CD3 Ab can overcome the block in RAG-$2^{-/-}$ DN cells, such treatment does not restore survivin-deficient DN thymocyte development (Fig. 3 D). Thus, the initiating elements of pre-TCR signaling are intact in survivin-deficient cells but survivin is required for the actual expansion of thymocytes in response to pre-TCR signaling.

Although survivin is a member of inhibitor of apoptosis family, its direct role in apoptosis is still controversial (29, 51, 52). In our system, survivin-deficient cells exhibited normal susceptibility to various external apoptotic stimuli in vitro (Fig. 4 B). In addition, survivin deficiency in DN3E cells did not induce spontaneous apoptosis (Fig. 4 A). However, a failure in survivin function triggers cell death of highly proliferating DN cells (Fig. 4 A). Based on these findings, we conclude that survivin primarily plays a critical role in mitosis progression but does not directly regulate apoptosis. Inactivation of survivin in proliferating cells induces severe defects in chromosome segregation and cytokinesis, and subsequent cell death with the morphology representing mitotic catastrophe (not depicted). On the other hand, cofactor-dependent caspase inhibition of survivin has recently been reported (24). Further analysis of survivin's cytoprotective functions in a tissue- or cell context–specific manner is therefore necessary.

p53 has a critical role in transducing signals from damaged DNA that control the cell cycle and apoptosis in mammalian cells (16, 17). In mutant mice with impaired pre-TCR expression, p53 deficiency can rescue the block in DN thymocyte development (11–15). This genetic evidence has suggested a model in which a p53-mediated checkpoint operates during pre–T cell development to sense and eliminate thymocytes that attempt to differentiate before the formation of a functional pre-TCR complex. In this scenario, the pre-TCR regulates the DN to DP transition by inactivating p53. However, others have suggested that the pre-TCR checkpoint may involve a p53-independent cell death pathway (11). In this study, we have shown that the death of survivin-deficient DN thymocytes involves such a pathway (Fig. 7) and we hypothesize that survivin controls critical aspects of the DN to DP transition. This hypothesis is consistent with recent reports that survivin is required for the maintenance of the spindle checkpoint. For example, in survivin-depleted cells, the spindle checkpoint molecules BubR1 and Mad2 are prematurely displaced from kinetochores and the spindle checkpoint cannot be maintained (53, 54).

We speculate that in exponentially growing DN thymocytes, survivin might be critical for maintaining the integrity of the rapidly forming spindles. Although the precise mechanism underlying the induction of p53 by spindle damage remains unknown, defects in centrosome duplication have been suggested to activate the p53 pathway (55). We found that survivin-deficient cells exhibited abnormal spindle formation (Fig. 5, A and B). Thus, the loss of survivin in the mutant cells may have activated the same p53 signaling pathway as seen in cells treated with microtubule-damaging agents (56). p53-mediated induction of p21 is required for the arrest of spindle-damaged cells at G1 (18). We also observed that p21 was highly up-regulated in survivin-deficient cells (Fig. 6 A) and that loss of p21 released the cell cycle arrest (Fig. 7 C). The p53 protein also has direct effects on other aspects of mitosis. For example, p53 controls centrosome duplication via a p21-dependent mechanism (57). p53 also binds directly to Aurora-A and suppresses Aurora-A–induced centrosome amplification (58). It is not hard to imagine that loss of survivin coupled with p53 inactivation might have cumulative devastating effects on mitosis.

Our work has generated useful genetic tools for the study of survivin functions in various tissues and pathological conditions. Further study of T cell–specific survivin-deficient mice and additional mutants should reveal much about pre-TCR signaling and the p53- and Bcl-2–independent mechanisms involved in cell death induced by loss of survivin. Research to define these pathways may shed new light on regulatory mechanisms governing cell death and proliferation, which could in turn ultimately lead to novel therapeutic targets.

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