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Yin Yang 1 promotes thymocyte survival by downregulating p53[1,2]

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Abstract

Yin Yang 1 (YY1) is a zinc finger protein that functions as a transcriptional activator or repressor and participates in multiple biological processes, including development and tumorigenesis. To investigate the role of YY1 in developing T cells, we used mouse models that depleted YY1 at two distinct stages of thymocyte development. When YY1 was depleted in CD4$^-$CD8$^-$ double negative (DN) thymocytes, development to the CD4$^+$CD8$^+$ double positive (DP) stage was impaired, due to increased apoptosis that prevented expansion of post–β-selection thymocytes. When YY1 was depleted in DP thymocytes, they underwent increased cell-autonomous apoptosis *in vitro* and displayed a shorter lifespan *in vivo*, as judged by their ability to undergo secondary Vα-to-Jα recombination. Mechanistically, we found that the increased apoptosis in YY1-deficient thymocytes was attributed to overexpression of p53, because concurrent loss of p53 completely rescued the developmental defects of YY1-deficient thymocytes. These results indicated that YY1 functions as a critical regulator of thymocyte survival and that it does so by suppressing the expression of p53.

Introduction

Effective T cell adaptive immunity depends upon efficient generation of T cells from intrathymic progenitors. CD4$^-$CD8$^-$ double negative (DN) thymocytes serve as precursors for both αβ and γδ T cells. DN thymocytes can be subdivided into four stages: DN1 (CD25$^-$CD44$^+$, which includes pluripotent early thymic precursors), DN2 (CD25$^+$CD44$^+$), DN3 (CD25$^+$CD44$^-$), and DN4 (CD25$^-$CD44$^-$). DN1 and DN2 thymocytes proliferate extensively in a TCR-independent, Notch-dependent manner before progression to the DN3 stage (1). DN3 cells can be further divided into DN3a and DN3b based on cell size and CD27 expression (2). During the DN3a stage, most cells become quiescent and undergo V(D)J recombination at the *Tcrg*, *Tcrd*, and *Tcrb* loci (3, 4). Thymocytes with in-frame rearrangements of *Tcrg* and *Tcrd* express the γδ TCR, and may commit to the γδ lineage and maintain a DN phenotype. Cells that successfully rearrange *Tcrb* produce a functional TCRβ

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protein, which can assemble with pTα and CD3 proteins to form pre-TCRs (1, 5). Expression of the pre-TCR drives a burst of proliferation and allows cells to progress from DN3a to DN3b in a process called β-selection (5). Only cells that pass the β-selection checkpoint develop through the DN4 and immature single-positive stages into $CD4^+CD8^+$ double-positive (DP) thymocytes. This developmental progression represents the hallmark of αβ T cell lineage commitment.

Failure to assemble a functional pre-TCR complex, as occurs in mice that are deficient for RAG1, RAG2, pre-Tα or CD3γ, leads to a severe block of αβ T cell development at the DN stage (5–8). Signals that rescue thymocytes from death and promote their proliferation are critical for β-selection. Known trophic signals for thymocytes at the β-selection checkpoint include those generated by the pre-TCR, Notch and the IL-7 receptor (9, 10). Notch promotes thymocyte survival by regulating glucose metabolism (9). The pro-apoptotic factor p53 has been suggested to eliminate thymocytes that fail to pass β-selection, because the concurrent loss of p53 can rescue developmental defects in pre-TCR-deficient mice (6, 7, 11). However, the mechanisms underlying p53 regulation during thymocyte development are not fully understood.

Regulated cell survival and apoptosis are also critical for the proper development of DP thymocytes. As thymocytes develop to the DP stage, they stop proliferating and survive for an average of 3–4 days. During this time, DP thymocytes undergo multiple rounds of Vα-to-Jα rearrangements, with Jα segments used sequentially from the 5' end to the 3' end of the Jα array (12). Because the lifespan of DP thymocytes impacts the progression of Vα-to-Jα rearrangements and positive selection of T cells, factors that regulate the survival of DP thymocytes (e.g., RORγ, an orphan nuclear receptor, and Bcl-xL, an anti-apoptotic Bcl-2 family protein) are essential regulators of TCR repertoire diversity (13, 14). Depleting RORγ shortens the lifespan of DP thymocytes and limits Vα-to-Jα rearrangements to the most 5'Jα segments, whereas extending the lifespan of DP thymocytes with a Bcl-xL transgene skews Vα-to-Jα rearrangement towards 3'Jα segments (14).

Yin Yang 1 (YY1) is a ubiquitously expressed, multi-functional transcription factor, which can activate or repress transcription through interactions with other transcriptional regulators (15). YY1 has been shown to regulate multiple physiological processes including embryogenesis, differentiation and cellular proliferation (16–22). YY1 also functions as potential tumor suppressor, because it can negatively regulate p53 (23, 24). In this regard, YY1 expression is elevated in various types of cancer (25). Although numerous studies have been devoted to understanding the roles of YY1 in B cell development and V(D)J recombination of the *Igh* and *Igk* loci (18, 21, 26–29), studies of YY1 in T-lineage cells have been limited to its role in regulating Th2 cytokine production (30).

To investigate the role of YY1 in early T cell development, we conditionally deleted YY1 in developing thymocytes. We found that early ablation of YY1 caused severe developmental defects in the DN compartment due to a dramatic increase in DN thymocyte apoptosis. Furthermore, YY1 emerged as a novel regulator of the lifespan of DP thymocytes, because late ablation of YY1 resulted in increased apoptosis of DP thymocytes and a restricted TCRα repertoire. Mechanistically, we showed that p53 was upregulated in both DN and DP

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YY1-deficient thymocytes. Eliminating p53 in YY1-deficient thymocytes rescued the survival and developmental defects, indicating that these YY1-dependent defects were p53-mediated. We conclude that YY1 is required to maintain cell viability during thymocyte development by thwarting the accumulation of p53.

Materials and Methods

Mice

All mice were used in accordance with protocols approved by the Duke University Animal Care and Use Committee. *Yy1*$^{f/f}$ mice (B6;129S4-Yy1^{tm2Yshi}/J) (16) obtained from The Jackson Laboratory were bred with *Lck*-Cre transgenic mice (B6.Cg-Tg(Lck-cre) 548Jxm/J), a gift from J. Rathmell (Duke University) (31), to generate *Yy1*$^{f/f}$ *Lck*-Cre mice and were further bred with *Rag2*$^{-/-}$ mice to generate *Yy1*$^{f/f}$ *Lck*-Cre *Rag2*$^{-/-}$mice. *Yy1*$^{f/f}$ *CD2*-Cre mice were a gift from A. Feeney (The Scripps Research Institute) and were further bred with *Rag2*$^{-/-}$ mice to generate *Yy1*$^{f/f}$ *CD2*-Cre *Rag2*$^{-/-}$ mice. *Trp53*$^{-/-}$ mice (B6.129S2-Trp53^{tm1Tyj}/J) were a gift from D. L. Silver (Duke University) and were bred with *Yy1*$^{f/f}$ *CD2*-Cre mice to generate *Yy1*$^{f/f}$ *CD2*-Cre *Trp53*$^{-/-}$ mice. The genetic background of mice used for experiments was a mixture of 129 and C57BL/6. Mice were analyzed at 4–5 weeks of age; those designated as wild-type carried floxed *Yy1* alleles but lacked Cre recombinase expression.

Flow cytometry and cell sorting

All reagents were purchased from Biolegend unless otherwise indicated. To sort DN3 thymocytes, total thymocytes were stained with anti-CD4 (GK1.5) and anti-CD8 (53–6.7), and sheep anti-rat IgG Dynabeads (Life Technologies) were used to remove CD4^{+} and CD8^{+} thymocytes. DN cells were then stained with 7-aminoactinomycin D (7AAD) and antibodies against CD44 (IM7), CD25 (PC61), and lineage (Lin) markers Gr-1 (RB6-8C5), CD3ε (145-2C11), Ter119 (TER-119), and CD11b (M1/70). 7AAD^{-}CD25^{+}CD44^{-}Lin^{-} cells were isolated by cell sorting and used for further analysis. To separate DN3a from DN3b thymocytes, the DN thymocytes were also stained with anti-CD28 (37.51). For intracellular staining with anti-TCRβ (H57-597) or anti-YY1 (H-414), cells were first stained with antibodies against surface markers before fixation and permeabilization (BD Cytofix/ Cytoperm™ Kit).

Anti-CD3ε treatment

Mice were injected i.p. with 150 μl of 1 mg/ml anti-CD3ε (145-2C11) or with an equal volume of PBS as previously described (32). Mice were euthanized 9 days after injection for isolation of DP thymocytes by cell sorting.

BrdU assay

Mice were injected i.p. with 1 mg BrdU at 2 or 4 hours prior to analysis. BrdU incorporation was detected by intracellular staining (FITC BrdU Flow kit; BD Pharmingen).

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OP9-DL1 culture

OP9-DL1 co-cultures were carried out as previously described (33). DN3a ($Lin^{-}CD25^{+}CD44^{lo}CD28^{lo}$ forward scatter^{hi}) or DN3b ($Lin^{-}CD25^{+}CD44^{lo}CD28^{hi}$ forward scatter^{lo}) thymocytes were sorted and stained with Celltrace Violet (Life Technologies) and were then placed on OP9-DL1 monolayers with 5 ng/ml IL-7. Cells were harvested on days 2, 3 and 4 to measure dilution of Celltrace Violet and expression of CD4, CD8 and CD25. Apoptotic cells were assayed by staining with Annexin V (Biolegend) on day 4.

Western blot

Antibodies specific for YY1 (H-414, Santa Cruz), p53 (1C12, Cell Signaling Technology), Bcl-xL (54H6, Cell Signaling Technology), Bim (559685, BD Biosciences), caspase-3 (8G10, Cell Signaling Technology), cleaved caspase-3 (5A1E, Cell Signaling Technology) and actin (I-19, Santa Cruz) were used according to the manufacturer's instructions.

PCR analysis of recombination and transcription

Tcra and *Tcrb* rearrangements were analyzed in genomic DNA isolated from sorted DP thymocytes and sorted intracellular (ic)$TCR\beta^{+}$ thymocytes, respectively. Analysis of *Cd14* was used for normalization. PCR primers are listed in Supplemental Table 1 or were described previously (34, 35). *Tcra* rearrangements were quantified by SYBR Green real-time PCR and *Tcrb* rearrangements were quantified by Taqman real-time PCR; conditions for both PCR reactions were described previously (36). Jα usage was also analyzed in cDNA prepared from total thymocytes by PCR with *Trav12* and Cα primers using the following program: 94°C for 2 min, 33–35 cycles of 92°C for 30 s, 55°C for 30 s and 72°C for 30 s, and 72°C for 4 minutes. PCR products were gel-purified, cloned with a TOPO TA Cloning Kit for Sequencing (Life Technologies), and sequenced using an internal Cα primer.

To analyze gene expression, total RNA was extracted with TRIzol reagent (Life Technologies) and reverse transcribed with SuperScript III First-Strand Synthesis System cDNA kit (Life Technologies) according to the manufacturer’s instruction. Amplification of *Hprt* or *Gapdh* was used for normalization. SYBR Green real-time PCR was conducted using PCR primers listed in Supplemental Table 1.

Statistics

Statistical analyses were performed using Graphpad Prism 6.0 software.

Results

Early ablation of YY1 severely blocks DN thymocyte development

To elucidate the role of YY1 in early T cell development, we analyzed $Yy1^{f/f}$ mice expressing a human *CD2*-Cre transgene. The human *CD2*-Cre transgene is active in common lymphoid progenitors (37) and should promote *Yy1* deletion in all T-lineage cells. These mice are hereafter referred to as $Yy1^{CD2}$ mice. $Yy1^{CD2}$ mice displayed a profound loss in thymocyte number, with thymus cellularity reduced to 1% of that of their wild-type ($Yy1^{f/f}$) littermates (Fig. 1A). Although all thymocyte subsets were reduced in number

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(Supplemental Fig. 1A), there was a dramatic increase in the proportion of DN thymocytes relative to DP thymocytes (Fig. 1B, Supplemental Fig. 1A), suggesting that the progression from the DN to the DP stage was compromised. The proportion of $CD4^+$ thymocytes was increased in $Yy1^{CD2}$ mice (Fig. 1B). However, these thymocytes did not express a surface TCRβ chain, indicating that they were immature (Fig. 1C). Because a similar population of $CD4^+$ thymocytes was not detected in $Rag2^{-/-}Yy1^{CD2}$ mice (Supplemental Fig. 1B), the population detected in $Yy1^{CD2}$ mice must represent bonafide, post–β-selection immature single positive thymocytes, rather than pre-β-selection thymocytes with dysregulated CD4 expression.

Consistent with impaired development of αβ-lineage precursors to the more mature DP and single positive stages, TCRβ-expressing thymocytes were significantly reduced in $Yy1^{CD2}$ mice (Fig. 1D). In contrast, the percentage of γδ T cells was substantially increased in $Yy1^{CD2}$ mice compared with wild-type littermates, indicating that the developmental defect was restricted to the αβ-lineage (Fig. 1D). Further delineation of DN thymocyte populations showed that the percentage of DN3 ($CD25^+CD44^-$) thymocytes was increased in $Yy1^{CD2}$ mice, whereas the percentage of DN4 ($CD25^-CD44^-$) thymocytes was markedly reduced (Fig. 1E). To exclude the possibility that the residual presence of DN4 thymocytes in $Yy1^{CD2}$ mice was due to incomplete deletion of *Yy1*, YY1 expression was measured by intracellular (ic) staining in DN3 and DN4 thymocytes from wild-type and $Yy1^{CD2}$ mice. YY1 protein was substantially reduced in both DN3 and DN4 thymocytes from $Yy1^{CD2}$ mice (Fig. 1F). Consistent with this, only 4% of *Yy1* alleles were intact in icTCRβ$^+$ thymocytes (Fig.1G). Moreover, intact *Yy1* alleles were essentially undetectable in TCRγδ $^+$ thymocytes (Fig. 1G). Hence, efficient γδ-lineage development and partial αβ-lineage development can occur in the absence of YY1. Taken together, our results demonstrated that $Yy1^{CD2}$ thymocytes have a severe, αβ-lineage–specific developmental defect, which impairs the DN3-to-DN4-to-DP progression of thymocytes.

A developmental defect at the β-selection checkpoint could reflect impaired *Tcrb* rearrangement in $Yy1^{CD2}$ mice. However, a substantial proportion of DN3 thymocytes expressed icTCRβ protein in $Yy1^{CD2}$ mice (Fig. 2A) and the TCRβ repertoire was minimally altered in these mice (Supplemental Fig. 1C). We then asked whether DN4 thymocytes in $Yy1^{CD2}$ mice had undergone a normal process of β-selection. We tested CD27 expression in DN3 and DN4 thymocytes, because upregulation of CD27 during the DN3-to-DN4 transition marks cells that pass the β-selection checkpoint (2). Although the mean fluorescence intensity of CD27 in $Yy1^{CD2}$ DN4 thymocytes was lower than in wild-type thymocytes (Fig. 2B), a substantial portion of $Yy1^{CD2}$ DN4 thymocytes had appropriately upregulated CD27, indicating that they represented bona-fide post–β-selection thymocytes. To rule out a possible defect in pre-TCR–driven proliferation, $Yy1^{CD2}$ mice and their wild-type littermates were pulsed with BrdU for 2 hours, and incorporation of BrdU into proliferating cells was measured by flow cytometry. Proliferating icTCRβ$^+$ DN thymocytes were slightly more abundant in $Yy1^{CD2}$ mice than in wild-type mice (Fig. 2C), perhaps reflecting a compensatory mechanism in the face of reduced cellularity. Nevertheless, this result indicated that the proliferative capacity of pre-TCR competent thymocytes was not impaired by loss of YY1. To further examine the dynamics of cell proliferation, purified

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DN3 thymocytes were stained with Celltrace Violet and co-cultured with OP9-Delta-like 1 (OP9-DL1) stromal cells, which provided the Notch signaling required for thymocyte development (38). $Yy1^{CD2}$ DN3 thymocytes proliferated with slower dynamics as compared to wild-type DN3 thymocytes (Fig. 2D). This could reflect a primary defect in proliferation, or alternatively, higher cell death during proliferation, resulting in lower cell numbers in successive generations.

To test whether increased cell death was responsible for the developmental defect in $Yy1^{CD2}$ mice, we analyzed the viability of YY1-deficient thymocytes *in vitro*, because apoptotic thymocytes are often undetected *in vivo* due to clearance by phagocytosis (39). Pre-β-selection DN3a thymocytes (7AAD$^-$CD25$^+$CD44$^-$Lin$^-$CD28$^-$) were sorted as previously described (40,41), stained with Celltrace Violet, and co-cultured with OP9-DL1 cells. After 4 days in culture, DN3a cells from wild-type mice developed into DP cells (Fig. 3A). In contrast, DN3a cells from $Yy1^{CD2}$ mice failed to generate DP cells (Fig. 3A), in accord with the developmental defects observed *in vivo* (Fig. 1B). During the same time-frame, wild-type DN3a thymocytes proliferated vigorously, as determined by dilution of Celltrace Violet, and gradually downregulated CD25 expression as they proliferated (Fig. 3B), consistent with previous analysis (40). By day 4, 75% of the cells had downregulated CD25 expression, indicating that these cells had adopted a DN4-DP phenotype (Fig. 3B). In contrast, although DN3a thymocytes from $Yy1^{CD2}$ mice proliferated, fewer CD25$^{low/-}$ cells were generated (Fig. 3B).

To further analyze population dynamics, thymocytes were harvest from culture on day 4, and apoptosis was assayed in gated proliferating cells. The percentages of early apoptotic cells (Annexin V$^+$7AAD$^-$) and late apoptotic cells (Annexin V$^+$7AAD$^+$) were determined in both the CD25^{hi} and CD25$^{low/-}$ populations. Apoptosis was higher in $Yy1^{CD2}$ than in wild-type thymocytes, particularly in the CD25$^{low/-}$ population (Fig. 3C). To rule out the possibility that defective differentiation and survival in these assay was an artifact of β-selection *in vitro*, we sorted post–β-selection DN3b thymocytes (7AAD$^-$CD25$^+$CD44$^-$Lin$^-$CD28$^+$) and subjected them to the same OP9-DL1 co-culture conditions. As expected (40), wild-type DN3b cells differentiated into DP thymocytes more rapidly and proliferated more vigorously in the co-cultures than did DN3a cells (Supplemental Fig. 2A,B). However, similar to $Yy1^{CD2}$ DN3a thymocytes, $Yy1^{CD2}$ DN3b thymocytes desplayed defects in DN-to-DP differentiation and increased apoptosis (Supplemental Fig. 2A-C). These results suggested that the developmental defects of $Yy1^{CD2}$ DN thymocytes were instrinsic properties of post–β-selection thymocytes. Together, these data indicate that YY1 is required for normal thymocyte development because it protects proliferating DN4 thymocytes from apoptosis.

YY1 is required for the normal life span of DP thymocytes

We next investigated whether YY1 regulates cell death in DP thymocytes. $Yy1^{f/f}$ mice were crossed with *Lck*-Cre transgenic mice to generate $Yy1^{Lck}$ mice. Although *Lck*-Cre has been reported to be active in DN2 and DN3 thymocytes (42), YY1 protein was not substantially depleted until the DP stage in $Yy1^{Lck}$ mice (Fig. 4A). We found that $Yy1^{Lck}$ mice possessed 30% of the total thymocytes detected in their wild-type littermates (Fig. 4B), with normal

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numbers of DN thymocytes and a normal DN1-to-DN4 progression (Fig. 4B, C). However, the percentage of DN thymocytes was higher in *Yy1*^{Lck} mice than in wild-type mice (Fig. 4D), and the absolute number of DP thymocytes was significantly reduced (Fig. 4B). To address whether the loss of DP thymocytes was due to a lower replenishment from proliferating precursors, we tracked DP thymocytes with a recent history of proliferation by labeling with BrdU *in vivo*. Four hours after BrdU injection, the percentage of BrdU$^+$ DP thymocytes in *Yy1*^{Lck} mice was comparable to that in wild-type mice (Fig. 4E). Thus, YY1 deficiency significantly decreased the number of DP thymocytes but did not affect the generation of DP thymocytes from proliferating precursors. To elucidate whether the DP thymocytes in *Yy1*^{Lck} mice were reduced because of a survival defect, we cultured wild-type and *Yy1*^{Lck} DP thymocytes *in vitro* to assess cell-autonomous apoptosis as previously described (13). After 24 and 48 hours of culture, there were far fewer viable cells (Annexin V$^-$7AAD$^-$) in cultures from *Yy1*^{Lck} mice than from wild-type mice (Fig. 4F). Reduced viability of *Yy1*^{Lck} DP thymocytes was due to increased apoptosis, because these cells exhibited higher levels of caspase 3 cleavage after 6 hours of *in vitro* culture (Fig. 4G).

Reduced survival of DP thymocytes *in vivo* should be apparent as a bias in the TCRα repertoire, since Jα usage follows a temporal progression during DP thymocyte development (14). Indeed, although rearrangements of Vα segments to 5’Jα segments were comparable in wild-type and *Yy1*^{Lck} DP thymocytes (Fig. 5A), rearrangements of Vα segments to more 3’Jα segments were underrepresented (Fig. 5B). Impaired *Tcra* rearrangement was not due to defective RAG expression (43), because *Rag1* and *Rag2* gene expression was normal in *Yy1*^{Lck} DP thymocytes (Fig. 5C). Therefore, we concluded that YY1 is required to protect DP thymocytes from apoptosis in order to generate a normal TCRα repertoire.

To assess whether increased death of DP thymocytes depended on the process of *Tcra* gene rearrangement or αβ TCR-dependent selection events, we crossed *Yy1*^{Lck} mice onto a *Rag2*$^{-/-}$ background to prevent the generation of αβ TCRs or RAG-dependent DNA breaks. *Rag2*$^{-/-}$ background mice were treated with anti-CD3ε to mimic pre-TCR signaling and drive DN-to-DP development in the absence of TCRβ (32). Nine days after injection of anti-CD3ε, YY1-sufficient *Rag2*$^{-/-}$ thymocytes expanded by over 100-fold, whereas YY1-deficient *Rag2*$^{-/-}$ thymocytes expanded only 30-40-fold (Fig. 6A), with fewer DP thymocytes (Fig. 6B). Moreover, YY1-deficient *Rag2*$^{-/-}$ thymocytes were more prone to apoptosis when cultured *in vitro* (Fig. 6C). Therefore, increased apoptosis of *Yy1*^{Lck} DP thymocytes was not caused by an altered response to V(D)J recombination-induced double-strand breaks or TCR-dependent selection signals.

YY1 regulates the p53-dependent apoptosis pathway

Because YY1-deficient thymocytes underwent cell-autonomous apoptosis in the absence of death receptor stimulation, we assessed involvement of the mitochondrial intrinsic apoptosis pathway. This pathway is primarily regulated by three groups of Bcl-2 family proteins: Bcl-2 homology 3-only apoptosis initiator proteins including PUMA and Bid; pro-survival cell guardians including Bcl-2, Bcl-xL and Mcl-1; and pro-apoptotic effector proteins including Bax and Bak (44). Additionally, p53, which is negatively regulated by YY1 (23, 24), has been connected to the intrinsic apoptosis pathway. p53 not only transactivates

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several genes encoding Bcl-2 family proteins but also antagonizes Bcl-2 and Bcl-xL and activates Bax and Bak through protein-protein interactions (45-49). We found that the transcription of *Bcl2l11* (encoding Bim), *Bmf*, *Bid*, *Bbc3* (encoding PUMA), *Bcl2*, *Mcl1*, *Bcl2l1* (encoding Bcl-xL) and *Bak1* (encoding Bak) was comparable in wild-type and $Yy1^{Lck}$ DP thymocytes (Fig. 7A). Transcription of *Birc5* (which encodes Survivin, another pro-survival factor in thymocytes), was also unchanged in $Yy1^{Lck}$ DP thymocytes (Fig. 7B), even though a previous report showed that YY1 represses *Birc5* transcription (50). However, the transcription of *Trp53* (encoding p53), *Cdkn1a* (encoding p21) and *Mdm2* was significantly upregulated in $Yy1^{Lck}$ DP thymocytes (Fig. 7B). These data were consistent with observations in other mammalian cells showing that *Cdkn1a* and *Mdm2* are direct targets of YY1 (24, 51). Furthermore, we found that the abundance of p53 protein was significantly increased in DN3 thymocytes from $Yy1^{CD2}$ mice (Fig. 7C), and in DP thymocytes from $Yy1^{Lck}$ mice (Fig. 7D), as compared to wild-type controls. However, expression of Bim and Bcl-xL proteins was normal in $Yy1^{Lck}$ DP thymocytes (Fig. 7D). These results indicated that YY1 is a negative regulator of p53 abundance in thymocytes.

To evaluate whether YY1 regulation of p53 expression could account for impaired thymocyte development in the YY1-deficient mice, we crossed $Yy1^{CD2}$ mice with $Trp53^{-/-}$ mice to generate $Yy1^{CD2}Trp53^{-/-}$ double-knockout mice. Absence of p53 complemented the defect in thymus cellularity in $Yy1^{CD2}$ mice (Fig. 8A), as well as the losses of DP thymocytes (Fig. 8B; left column) and DN4 thymocytes (Fig. 8B; middle and right columns). Absence of p53 also complemented the defect in Jα usage in YY1-deficient thymocytes (Supplemental Fig. 3). Together, these data demonstrated that overexpression of p53 is responsible for impaired development of YY1-deficient thymocytes.

Discussion

By disrupting *Yy1* gene expression at two distinct stages of thymocyte development, we identified a novel function for YY1 in the generation of αβT lymphocytes. $Yy1^{CD2}$ mice displayed increased apoptosis of post–β-selection DN4 thymocytes, leading to severe developmental arrest at the DN4 stage. $Yy1^{Lck}$ mice had fewer DP thymocytes due to reduced DP thymocyte lifespan *in vivo*; consistent with this, YY1-deficient DP thymocytes were prone to cell-autonomous apoptosis when cultured *in vitro*. Further analysis revealed elevated levels of p53 protein in both $Yy1^{CD2}$ DN and $Yy1^{Lck}$ DP thymocytes, and *Trp53* gene deletion corrected the blockade of T cell development in $Yy1^{CD2}$ mice. Taken together, these data suggested that YY1 plays a critical cell-intrinsic role in thymocyte development by suppressing the level of p53. This function of YY1 is specific to the αβ T cell lineage, because γδ T cell development was normal in $Yy1^{CD2}$ mice.

YY1 is likely to regulate the accumulation of p53 protein in two ways. First, we showed that YY1 suppresses *Trp53* transcription. In addition, YY1 has been shown to negatively regulate p53 post-transcriptionally by stabilizing the interaction of p53 with Mdm2, an E3 ubiquitin ligase (23, 24). This interaction is required for ubiquitination and proteasomal degradation of cytoplasmic p53 (52, 53). YY1 has also been documented to inhibit transcriptional activation by p53 by blocking its interaction with co-activator p300 (24). However, we do not believe that this mechanism contributes significantly to the phenotype

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of YY1-deficient thymocytes, since we found no change in the transcription of a number of genes that are direct transcriptional targets of p53 (e.g., *Bbc3*, *Bak1*, *Mdm2*, *Bcl2*, and *Birc5*) (45, 54–59). Thus, transcriptional activation by p53 is largely unchanged in YY1-deficient thymocytes. We did detect increased transcription of one p53 target, *Cdkn1a*, in $Yy1^{Lck}$ DP thymocytes. However, YY1 has been shown to repress *Cdkn1a* transcription by blocking Sp1 binding to the *Cdkn1a* promoter in smooth muscle cells (51). Thus, we speculate that *Cdkn1a* expression may be regulated by YY1 directly, rather than by p53. However, we think it unlikely that p21 contributes to the developmental defect in $Yy1^{Lck}$ DP thymocytes because most DP thymocytes are quiescent, non-cycling cells.

Pre-TCR signals promote the differentiation, proliferation and survival of thymocytes at the β-selection checkpoint (2, 5). Previous studies have addressed the role of p53 at this stage. RAG2-deficient thymocytes do not differentiate to the DP stage because they fail to assemble a pre-TCR. In these mice, *Trp53* gene deletion suppressed apoptosis to reveal limited DP differentiation, but in the absence of pre-TCR signals, these cells did not expand (7). CD3γ-deficient mice have small numbers of DP thymocytes due to compromised pre-TCR signaling. In these mice, loss of p53 fully restored the DP compartment by suppressing apoptosis in cells that were differentiating and proliferating in response to CD3γ-independent pre-TCR signals (6). Together, these studies suggest that p53 normally enforces β-selection by inducing apoptosis in pre-TCR negative thymocytes, and that pre-TCR signaling promotes cell survival by downregulating p53 (6, 7). With this in mind, quantitative rescue of the DP compartment by p53-deficiency in YY1-deficient thymocytes (Fig. 8A, B) implies that pre-TCR signaling pathways that promote differentiation and proliferation are largely intact in YY1-deficient thymocytes. We note that YY1-deficient thymocytes closely phenocopy Rpl22-deficient thymocytes (60). This makes sense, because YY1 and Rpl22 both function to suppress the accumulation of p53 in post–β-selection thymocytes.

Because transcription of pro- and anti-apoptotic genes was normal, increased apoptosis of DN and DP thymocytes from $Yy1^{CD2}$ and $Yy1^{Lck}$ mice, respectively, was likely due to mitochondrial damage induced by accumulated cytoplasmic p53 (61, 62). p53 can rapidly associate with the mitochondrial outer membrane upon cell death signaling and form a complex with Bcl-2 and Bcl-xL (63). The Bcl-xL-p53 complex is not apoptotic; rather, apoptosis is induced only after PUMA releases p53 from Bcl-xL (45). Free p53 protein can then further interact with and activate Bak and Bax, thus inducing mitochondrial outer membrane permeabilization and the release of cytochrome c and other pro-apoptotic factors (45, 46). Recently, p53 has been shown to activate programmed necrosis (or necroptosis) in response to oxidative stress and ischemia, which involve opening the mitochondrial permeability transition pore (64). However, increased cleavage of caspase 3 in cultured $Yy1^{Lck}$ thymocytes indicated that YY1-deficient thymocytes underwent apoptosis rather than necroptosis (Fig. 4G).

It has been shown that YY1 plays critical roles during B cell development (18). YY1 binds to multiple sites in the *Igh* locus and regulates *Igh* recombination through effects on locus transcription, conformation and long-distance chromatin interactions (18, 26, 27, 29). YY1 also binds to multiple sites across the *Igk* locus and regulates *Igk* recombination (28). Based

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on these results, the ubiquitously expressed YY1 protein might also have been considered a candidate regulator of TCR loci. However, we detected no substantial changes in TCR locus rearrangement in our study. Although there was a modest reduction in the proportion of DN3 thymocytes that were icTCRβ^{+} in *Yy1*^{CD2} mice (Fig. 2A), we observed normal numbers of icTCRβ^{+} DN3 thymocytes in *Yy1*^{CD2} *Trp53*$^{-/-}$ mice (Fig. 8B), suggesting that any reduction in *Tcrb* rearrangement may reflect increased p53-dependent sensitivity to V(D)J recombination-induced double strand breaks (11). Moreover, we discerned, at best, only a minor effect on the TCRβ repertoire in *Yy1*^{CD2} mice (Supplemental Fig. 1C). We similarly observed no effect of YY1 on Jα usage when YY1-deficiency was analyzed on a *Trp53*$^{-/-}$ background (Supplemental Fig. 3). Further, we detected no obvious effect on γδ T cell development (Fig. 1D). Although influences of YY1 on TCR repertoires may have gone undetected in our assays, we suggest that any direct effects of YY1 on TCR loci are likely to be relatively subtle as compared to those at Ig loci.

In summary, we identified YY1 as a critical regulator of thymocyte development, and showed that YY1 regulates thymocyte development by setting the threshold of p53 expression and p53-dependent apoptosis. The influence of YY1 is apparent during αβ T cell development, but is not mirrored in γδ T cells. This may reflect a more limited role for p53 in γδ T cells, given that they undergo limited proliferation as compared to αβ T cells (2, 65).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

7AAD	7-aminoactinomycin-D
DN	double negative
DP	double positive
ic	intracellular
Lin	lineage
YY1	Yin Yang 1

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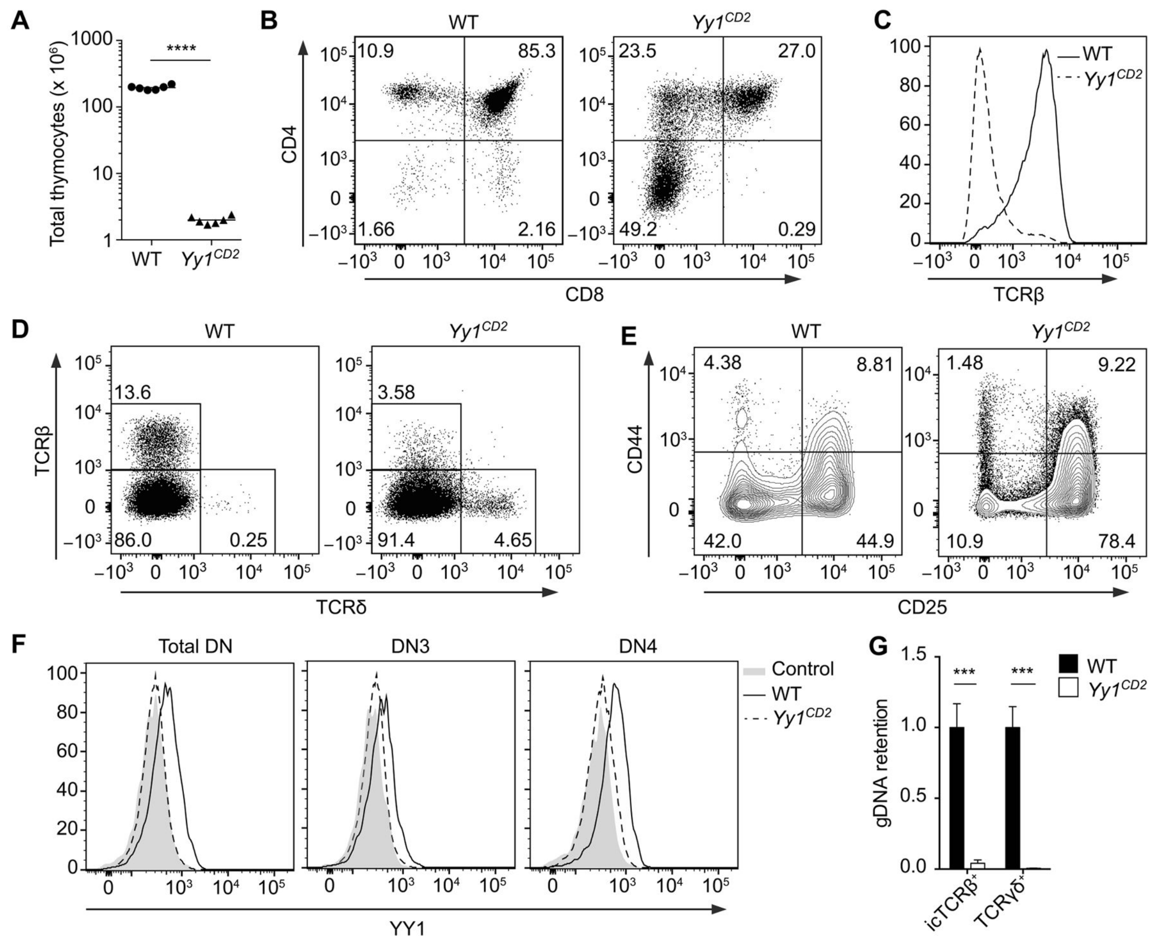

Figure 1. Early ablation of *Yy1* severely blocks T cell development

(A) Number of total thymocytes in *Yy1*$^{f/f}$ (WT) and *Yy1*$^{f/f}$ *CD2*-Cre (*Yy1*^{CD2}) mice. Each data point represents an individual mouse and the horizontal line indicates the mean. Statistical significance was evaluated by unpaired Student's *t*-test. (B–F) Flow cytometry analysis of thymocytes from WT and *Yy1^{CD2}* littermates. (B) CD4 and CD8 staining is shown for total thymocytes. (C) TCRβ staining is shown for pre-gated CD4^{+}CD8$^{−}$ thymocytes. (D) TCRβ and TCRδ staining is shown for total thymocytes. (E) CD44 and CD25 staining is shown for pre-gated CD4$^{−}$CD8$^{−}$Lin$^{−}$ thymocytes. (F) Intracellular staining of YY1 in pre-gated DN (CD4$^{−}$CD8$^{−}$Lin$^{−}$), DN3 (CD4$^{−}$CD8$^{−}$Lin$^{−}$CD25^{+}CD44$^{−}$) and DN4 (CD4$^{−}$CD8$^{−}$Lin$^{−}$CD25$^{−}$CD44$^{−}$) thymocytes. The control consists of WT thymocytes incubated with anti-YY1 without fluorescent secondary antibody. Data are representative of three (B–E) or two (F) independent experiments. (G) Genomic DNA was extracted from sorted icTCRβ^{+} or TCRγδ^{+} thymocytes and deletion of *Yy1* exon1 was measured by real-time PCR with normalization to *Cd14*. Data represent the mean ± SEM of 3 samples for each genotype. Statistical significance was evaluated by unpaired Student's *t*-test with Holm-Sidak correction for multiple comparisons. ***$P < 0.001$, ****$P < 0.0001$.

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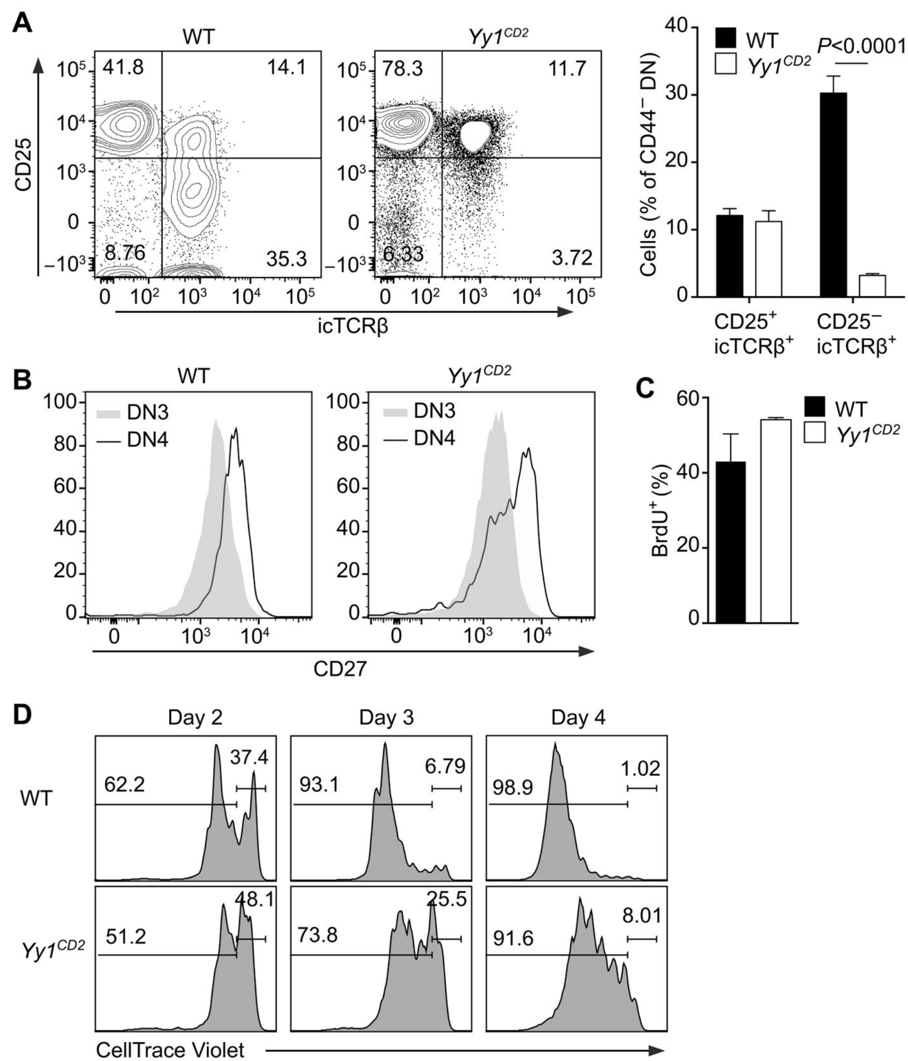

Figure 2. β-selection in YY1-deficient mice

(A) icTCRβ staining is shown for pregated CD4⁻CD8⁻Lin⁻CD44⁻ (DN3 and DN4) thymocytes of *Yy1*$^{f/f}$ (WT) and *Yy1*$^{f/f}$ *CD2*-Cre (*Yy1*^{CD2}) mice (left panels). Mean ± SEM of three independent experiments (right panel). Statistical significance was evaluated by two-way ANOVA with Sidak's multiple-comparison test. (B) Cell surface expression of CD27 analyzed in pregated DN3 and DN4 thymocytes. Data are representative of two independent experiments. (C) WT and *Yy1*^{CD2} mice were pulsed with BrdU for 2 h and the percentage of BrdU$^+$ cells was measured in icTCRβ$^+$ DN thymocytes. Data are presented as the mean ± SEM of two independent experiments. (D) sorted DN3a thymocytes were stained with Celltrace Violet and cultured on OP9-DL1 stromal cells (Day 0). The dilution of Celltrace Violet was measured at the indicated time points. Data are representative of three independent experiments.

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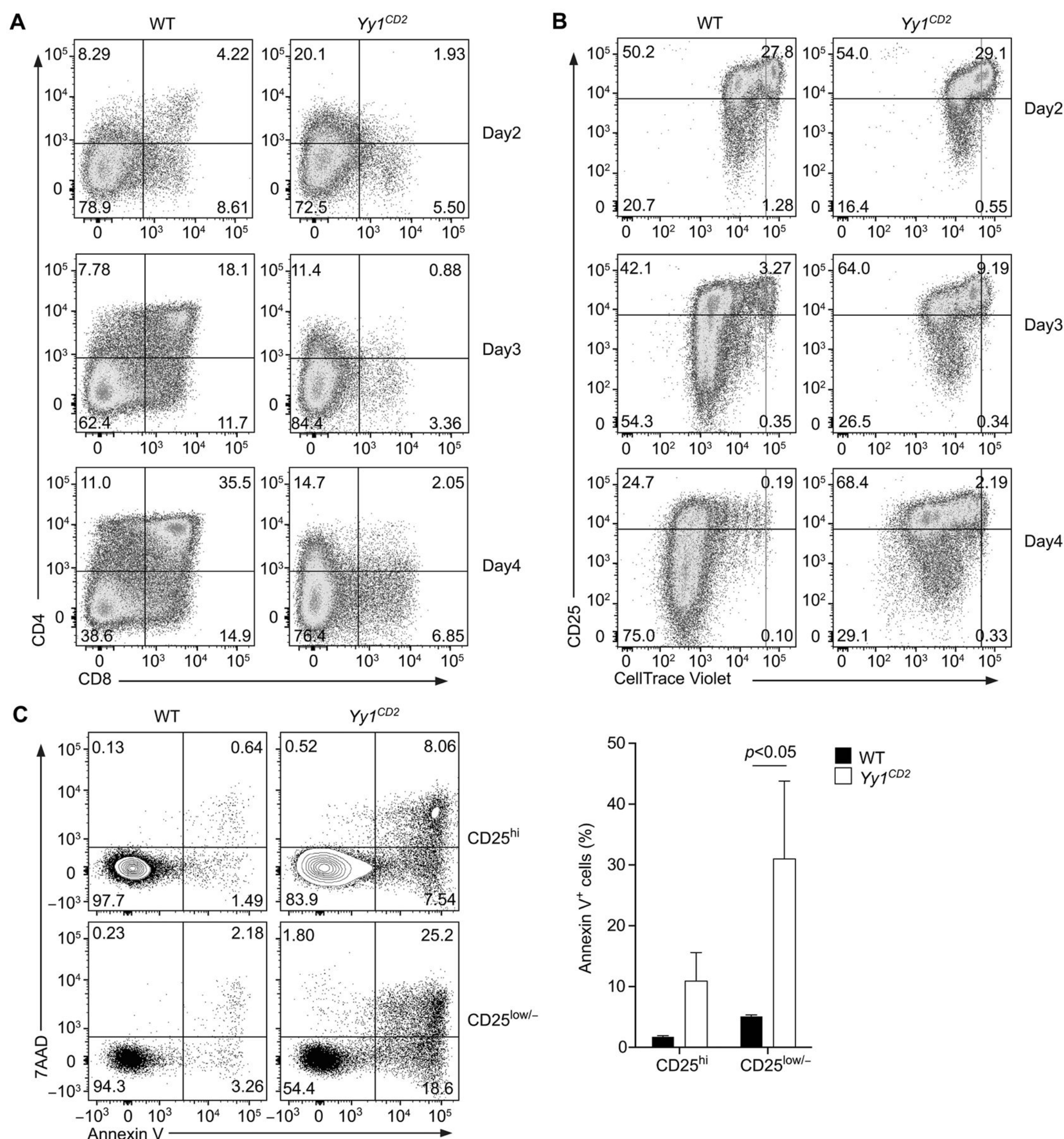

Figure 3. Increased cell death in YY1-deficient DN thymocytes

Sorted DN3a thymocytes from *Yy1*$^{f/f}$ (WT) and *Yy1*$^{f/f}$ *CD2*-Cre (*Yy1*^{CD2}) mice were labeled with Celltrace Violet and placed in OP9-DL1 co-cultures. CD4 and CD8 expression (A) and CD25 expression and dilution of Celltrace Violet (B) were analyzed at the indicated time points. The results are representative of three independent experiments. (C) Annexin V and 7AAD staining of CD25^{hi} and CD25$^{low/-}$ proliferating cells (a combination of the left upper and lower quadrants in (B)) at day 4 of culture (left). Mean ± SEM of two independent experiments analyzing the results of three WT and two *Yy1*^{CD2} cultures (right). Statistical significance was evaluated by two-way ANOVA with Sidak's multiple-comparison test.

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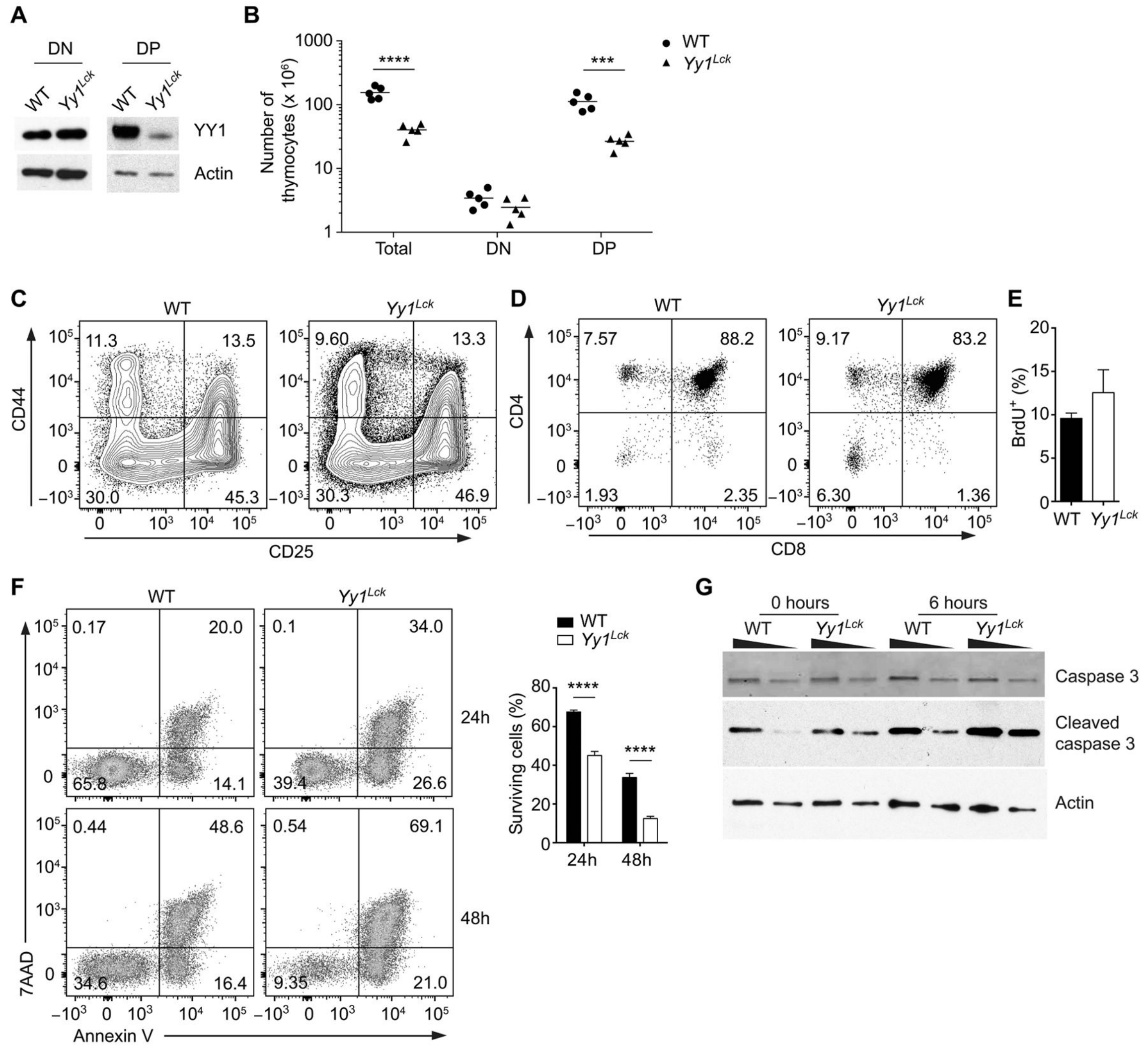

Figure 4. YY1 regulates the survival of DP thymocytes

(A) Western blot of YY1 and actin in purified DN (CD4$^-$CD8$^-$Lin$^-$) and DP thymocytes of *Yy1*$^{f/f}$ (WT) and *Yy1*$^{f/f}$ *Lck*-Cre (*Yy1*^{Lck}) mice. Results are representative of two independent experiments. (B) Numbers of total, DN and DP thymocytes in WT and *Yy1*^{Lck} mice. Each data point represents an individual mouse and the horizontal line indicates the mean. Statistical significance was evaluated by unpaired Student's *t*-test with Holm-Sidak correction for multiple comparisons. (C) CD44 and CD25 staining of WT and *Yy1*^{Lck} thymocytes pre-gated as CD4$^-$CD8$^-$Lin$^-$. Results are representative of three independent experiments. (D) CD4 and CD8 staining of total thymocytes of WT and *Yy1*^{Lck} mice. (E) Frequency of BrdU$^+$ WT and *Yy1*^{Lck} DP thymocytes following a 4 h pulse with BrdU. The mean ± SEM of three independent experiments is shown. (F) Sorted DP thymocytes were cultured *in vitro* for 24 or 48 h and stained with Annexin V and 7AAD (left). Mean ± SEM survival is presented for three WT and four *Yy1*^{Lck} cultures (right). Statistical significance was evaluated by two-way ANOVA with Sidak's multiple-comparison test. (G) Sorted DP thymocytes were analyzed for caspase 3 and cleaved caspase 3 by western blot either immediately *ex vivo* or after 6 h of *in vitro* culture. The wedges indicate 2-fold dilutions of

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cell extract. Data are representative of two independent experiments. ***$P < 0.001$, ****$P < 0.0001$.

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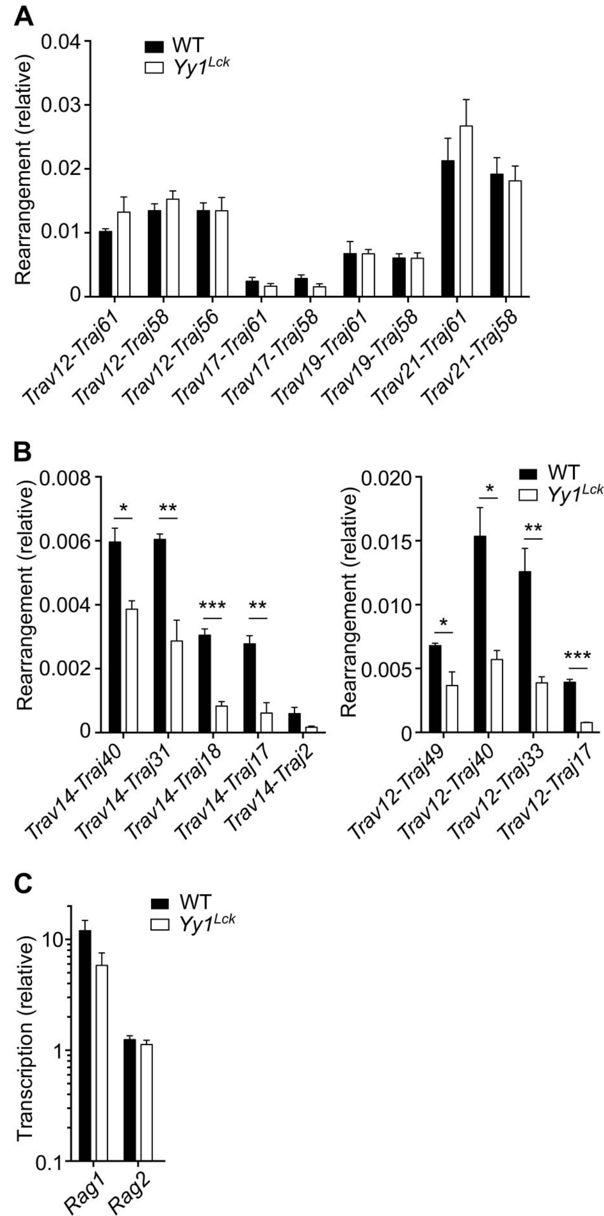

Figure 5. YY1 regulates the TCRα repertoire

Genomic DNA extracted from DP thymocytes from *Yy1*$^{f/f}$ (WT) and *Yy1*$^{f/f}$ *Lck*-Cre (*Yy1*^{Lck}) mice was analyzed for rearrangement of Vα segments to 5'Jα segments (A) and to 3'Jα segments (B) by real-time PCR with normalization to *Cd14*. Data represent the mean ± SEM of three DNA preparations for each genotype, each preparation representing a different mouse. (C) *Rag1* and *Rag2* transcription in DP thymocytes was analyzed by real-time PCR with normalization to *Hprt*. Data represent the mean ± SEM of three cDNA preparations for each genotype, each preparation representing a different mouse. Statistical significance was evaluated by unpaired Student's *t*-test with Holm-Sidak correction for multiple comparisons. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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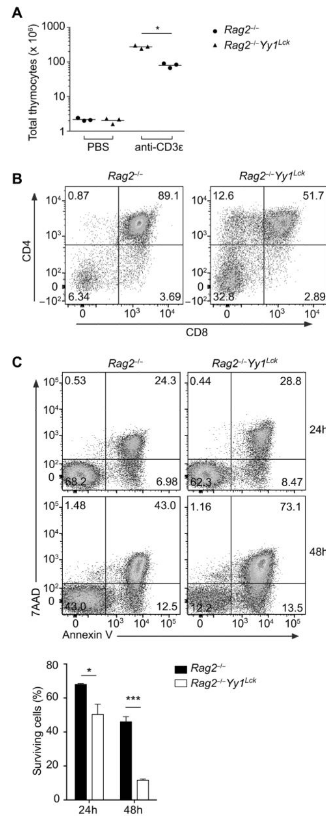

Figure 6. YY1 regulates of DP thymocyte survival independent of V(D)J recombination or TCR expression

Rag2$^{-/-}$ *Yy1*$^{f/f}$ (*Rag2*$^{-/-}$) or *Rag2*$^{-/-}$ *Yy1*$^{f/f}$ *Lck*-Cre (*Rag2*$^{-/-}$ *Yy1*^{Lck}) mice were injected with anti-CD3ε or PBS and thymocytes were analyzed 9 d later. (A) Total number of thymocytes. Each data point represents an individual mouse and the horizontal line indicates the mean. Statistical significance was evaluated by unpaired Student's *t*-test with Holm-Sidak correction for multiple comparisons. (B) CD4 and CD8 staining. Results are representative of three independent experiments. (C) Annexin V and 7AAD staining of DP thymocytes cultured *in vitro* for the indicated time points (top). Mean ± SEM survival (Annexin V^{-}7AAD^{+}) is shown for three WT and three *Yy1*^{Lck} cultures, each from a different mouse (bottom). Statistical significance was evaluated by two-way ANOVA with Sidak's multiple-comparison test. *$P < 0.05$, ***$P < 0.001$.

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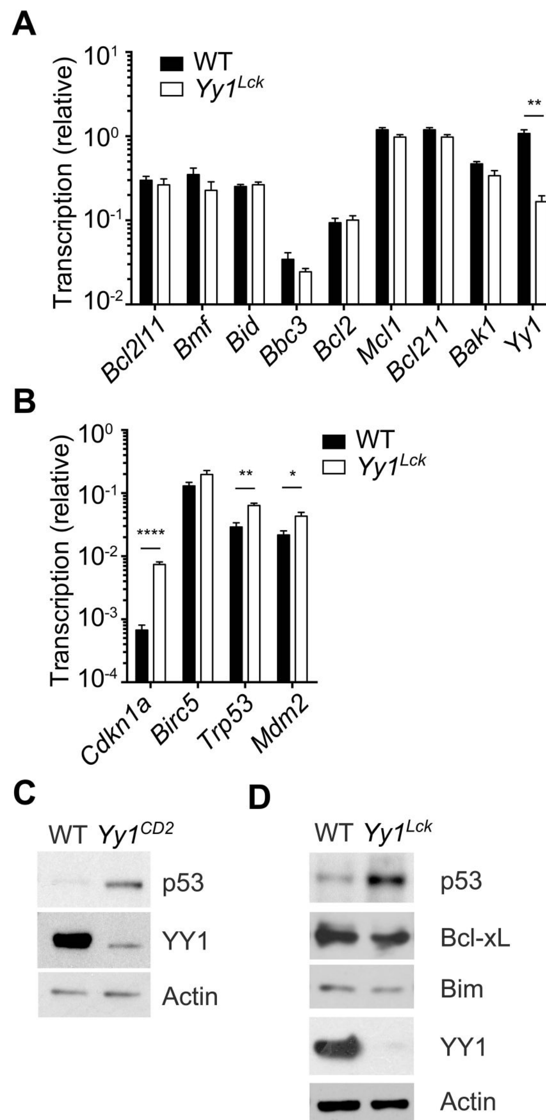

Figure 7. YY1 negatively regulates p53

The abundance of transcripts encoding pro- and anti-apoptotic proteins, YY1, and p21 were analyzed in DP thymocytes of *Yy1*$^{f/f}$ (WT) and *Yy1*$^{f/f}$ *Lck*-Cre (*Yy1*^{Lck}) mice, with normalization to *Hprt* (A) or *Gapdh* (B). Data represent the mean ± SEM of three WT and three *Yy1*^{Lck} preparations, each from a different mouse. Statistical significance was evaluated by unpaired Student's *t*-test with Holm-Sidak correction for multiple comparisons. Sorted DN3 thymocytes from WT or *Yy1*^{CD2} mice (C) or DP thymocytes from WT or *Yy1*^{Lck} mice (D) were analyzed by western blot. Data are representative of three independent experiments. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.

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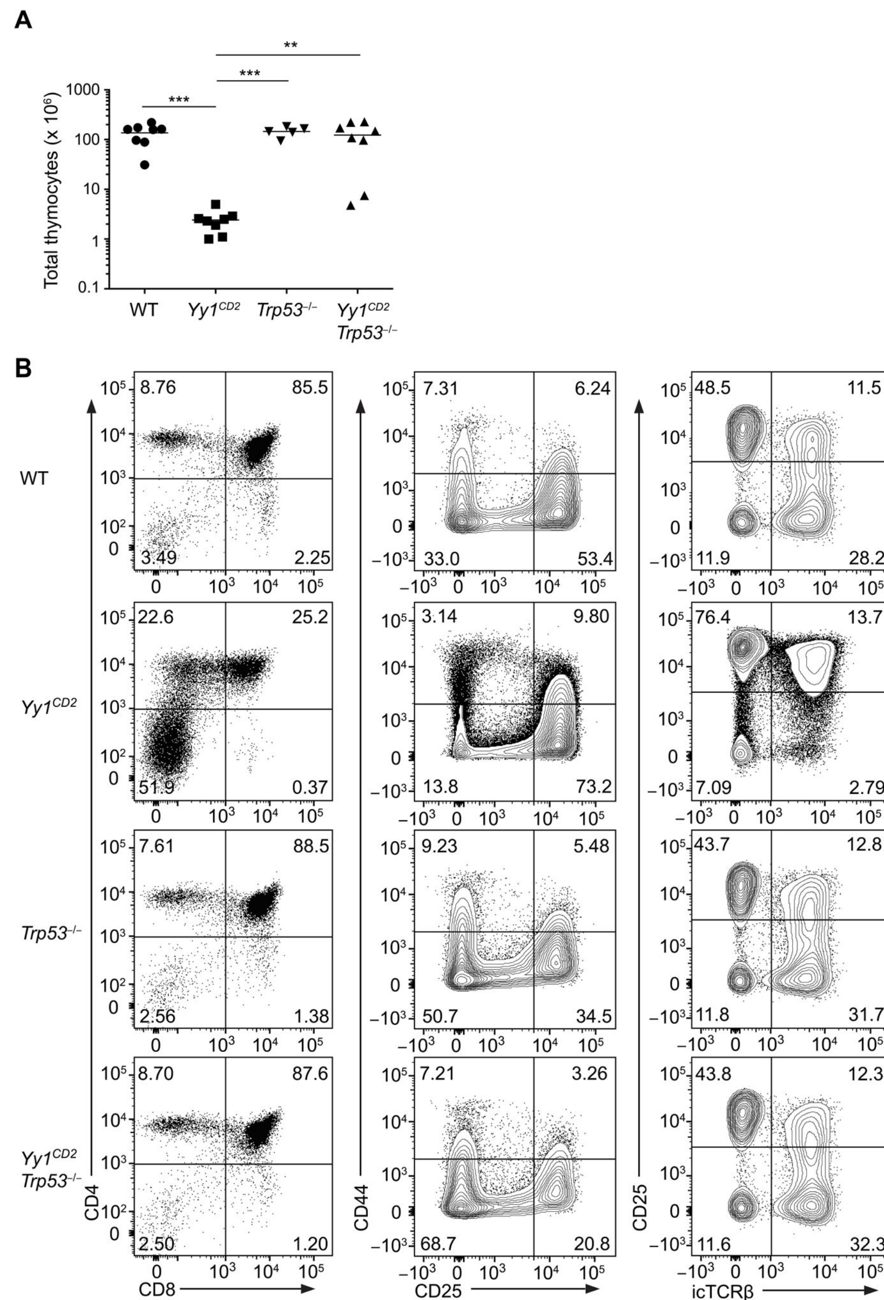

Figure 8. Absence of p53 rescues the developmental defect in *YY1*^{CD2} mice

(A) Number of total thymocytes in *Yy1*$^{f/f}$ (WT), *Yy1*$^{f/f}$ *CD2*-Cre (*Yy1*^{CD2}), *Trp53*$^{-/-}$ and *Yy1*$^{f/f}$ *CD2*-Cre *Trp53*$^{-/-}$ (*Yy1*^{CD2} *Trp53*$^{-/-}$) mice. Each data point represents an individual mouse and horizontal lines indicate the mean. Statistical significance was evaluated by one-way ANOVA with Tukey's multiple-comparison test. (B) Staining of CD4 and CD8 in total thymocytes (left column), of CD44 and CD25 in DN thymocytes (CD4^{-}CD8^{-}Lin^{-}) (middle column), and of CD25 and icTCRβ in DN thymocytes (CD4^{-}CD8^{-}Lin^{-}) (right column) are

shown. The results are representative of two independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$.

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