Sonic hedgehog negatively regulates pre-TCR–induced differentiation by a Gli2-dependent mechanism

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Hedgehog signaling regulates differentiation, survival, and proliferation of the earliest double-negative (DN) thymocytes, but its importance at later stages of T-cell development is controversial. Here we use loss- and gain-of-function mouse models to show that Shh, by signaling directly to the developing thymocyte, is a negative regulator of pre-TCR–induced differentiation from DN to double-positive (DP) cell. When hedgehog signaling was reduced, in the Shh$^{-/-}$ and Gli2$^{-/-}$ thymus, or by T lineage–specific transgenic expression of a transcriptional-repressor form of Gli2 (Gli2ΔC$_2$), differentiation to DP cell after pre-TCR signal transduction was increased. In contrast, when Hh signaling was constitutively activated in thymocytes, by transgenic expression of a constitutive transcriptional-activator form of Gli2 (Gli2ΔN$_2$), the production of DP cells was decreased. Gene expression profiling showed that physiologic Hh signaling in thymocytes maintains expression of the transcription factor FoxA2 on pre-TCR signal transduction. (Blood. 2009;113:5144-5156)

Introduction

The thymus provides a specialized environment that supports the maturation of lymphocyte precursors into functional T cells, and one factor from the thymic epithelium that signals to developing thymocytes is the secreted intercellular signaling protein Sonic hedgehog (Shh).[1] Shh, one of 3 mammalian hedgehog (Hh) proteins, is a morphogen essential for embryogenesis,[2-4] and homeostasis of adult tissues.[5,6] The mammalian Hh proteins trigger a common signaling pathway: They bind their cell-surface receptor Patched, which on Hh interaction releases the Hh-signal transduction molecule Smoothened (Smo) from inhibition, and Smo activates members of the Gli family of transcription factors, Gli1, Gli2, and Gli3.[3,7,8] The Gli proteins bind DNA in a sequence-specific manner and have specialized functions and distinct temporal and spatial expression patterns.[9,10] On Hh signal transduction, they are transported to the nucleus to promote target-gene transcription. Gli1 acts only as a transcriptional activator and is not essential for mouse development.[11] Gli2 and Gli3 can be processed to function as transcriptional activators or transcriptional repressors.[12,13] In the absence of Hh signaling, Gli2 and Gli3 undergo modification by phosphorylation and cleavage of their C-terminal transactivation domain to function as transcriptional repressors.[9,10]

Gli2 and Gli3 are essential for mouse development and have distinct, and partially overlapping, functions.[14] Although Gli2 and Gli3 are bifunctional, Gli2 acts in vivo primarily as a transcriptional activator and is necessary to initiate the first transcriptional changes on Hh signal transduction,[15] whereas Gli3 functions mainly as a negative regulator of transcription.[15,16]

During T-cell development, thymocytes pass through developmental stages defined by expression of cell-surface markers. CD4$^-$CD8$^-$ double-negative (DN) thymocytes differentiate into CD4$^+$CD8$^+$ double-positive (DP) thymocytes, which then mature into CD4$^+$ or CD8$^+$ single-positive cells. The DN population can be further subdivided: the most immature population expresses CD44 but not CD25 (DN1). As these thymocytes mature they gain CD25 expression (DN2), then lose CD44 expression (DN3), eventually becoming negative for both CD44 and CD25 (DN4) before differentiating into DP, often via an immature CD8$^+$ intermediate (CD8ISP).

During the DN3 stage, the TCRβ chain locus is rearranged. Cells that successfully rearrange a TCRβ allele and express a functional TCRβ chain signal through the pre-TCR, causing rearrangement of the other TCRβ locus to be suppressed (allelic exclusion), rescue from apoptosis, proliferation. and up-regulation of CD4 and CD8 expression. This process, known collectively as β-selection, is necessary for the transition from DN to DP thymocyte.[17]

In the mouse thymus, Shh is expressed by some epithelial cells in the subcapsular region, medulla, and corticomedullary junction, and the molecules that allow a cell to respond to Shh are expressed by both lymphoid and stromal components.[1,6,18-23] Analysis of mice mutant for Shh, Gli3, and Smo have shown that Hh signaling is necessary for efficient survival, proliferation, and differentiation of thymocytes at the transition from DN1 to DN2,[18,21,22] but the role of Hh signaling at the transition from DN to DP thymocytes is controversial. Different experimental approaches have produced conflicting results that have led to 3 different interpretations: that Hh is a negative regulator of the pre-TCR signal and differentiation to DP[6,21,24]; that Shh is a positive regulator of the DN to DP transition[18]; or that Hh signaling does not influence thymocyte differentiation after the DN2 stage.[22]

In vitro studies first demonstrated that Hh signaling influences thymocyte development[6,24] and suggested that Hh signaling was a negative regulator of differentiation to DP cell. Treatment of mouse fetal thymus organ cultures (FTOCs) with recombinant Shh protein arrested thymocyte development at the DN3 stage after TCRβ

chain rearrangement, whereas neutralization of endogenous Hh signaling by anti-Hh antibody treatment increased DP production.[6,18] In addition, in anti-CD3–treated Rag1^{−/−} FTOCs, differentiation to DP was arrested or enhanced by Shh or anti-Hh treatment, respectively.[6]

In contrast to these in vitro experiments, ex vivo analysis of Shh^{−/−} fetal thymi on E16.5 suggested that Shh promotes differentiation to the DP stage,[18] as both thymus size and the proportion of DP cells were greatly reduced. Analysis of Gli3 mutants showed that regulation of Hh signaling after pre-TCR signal transduction, via Gli3, is necessary for efficient transition from DN to DP.[21] Depending on whether Gli3 is acting as an activator or repressor, the latter data support either a positive or negative role, respectively, for Hh signaling after pre-TCR signal transduction.

Finally, consistent with no role for Hh at the transition to DP cell, analysis of a conditional T lineage–specific Smo knockout model failed to reveal an influence of Hh after the DN2 stage.[22] Lck-Cre–induced deletion of Smo reduced both the number and relative proportion of DP cells in the adult, but this reduction in DP cells was interpreted as the result of the earlier effects observed on the DN1 and DN2 populations, rather than an effect on or after pre-TCR signaling. In addition, in vivo anti-CD3–induced differentiation of Rag1^{−/−} thymocytes produced similar numbers of DP cells, in both conditional Smo-deficient mice and littermates.[22]

These conflicting interpretations could reflect functional differences in the experimental models used, such as differences between the adult and fetal thymus, or in the case of the Shh^{−/−}, systemic effects of the mutation on the developing embryo. Alternatively, the action of Hh signaling on differentiation to DP could be indirect through another cell type (eg, epithelium), so that when T lineage–specific mutants, such as the conditional Smo knockout, were analyzed no effect was observed. To reconcile all experimental data, we have proposed a model in which Shh promotes thymocyte differentiation before pre-TCR signaling, but negatively regulates differentiation to DP after pre-TCR signal transduction.[1]

Here, to resolve this controversy and provide further experimental evidence, we re-examine the transition from DN to DP in novel fetal and adult mouse models. By differentiating between events before and after pre-TCR signal transduction, we show that Shh, by signaling directly to the developing thymocyte, is a negative regulator of pre-TCR–induced differentiation to DP cell.

Methods

Mice

Gli2^{+/−} mice[14] and Shh^{+/−} mice[25] were on C57BL/6 background. lck-Gli2ΔN₂ and lck-Gli2ΔC₂ transgenic mice were as described.[20,26] For hydrocortisone (HC) treatment, mice were injected intraperitoneally with 0.03 mg per gram of body weight HC (Sigma-Aldrich, St Louis, MO) in PB, and analyzed after 2 to 7 days.[27] The United Kingdom Home Office (London, United Kingdom) approved mouse studies.

PCR analysis

DNA was extracted from tissue by digesting in lysis buffer containing 50 mM KCl, 1.5 mM MgCL₂, 10 mM Tris HCL (pH 8.5), 0.01% gelatin, 0.45% Nonidet P-40, 0.45% Tween20, and 0.5 μg/mL proteinaseK in water. Approximately 1 μg DNA was used per polymerase chain reaction (PCR). Primers for Gli2 gene[14] were oGli2 (Gli2WT) (5′)AAACAAAGCTCCTGT-CACG, (3′)CACCCCAAAGCATGTGTTTT, OpPNT (Gli2mutated) (5′)CACCCCAAAGCATGTGTTTT, (3′)ATGCCTGCTCTTTACTGAAG.

Analysis of TCRβ gene rearrangement by quantitative PCR (Q-PCR), using 5′ primers to Vβ8.2 and Vβ5.1 and a 3′ primer to Jβ2.7, was as described.[28]

Cell sorting

DN1-4 populations were sorted, using anti-CD25[FITC], -CD4/8[PE], and -CD44[Cychrome]. Staining with anti-CD4[PE] and -CD8[Cychrome] allowed sorting of DP and SPs, on MoFlo (Cytomation, Fort Collins, CO).

QRT-PCR for Gli2 RNA expression

RNA was extracted using Absolutely-RNA Miniprep kit (Stratagene, La Jolla, CA) from fluorescence-activated cell sorting (FACS)–sorted cells. cDNA was synthesized using Superscript-II (Invitrogen, Carlsbad, CA). Quantitative reverse-transcription (QRT)–PCR was as described,[21] using primers Gli2F AGAACCTGAAGACACACCTGCG, Gli2R GAGGCAT-TGGAGAAGGCTTTG.

Antibodies and flow cytometry

Cells were stained as described[21] using directly conjugated antibodies from BD PharMingen (San Diego, CA) and analyzed on a FACScan (BD Biosciences). Live cells were gated by FSC/SSC profiles. Data represent more than 3 experiments.

Intracellular staining for TCRβ[27] and phospho-Erk[29] were as described.

FTOC

FTOC was as described[6] with azide-free anti-CD3ε monoclonal antibody (mab) treatment where stated.

PCR arrays with anti-CD3–treated Rag1^{−/−} and Gli2ΔC₂Rag1^{−/−} thymocytes

One lobe of each thymus was cultured overnight in AIM-V medium with 5 μg/mL anti-CD3ε. Thymocytes were extracted from the second, non-treated, control lobe. Thy1.2^{+} cells were separated using the EasySep system (StemCell Technologies, Vancouver, BC), following the manufacturer's instructions. RNA was extracted using Absolutely-RNA kit (Stratagene) following the manufacturer's instructions.

Approximately 250 ng RNA was used for cDNA synthesis using the RT² First strand kit (SuperArray Bioscience, Frederick, MD) following the manufacturer's instructions. cDNA synthesized was used for Mouse Signal Transduction PathwayFinder RT² Profiler PCR Array (SA Biosciences, Frederick, MD). Three arrays were performed with preparations from each of Rag1^{−/−}, Gli2ΔC₂Rag1^{−/−}, Rag1^{−/−} + anti-CD3–treated, and Gli2ΔC₂Rag1^{−/−} + anti-CD3–treated thymocyte samples. ΔCt values for each gene were obtained by subtracting the mean threshold cycle (Ct) of the housekeeping genes (Gusb, HPRT, Hsp90ab1, and Actb) from the Ct value of the gene. Average ΔCt value for 3 experiments was calculated, for each gene, and average normalized transcription was calculated as follows: $2^{-\text{average}\Delta Ct}$. Fold up- or down-regulation of transcription after anti-CD3 treatment was calculated by dividing the average normalized transcription of each gene in the test samples (Rag1^{−/−} + anti-CD3 or Gli2ΔC₂Rag1^{−/−} + anti-CD3) by the corresponding control (Rag1^{−/−} or Gli2ΔC₂Rag1^{−/−}) value. Statistical significance in up- or down-regulation of transcription was determined by Student t test (paired, 2-tailed), comparing the ΔCt values, for each gene, before and after treatment from the 3 experiments performed for Rag1^{−/−} or Gli2ΔC₂Rag1^{−/−} genotypes. To determine significant differences in the response in anti-CD3 treatment between Rag1^{−/−} and Gli2ΔC₂Rag1^{−/−} thymocytes, we performed Student t test (2-sample, equal variance, 2-tailed distribution) for each gene comparing the ΔΔCt ($\Delta\Delta Ct = \Delta Ct_{\text{anti-CD3 treated}} - \Delta Ct_{\text{control}}$) values from the 3 experiments. Significant differences were identified when P was less than .05.
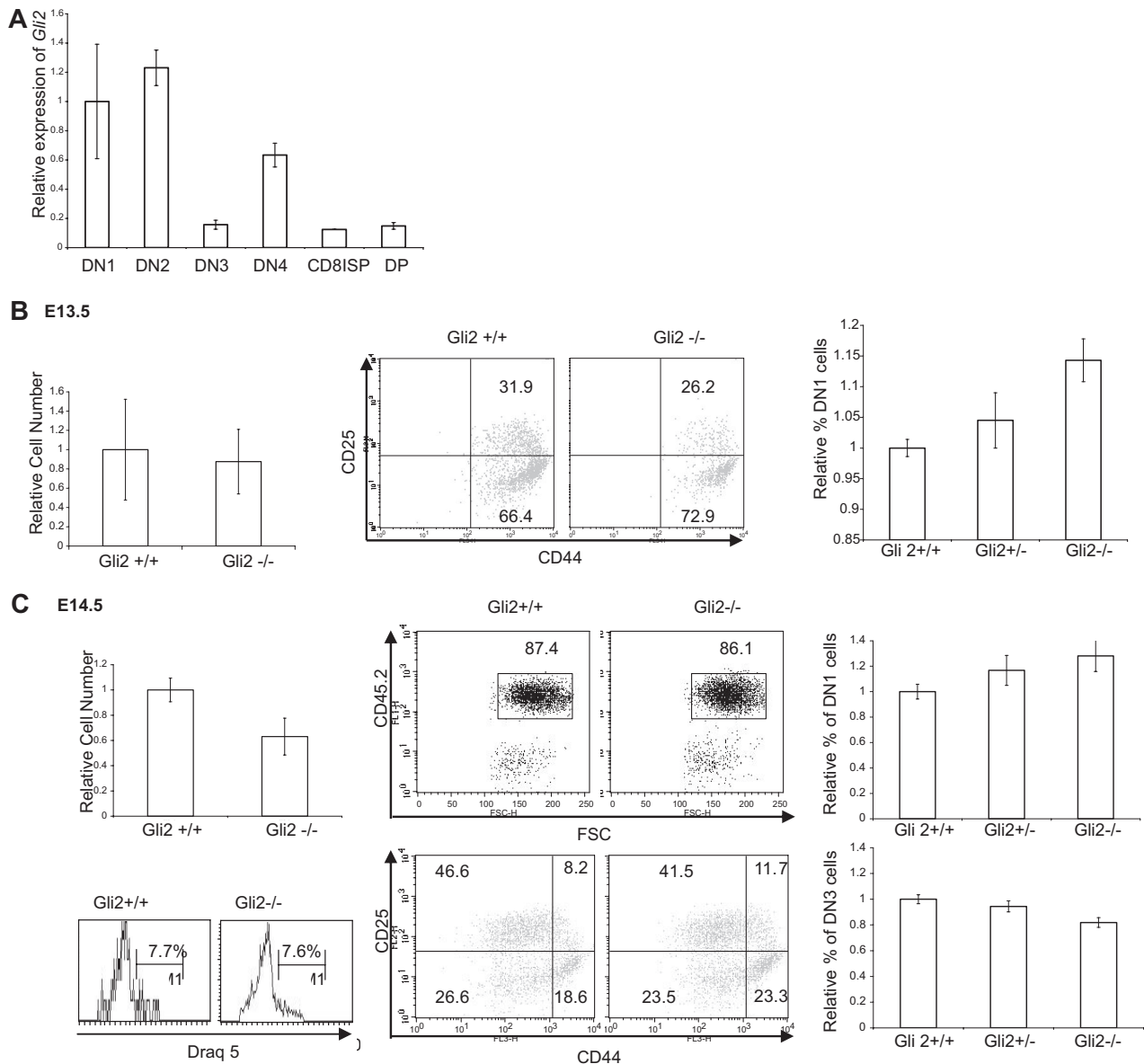
Figure 1. *Gli2* expression in thymocytes on E16.5, and function in E13.5 and E14.5 thymus. (A) *Gli2* expression in sorted C57BL/6 E16.5 thymocytes. Error bars represent SD. (B) Gli2 affects the transition from DN1 to DN2 in E13.5 embryos (WT, n = 5; Gli2^{+/−}, n = 12; Gli2^{−/−}, n = 8). Relative number of thymocytes on E13.5. To allow comparison between litters, the number of cells in each thymus was divided by the mean of WT littermates to give a relative cell number for each thymus. Dot plots show DN subset composition of E13.5 thymi. Cells were gated for CD45.2[+], and analyzed by CD25 and CD44 expression. Bar chart shows the relative percentage of DN1 cells in E13.5 thymi. The difference between WT and Gli2^{−/−} is statistically significant (*P* = .02). (C) Thymocyte development in E14.5 Gli2^{−/−} and littermate embryos (WT, n = 17; Gli2^{+/−}, n = 27; Gli2^{−/−}, n = 15). Bar chart shows the relative thymocyte number on E14.5. The difference between WT and Gli2^{−/−} is significant (*P* = .04). Top dot plot shows E14.5 thymocytes stained with anti-CD45.2. Cells positive for CD45.2[+] were analyzed for CD25 and CD44 expression. Righthand bar charts show the relative percentage of DN1 and DN3 cells in E14.5 thymi. Differences in mean relative percentages of DN1 and DN3 populations were statistically significant between WT and Gli2^{−/−} (*P* = .04 and *P* = .002, respectively). Histograms show Draq5 staining of E14.5 thymocytes. The percentage of cells in G2+S/M is given.

Results

Gli2 is expressed differentially in fetal DN subsets

Gli2 is essential to initiate the Hh signal and activate transcription of target genes.[15] Therefore, to investigate the function of Hh pathway activation in developing DN cells, we followed thymocyte development in Gli2^{−/−} embryos on sequential embryonic days. Gli2-deficient embryos die just before birth, and in contrast to Shh^{−/−} embryos,[25] are of fairly normal size and appearance,[14] so effects on thymocyte development are unlikely to be due to systemic differences in embryo growth or survival.

We analyzed the expression pattern of *Gli2* in developing fetal thymocyte populations. Components of the Hh signaling pathway, including the Gli proteins, are differentially expressed in adult thymocyte populations.[6,19-22] In the fetal thymus, *Gli3* is differentially expressed in DN cells, with expression in the DN1 population, down-regulation in the subsequent DN2 and DN3 stages, and highest expression in the DN4 population.[21]

Thymocyte populations were FACS-sorted from E16.5 fetal thymi and transcription of *Gli2* was analyzed by QRT-PCR (Figure 1A). *Gli2* was most highly expressed in DN1 and DN2 cells, down-regulated at the DN3 stage, and up-regulated more than 3-fold in the DN4 population. This expression pattern suggests that
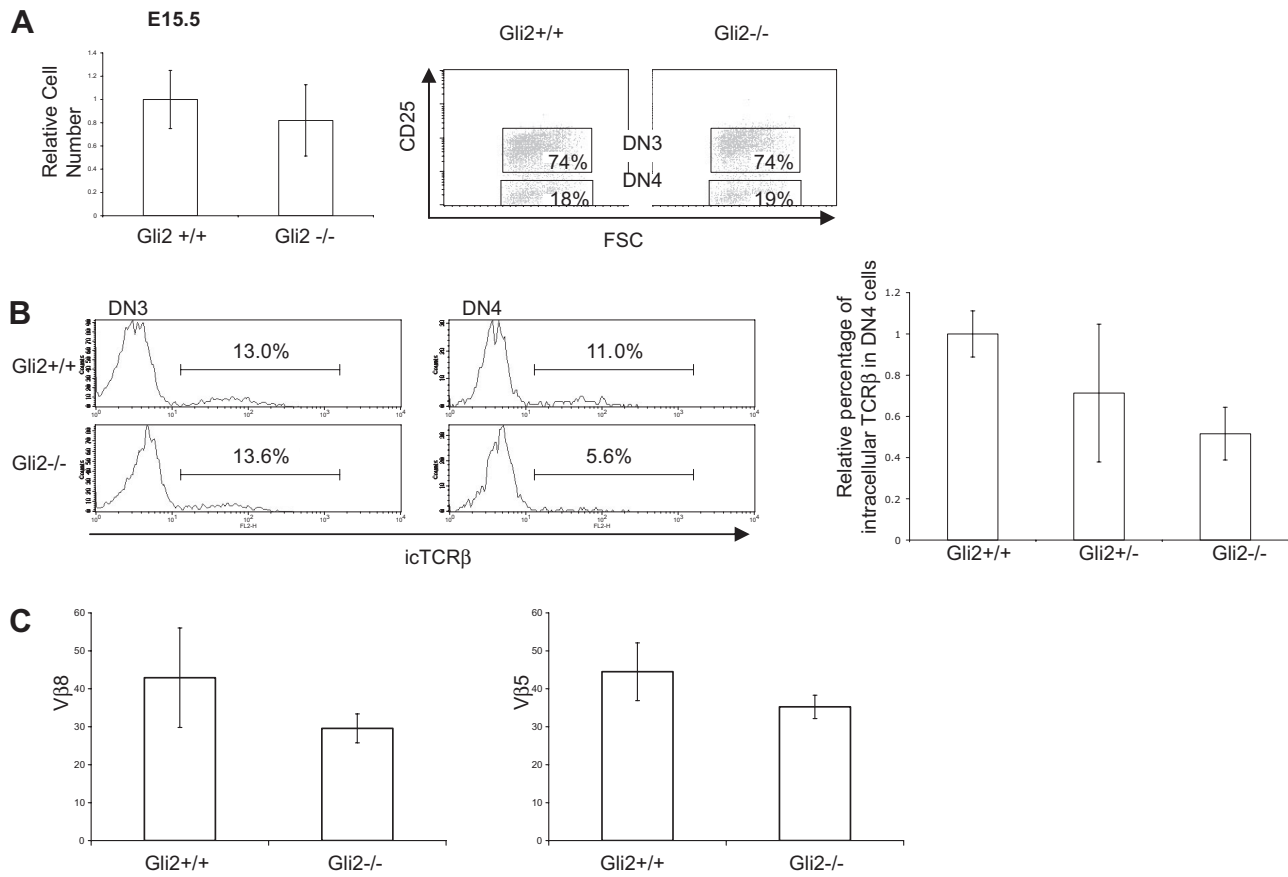
Figure 2. Gli2 function in the E15.5 thymus. (A) Phenotype of E15.5 Gli2$^{-/-}$ thymi (WT, n = 8; Gli2$^{+/-}$, n = 9; Gli2$^{-/-}$, n = 7). Bar chart shows the relative number of thymocytes on E15.5; error bars are SD. The difference between WT and Gli2$^{-/-}$ was not statistically significant. Dot plot shows the relative proportions of DN3 and DN4 populations: Cells negative for CD3, CD4, CD8, and CD44 were analyzed for CD25 expression. Cells positive for CD25 (DN3) and negative for CD25 (DN4) were gated separately. There were no significant differences in the proportion of DN3 and DN4 cells on E15.5. (B) Intracellular TCRβ (icTCRβ) expression in the E15.5 WT and Gli2$^{-/-}$ thymus. Histogram plots of icTCRβ staining gated on DN3 (left) and DN4 (right) cells. Bar chart shows the relative percentage of icTCRβ on DN4 cells in E15.5 thymi. The difference between WT and Gli2$^{-/-}$ was statistically significant (P = .02). (C) Bar charts to show degree of *Vβ8* to *Jβ2.7* (left chart) and *Vβ5* to *Jβ2.7* (right chart) rearrangement in E15.5 WT and Gli2$^{-/-}$ thymocytes, assessed by Q-PCR.

Gli2 is likely to function early in thymocyte development at DN1/DN2 (as has been shown for Shh, Gli3, and Smo[18,21,22]) and in the DN4 population. We therefore assessed the impact of *Gli2* mutation on these 2 developmental stages by analyzing thymocyte development from E13.5 to just before birth.

Inefficient differentiation from DN1 to DN2 in Gli2$^{-/-}$ thymus

In embryos, thymocyte development occurs in a more or less synchronized wave facilitating analysis of specific stages of development on different embryonic days, and on E13.5 the majority of cells are DN1 and DN2 (Figure 1B). On E13.5, the difference in cell number between Gli2$^{-/-}$ and WT littermate thymi was not significant (Figure 1B), but the proportion of DN1 cells was significantly increased (P = .02) in the Gli2$^{-/-}$ compared with WT (Figure 1B), and the proportion of DN2 cells was concomitantly decreased. There were no differences in anti-ckit or anti-B220 staining on the CD45$^+$CD44$^+$ population between WT and Gli2$^{-/-}$ (data not shown).

We assessed thymocyte development on E14.5, to ask whether the inefficient DN1 to DN2 transition observed on E13.5 impacted on subsequent developmental stages. On E14.5, thymocyte number was significantly reduced, and in addition to the increase in the DN1 population, the proportion of DN3 cells was significantly reduced, indicative of a developmental delay (Figure 1C). There

was no significant difference in the proportion of thymocytes in G2+S/M as assessed by Draq5 staining (Figure 1C).

On E15.5 there was no significant difference in cell number, or in the proportions of DN3 or DN4 cells (Figure 2A). To test for TCRβ expression, we measured intracellular TCRβ (icTCRβ) protein in DN populations. In the DN3 population, the proportion of icTCRβ$^+$ was similar for both the Gli2$^{-/-}$ and WT littermates, typically around 13% (Figure 2B). In contrast, the proportion of icTCRβ$^+$ cells was reduced in the Gli2$^{-/-}$DN4 population compared with WT (Figure 2B). During fetal thymocyte development, TCRβ expression is not necessary for transition from DN3 to DN4 and the majority of the DN4 population do not express icTCRβ until E17.5.[27] We therefore measured *TCRβ* gene rearrangement using a Q-PCR assay,[28] using 5′ primers to *Vβ8.2* and *Vβ5.1* and a 3′ primer to *Jβ2.7* (Figure 2C). We did not detect a statistically significant reduction in either *Vβ8.2* to *Jβ2.7* or *Vβ5.1* to *Jβ2.7* rearrangement in the Gli2$^{-/-}$ DN cells, relative to WT, suggesting that the reduction in icTCRβ staining in the DN4 population was most likely the consequence of delayed progression of thymocyte development.

Reduced transition from DN to DP in E16.5 Gli2$^{-/-}$ mice

DP cells first appear on E16.5, so to assess the impact of *Gli2* mutation on differentiation to DP we analyzed E16.5 Gli2$^{-/-}$
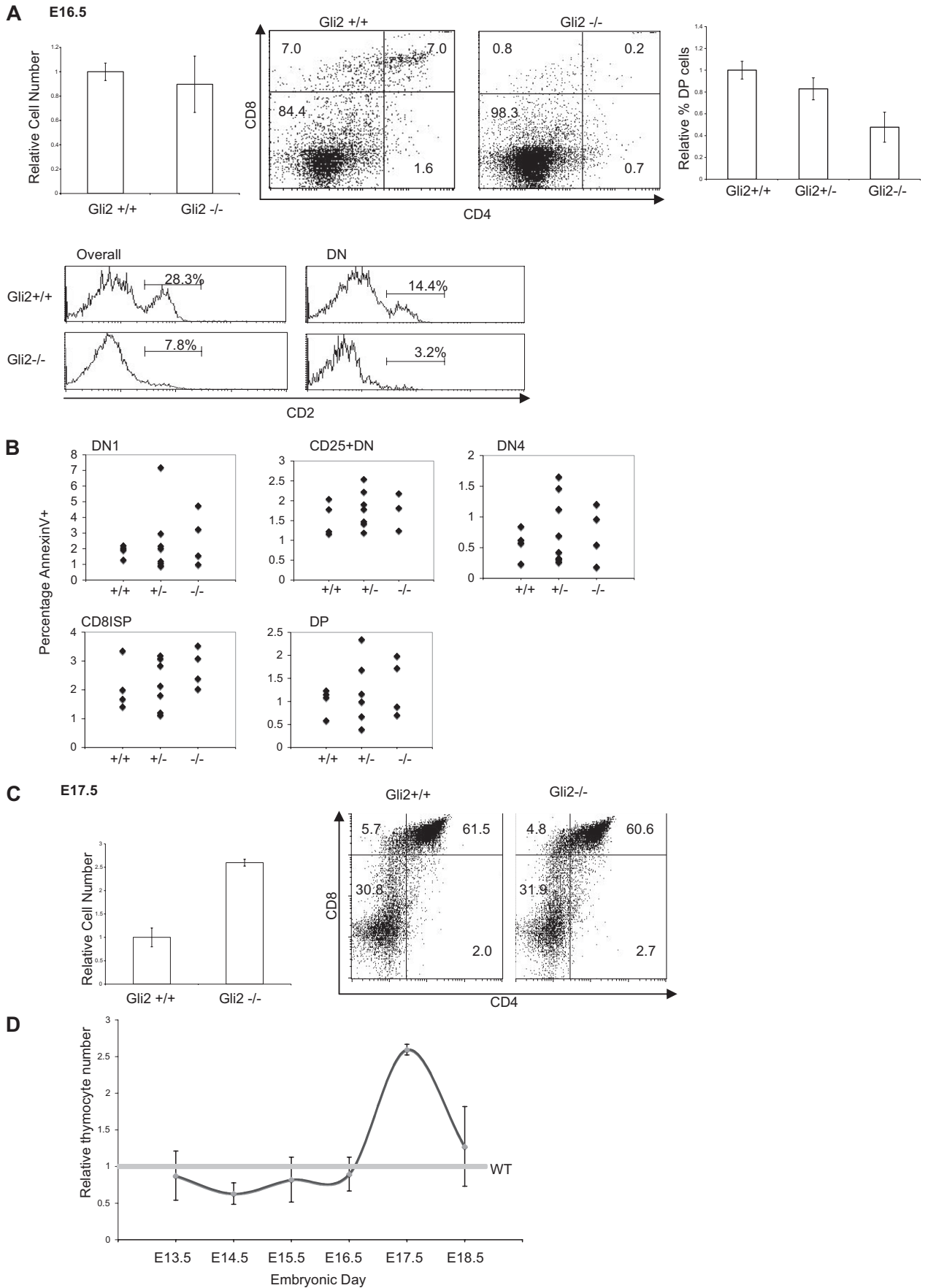
Figure 3.

thymi. There was no significant difference in thymocyte number on E16.5 between the Gli2$^{-/-}$ and WT littermates, but the proportions of DP and ISP cells were significantly reduced (Figure 3A), and the DN population was increased.

We also found a reduction in the developmentally regulated marker CD2.[30,31] In Gli2$^{-/-}$ thymocytes, cell-surface expression of CD2 was reduced from 28.3% in WT to 7.8%. This reduction did not simply reflect the decrease in DP cells, as when we gated on the DN population CD2 expression was reduced from 14.4% to 3.2% (Figure 3A).

Given that we were unable to detect a difference in cell-cycle status between Gli2$^{-/-}$ and WT in the DN populations (Figure 1C), but the number of DP and ISP cells was reduced relative to WT in the E16.5 Gli2$^{-/-}$ thymus, we asked whether Gli2 is necessary for efficient survival of DN, ISP, or DP populations. We assessed apoptosis by annexin V staining in DN1, CD25$^+$DN, DN4, CD8ISP, and DP populations on E16.5 (Figure 3B). We found no significant differences in the mean percentages of annexin V$^+$ cells between E16.5 WT, Gli2$^{+/-}$, and Gli2$^{-/-}$ littermates in any thymocyte population. This is in contrast to the Shh$^{-/-}$ thymus, in which apoptosis is increased relative to WT in the E16.5 DN4 population,[18] and suggests that the functions of Gli2 downstream of Shh signaling in thymocytes are partially redundant with Gli1 or Gli3, as has been shown in other tissues.[14]

Accelerated differentiation to DP population after pre-TCR signaling

As we found that Gli2 was not necessary for thymocyte survival (Figure 3B), it seemed likely that the reduction in the DP population observed on E16.5 was a consequence of an earlier requirement of Gli2 (and Hh signaling). We therefore asked whether the DP population could recover. On E17.5 there were approximately 2.5 times more DP thymocytes in the Gli2$^{-/-}$ thymus than in WT ($P = .025$; Figure 3C), indicating that after pre-TCR signal transduction, Gli2$^{-/-}$ thymocytes expand and differentiate more quickly than their WT counterparts.

From E13.5 to E16.5 the Gli2$^{-/-}$ thymus was on average slightly smaller than that of WT littermates, but by E17.5 it rapidly expanded to contain 2.5-times more thymocytes than WT, and remained on average larger on E18.5 (Figure 3D). The expansion of the Gli2 deficient thymus corresponded to the day of embryonic development on which icTCRβ chain expression in the DN populations was approaching adult levels[27] and capable of pre-TCR signal transduction. This presumably could not be detected on E16.5, because of low pre-TCR complex expression[27] and the earlier developmental delay. These data indicate that Hh signaling, via Gli2, is a negative regulator of thymocyte expansion and differentiation to DP cell, after pre-TCR signal transduction, during fetal development (Figure 3D).

Hh pathway activation impedes reconstitution of the DP pool in adult mice

Analysis of T-lineage–specific Smo conditional knockout mice has not revealed an influence of Hh signaling at the transition from DN to DP in the adult thymus,[22] but this ex vivo analysis of Gli2$^{-/-}$ embryonic thymus and previous in vitro experiments using fetal thymus have indicated that Hh signaling is a negative regulator of the transition.[6,21,24] Differences in the molecular regulation of thymocyte development between adult and fetus might account for this discrepancy. We therefore investigated the impact of Hh signaling on the production of DP cells in the adult thymus. Gli2-deficient embryos die before birth,[14] so we used mouse models in which Hh signaling is specifically repressed or activated in T-lineage cells, by transgenic expression of activator-only or repressor-only truncated forms of Gli2, under the control of the *lck* promotor.[13,20,26,32] Our earlier work showed that these transgenics were successful at up-regulating (Gli2ΔN$_2$ [constitutively active Hh signal]) or down-regulating (Gli2ΔC$_2$ [constitutive repressor of Hh signaling]) Hh target genes in T-lineage cells. However, our previous studies of the Gli2ΔN$_2$ and Gli2ΔC$_2$ thymi had focused on later stages of T-cell development, and relative differences in the proportion of the DP population were small and were effected by changes at the DP to SP transition.[20,26]

Therefore, to examine differentiation from DN to DP in a synchronized wave, we used treatment with hydrocortisone (HC) to deplete the adult thymus by inducing apoptosis in all but the most mature cells.[27] We then followed the recovery of the DP population, as the thymus grows to recover its normal size and subset distribution during the week after treatment. This strategy has previously been used to analyze the developmental sequence and timing of transition between thymocyte populations,[27,33] allowing analysis of adult thymocyte differentiation in a synchronized wave, without resorting to the production of radiation chimeras. It facilitated detection of differences in the rate of differentiation to DP, which in adult mice is complicated by the fact that the adult thymus has reached steady state, so the transition is no longer synchronized, and there are pre-existing cells at all stages of development and homeostasis between populations. Adult Gli2ΔN$_2$, Gli2ΔC$_2$, and their respective WT littermates were injected with HC and analyzed after 2 to 6 days (Figure 4).

In the case of the Gli2ΔN$_2$ experiment, the DP population was depleted in both Gli2ΔN$_2$ and WT littermates, to approximately 4% DP, with 7% to 8% DN, 2 days after HC treatment (Figure 4A). The Gli2ΔN$_2$ thymi then produced DP cells at a slower rate than those of their WT littermates. Four days after HC treatment, the Gli2ΔN$_2$ thymus contained 16.1% DP cells compared with 31.4% DP cells in the WT. After 5 days, production of DP cells in the Gli2ΔN$_2$ transgenic was still significantly impeded ($P = .01$) with the WT

Figure 3. Reduced transition from DN to DP in E16.5 Gli2$^{-/-}$ mice. (A) Thymocyte development on E16.5 (WT, n = 7; Gli2$^{+/-}$, n = 15; Gli2$^{-/-}$, n = 7). Left bar chart shows relative number of thymocytes on E16.5. Error bars show SE. The difference between WT and Gli2$^{-/-}$ was not statistically significant. Dot plots show anti-CD4 and anti-CD8 staining on E16.5 thymocytes. Right bar chart shows the relative percentage of DP cells in E16.5 thymi. The difference between WT and Gli2$^{-/-}$ was statistically significant ($P = .007$). Histograms show CD2 expression in E16.5 thymi. Left panels: CD2 expression on all thymocytes. Right panels: CD2 expression on DN cells. The difference in percentage of CD2$^+$ cells overall and on DN cells between Gli2$^{+/+}$ and Gli2$^{-/-}$ thymi were statistically significant ($P = .01$ and $P = .04$, respectively). (B) Apoptosis in thymocyte populations on E16.5, measured by annexin V staining. Scatterplots show the percentage of annexin V$^+$ cells in DN1, CD25$^+$DN, DN4, CD8ISP, and DP thymocyte populations in WT (n = 4), +/− (n = 8), and −/− (n = 4) embryos. Mean percentage positive was: DN1 WT, 1.85; +/−, 2.29; −/−, 2.62. CD25$^+$DN WT, 1.55; +/−, 1.79; −/−, 2.0. DN4 WT, 0.57; +/−, 0.78; −/−, 0.72. CD8ISP WT, 2.10; +/−, 2.19; −/−, 2.75. DP WT, 1.01; +/−, 1.21; −/−, 1.32. There were no significant differences in the mean number of annexin V$^+$ cells between genotype of mice in any thymocyte population. (C) Thymocyte development on E17.5 (WT, n = 7; Gli2$^{+/-}$, n = 4; Gli2$^{-/-}$, n = 7). Relative thymocyte number on E17.5. The difference between WT and Gli2$^{-/-}$ was statistically significant ($P = .02$). Dot plots show anti-CD4 and anti-CD8 staining for E17.5 thymocytes. (D) Relative thymocyte number from E13.5 to E18.5, normalized to mean WT littermate value of 1.0. (For E18.5: WT, n = 5; Gli2$^{+/-}$, n = 5; Gli2$^{-/-}$, n = 5.) Error bars show SD.
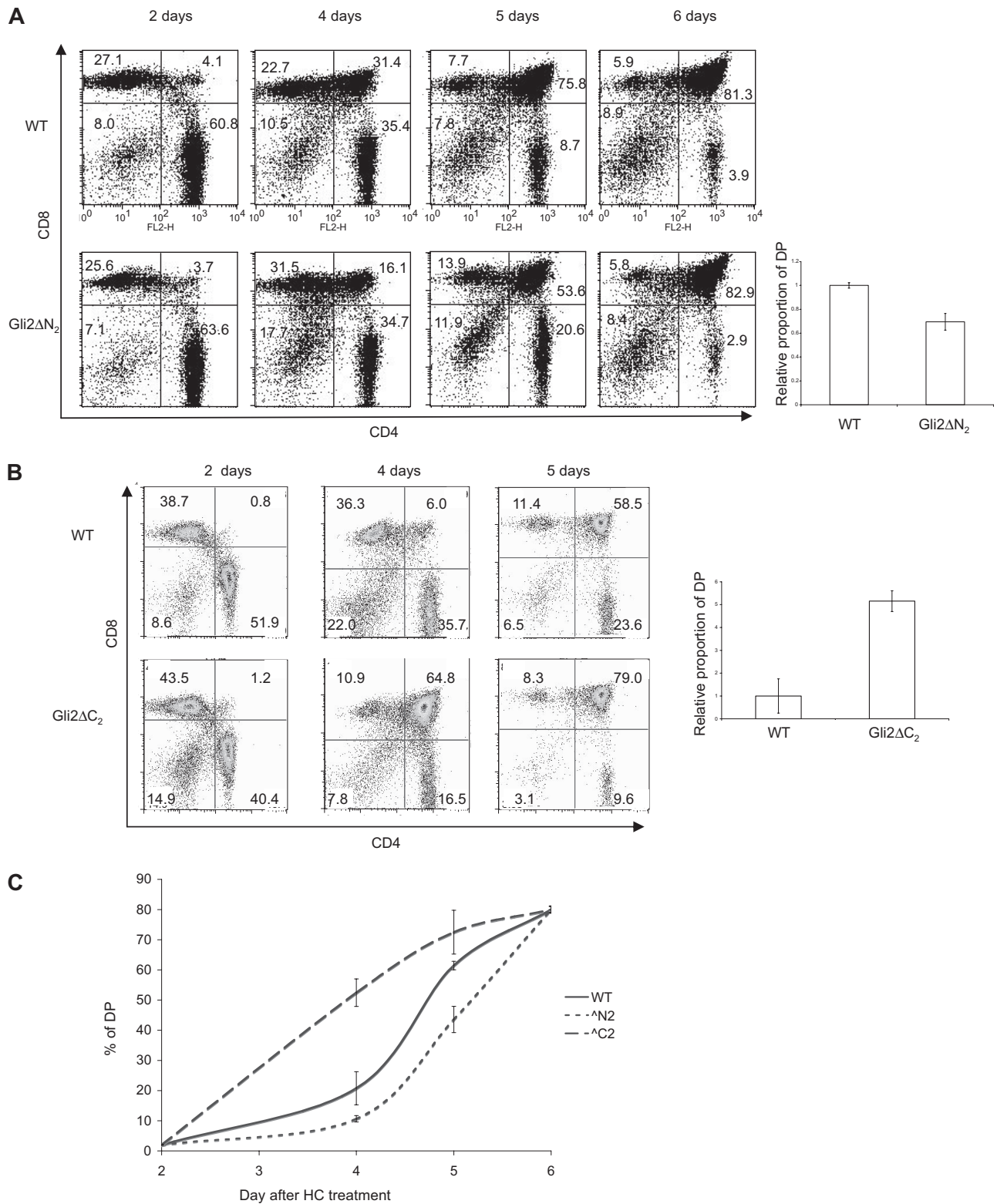
Figure 4. Thymocyte recovery after hydrocortisone treatment in Gli2ΔN₂ and Gli2ΔC₂ transgenic mice. (A) Dot plots show anti-CD4 and anti-CD8 staining for Gli2ΔN₂ transgenic thymocytes and WT littermate thymocytes 2 to 6 days after treatment with HC. Bar chart shows the mean relative proportion of DP cells 5 days after HC treatment for WT and Gli2ΔN₂ transgenic mice. The difference in mean percentage of DP cells was statistically significant (P = .01). (B) Dot plots show anti-CD4 and anti-CD8 staining for Gli2ΔC₂ transgenic thymocytes and WT littermate thymocytes 2 to 5 days after HC treatment. Bar chart shows the mean relative proportion of DP cells 4 days after HC treatment. The difference in DP percentage was statistically significant (P = .004). (C) Graph of relative kinetics of DP recovery after HC treatment in WT, Gli2ΔN₂, and Gli2ΔC₂. Error bars represent SD. (Day 2: WT, n = 6; Gli2ΔN₂, n = 3; Gli2ΔC₂, n = 3. Day 4: WT, n = 7; Gli2ΔN₂, n = 3; Gli2ΔC₂, n = 3. Day 5: WT, n = 7; Gli2ΔN₂, n = 3; Gli2ΔC₂, n = 3. Day 6: WT, n = 5; Gli2ΔN₂, n = 3; Gli2ΔC₂, n = 2.)

DP population recovering to around 75.7%, compared with 53.7% in the Gli2ΔN$_2$ transgenic (Figure 4A).

In contrast, the Gli2ΔC$_2$ transgenic thymi produced DP cells more quickly than WT. In a typical experiment, the DP population was depleted 2 days after HC treatment, but after 4 days 64% of Gli2ΔC$_2$ thymocytes were DP cells, compared with 6% of WT cells ($P = .004$; Figure 4B). After 5 days, the Gli2ΔC$_2$ thymus contained a normal proportion of DP cells, compared with 58.5% in the WT (Figure 4B).

Six days after HC treatment, the DP populations in all genotypes of mice in all experiments had recovered to around 80% (Figure 4C). The relative kinetics of DP reconstitution after HC treatment (Figure 4C) demonstrated that whereas constitutive activation of Hh signaling (Gli2ΔN$_2$) impeded differentiation to DP, repression of Hh signaling in thymocytes (Gli2ΔC$_2$) enhanced DP cell production, in the adult thymus in vivo.

Hh signaling is a negative regulator of pre-TCR signal, reducing ERK phosphorylation

Our data indicate that the Hh signaling pathway is a negative regulator of the transition from DN to DP in vivo in both the fetal and adult thymus. As pre-TCR signal transduction is essential for this stage of development, we used Rag1$^{-/-}$ E15.5 FTOC in which anti-CD3 mab treatment mimics the pre-TCR signal,[34,35] to investigate the impact of Hh signaling on pre-TCR signal transduction. We treated Shh$^{-/-}$Rag1$^{-/-}$ and Rag1$^{-/-}$ littermate FTOC with anti-CD3 and analyzed differentiation to DP after 3 days. Consistent with the phenotype of E13.5 Shh$^{-/-}$Rag$^{+/+}$ embryos,[18] before culture the Shh$^{-/-}$Rag1$^{-/-}$ thymi were smaller and contained a greater proportion of DN1 cells than Rag1$^{-/-}$ littermate thymi (Figure 5A). Propidium iodide staining showed that fewer thymocytes were in S+G2/M in the Shh$^{-/-}$Rag1$^{-/-}$ (12.7%) than in Rag1$^{-/-}$ (20.3%) littermate thymus (Figure 5B). After anti-CD3 treatment, the Shh$^{-/-}$Rag1$^{-/-}$ thymocytes expanded and differentiated to DP faster than the anti-CD3–treated control Rag1$^{-/-}$ thymocytes (Figure 5C). The proportion of DP cells was increased from 16.1% in the Rag1$^{-/-}$ FTOC to 25.3% in the Shh$^{-/-}$Rag1$^{-/-}$ FTOC. As expected, anti-CD3 treatment stimulated proliferation, and the proportion of thymocytes in S+G2/M was increased to 30.8% in the anti-CD3–treated Shh$^{-/-}$Rag1$^{-/-}$ FTOC and 27.3% in the anti-CD3–treated Rag1$^{-/-}$ littermate FTOC. To test whether Gli2 functions downstream of Shh at this stage of thymocyte development, we also compared differentiation to DP cell in anti-CD3–treated Gli2$^{-/-}$Rag1$^{-/-}$ FTOC and littermate Rag1$^{-/-}$ FTOC. The anti-CD3–treated Gli2$^{-/-}$Rag1$^{-/-}$ FTOC differentiated more quickly than their Rag1$^{-/-}$ littermate counterparts, and in a typical experiment, 28.9% of thymocytes were DP in the Gli2$^{-/-}$Rag1$^{-/-}$ FTOC, compared with 16.4% in littermate Rag1$^{-/-}$ FTOC (Figure 5D), confirming that Gli2 is downstream of Shh as a negative regulator of differentiation and that it functions as an activator of transcription at this developmental stage.

The accelerated differentiation to DP in the anti-CD3–treated Shh$^{-/-}$Rag1$^{-/-}$ FTOC appeared to contrast with the phenotype of the E16.5 Shh$^{-/-}$ thymus, which like the E16.5 Gli2$^{-/-}$ thymus, contained fewer DP cells than WT.[18] It demonstrates that Shh is a negative regulator of pre-TCR–induced differentiation to DP, and therefore indicates that in the E16.5 Shh$^{-/-}$ thymus, as seen in the E16.5 Gli2$^{-/-}$ thymus, the reduction in DP cells was the result of an earlier effect on thymocyte development.

Given that T lineage–specific conditional deletion of Smo from Rag-deficient thymocytes has been previously shown to have no effect on differentiation to DP on anti-CD3 treatment,[22] it was possible that the effect of Shh deletion on thymocyte differentiation was indirect via

another cell type, and not the result of reduction of Hh pathway activation in developing thymocytes. To test this, we treated FTOC from Gli2ΔN$_2$Rag1$^{-/-}$ or Gli2ΔC$_2$Rag1$^{-/-}$ embryos and their respective Rag1$^{-/-}$ littermates for 3 days with anti-CD3, to follow the differentiation of thymocytes in which Hh signal transduction was either specifically activated or specifically repressed.

In the case of the Gli2ΔN$_2$Rag1$^{-/-}$ FTOC, where Hh signaling is constitutively active in thymocytes, after treatment with anti-CD3 both thymocyte number and pre-TCR–induced differentiation to DP cell were significantly reduced compared with Rag1$^{-/-}$ littermates (Figure 6A). In the Gli2ΔN$_2$Rag1$^{-/-}$ FTOC, 7.2% of thymocytes were DP compared with 23.3% in the Rag1$^{-/-}$ control FTOC (Figure 6A). This reduction in anti-CD3–induced differentiation showed that Hh pathway activation inhibited pre-TCR signaling in a thymocyte-autonomous manner.

We measured MAPKinase pathway activation on pre-TCR signal transduction, using a FACS-based assay[29] to compare icERK phosphorylation between Gli2ΔN$_2$Rag1$^{-/-}$ and Rag1$^{-/-}$ thymocytes stimulated with anti-CD3 (Figure 6B). On pre-TCR stimulation, there was a clear shift in icphospho-ERK levels in the Rag1$^{-/-}$ thymocytes, with an approximately 2-fold increase in mean fluorescence intensity. In contrast, the shift in anti–icphospho-ERK fluorescence was negligible in the Gli2ΔN$_2$Rag1$^{-/-}$ thymocytes (Figure 6B) on pre-TCR stimulation.

Thymocyte-specific repression of Hh signaling in the Gli2ΔC$_2$Rag1$^{-/-}$ FTOC had the opposite effect on pre-TCR–induced differentiation to DP, and promoted the transition. The proportion of DP cells in the anti-CD3–treated Gli2ΔC$_2$Rag1$^{-/-}$ FTOC was increased to 39.4%, compared with 28.1% in the control anti-CD3–treated Rag1$^{-/-}$ FTOC (Figure 6C), but there was no significant difference in thymocyte number between the 2 genotypes of FTOC (Figure 6D). As expected from the Gli2ΔN$_2$Rag1$^{-/-}$ experiment, icERK phosphorylation was increased on pre-TCR stimulation of Gli2ΔC$_2$Rag1$^{-/-}$ thymocytes compared with Rag1$^{-/-}$ littermate thymocytes (Figure 6E).

Thus, the fact that constitutive repression or activation of Hh signaling in thymocytes has opposing effects on pre-TCR–induced differentiation to DP confirms that Hh signaling is a direct negative regulator of pre-TCR–induced differentiation via the MAPkinase cascade.

Identification of transcriptional targets of pre-TCR signal transduction

To identify genes that are transcriptionally targeted on pre-TCR signal transduction under physiologic conditions, or on repression of Hh signaling, we used a QRT-PCR array approach to profile the expression of 84 genes representative of different signal transduction pathways. RNA was prepared from control and anti-CD3–treated thymocytes purified from Gli2ΔC$_2$Rag1$^{-/-}$ and Rag1$^{-/-}$ littermate thymi for QRT-PCR analysis. We repeated this experiment 3 times, with independent groups of littermate mice, and identified genes whose transcription was significantly altered by pre-TCR signal transduction (Figure 7). This strategy identified 6 genes that were up-regulated on anti-CD3 treatment, and that were not differentially regulated between Gli2ΔC$_2$Rag1$^{-/-}$ and Rag1$^{-/-}$ thymocytes: the TCR signal modulator *CD5*, the transcription factor *Tcf7*, the EGR1-binding protein, *Nab2*, the antiapoptotic factor *Birc3*, *Fas ligand*, and the transcriptional regulator *Nrip1* (Figure 7A). The identification of *CD5* and *Tcf7* thus validated the experimental approach, as cell-surface CD5 is known to be up-regulated on pre-TCR signal transduction[36] and Tcf7 expression is increased on pre-TCR signaling.[37] The up-regulation of the EGR1-binding protein, *Nab2,* is of interest, given that EGR1 promotes thymocyte development at β-selection.[38,39]
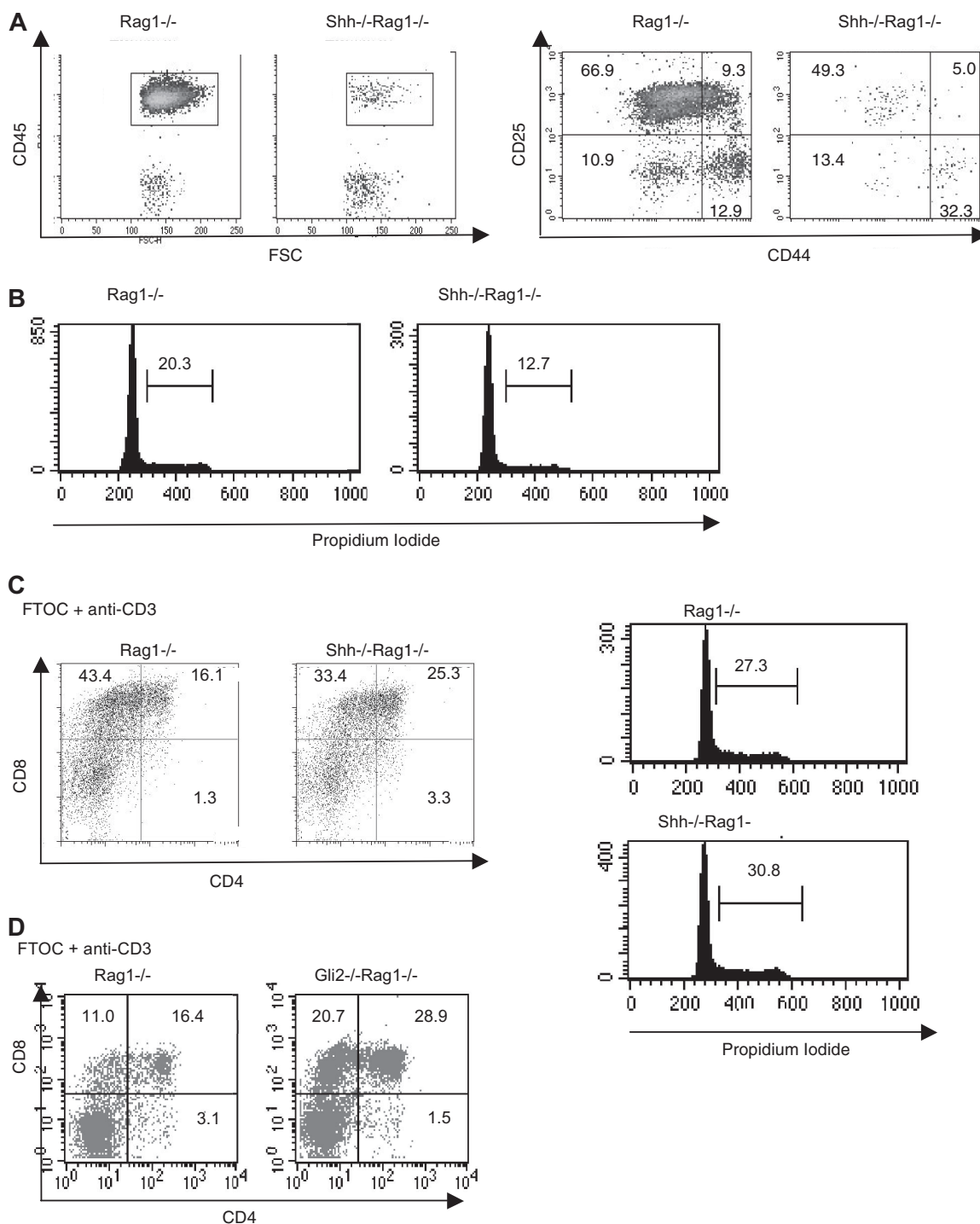
Figure 5. The effect of Shh deficiency and Gli2 deficiency on Rag1$^{-/-}$ FTOC stimulated with anti-CD3. (A) Phenotype of E15.5 Shh$^{-/-}$Rag1$^{-/-}$ and littermate Rag1$^{-/-}$ thymi. (Shh$^{-/-}$Rag1$^{-/-}$, n = 5; littermate Rag1$^{-/-}$, n = 9). Cells positive for CD45.2$^+$ (left dot plot) were analyzed for CD25 and CD44 expression (right dot plot). (B) Propidium iodide staining of thymocytes from Shh$^{-/-}$Rag1$^{-/-}$ and littermate Rag1$^{-/-}$ thymi as in panel A. The percentage of cells in G2+M/S is given. (C) E15.5 Shh$^{-/-}$Rag1$^{-/-}$ and littermate Rag1$^{-/-}$ fetal thymus organ cultures (FTOCs) were treated for 3 days with 1 μg/mL anti-CD3 mab. Dot plots show CD4 and CD8 profiles. (Shh$^{-/-}$Rag1$^{-/-}$, n = 5; littermate Rag1$^{-/-}$, n = 9). Histograms show propidium iodide staining on thymocytes from the anti-CD3–treated Shh$^{-/-}$Rag1$^{-/-}$ and littermate Rag1$^{-/-}$ FTOC. The percentage of cells in G2+M/S is given. (D) E17.5 Gli2$^{-/-}$Rag1$^{-/-}$ and littermate Rag1$^{-/-}$ FTOCs were treated for 5 days with 0.5 μg/mL anti-CD3 mab. Dot plots show CD4 and CD8 profiles. (Gli2$^{-/-}$Rag1$^{-/-}$, n = 4; littermate Rag1$^{-/-}$, n = 8.)

We also identified 9 genes that were significantly down-regulated on anti-CD3 treatment, and that were not differentially regulated between Gli2ΔC$_2$Rag1$^{-/-}$ and Rag1$^{-/-}$ thymocytes (Figure 7B). These included *CD25, TRp53 (p53), Wnt-1,* and *Brca1.* Down-regulation of CD25 is an early phenotypic change on pre-TCR signal transduction, and therefore serves as an internal control for detection of transcriptional down-

regulation. Likewise consistent with its down-regulation on pre-TCR signal transduction, p53 deficiency allows Rag-deficient thymocytes to differentiate to DP.[40] Both *Brca1* and *Wnt-1* are necessary for thymocyte development and proliferation[41,42] but their transcriptional regulation has not previously been linked to pre-TCR signal transduction or the transition from DN to DP. We also found down-regulation of *Myc,*
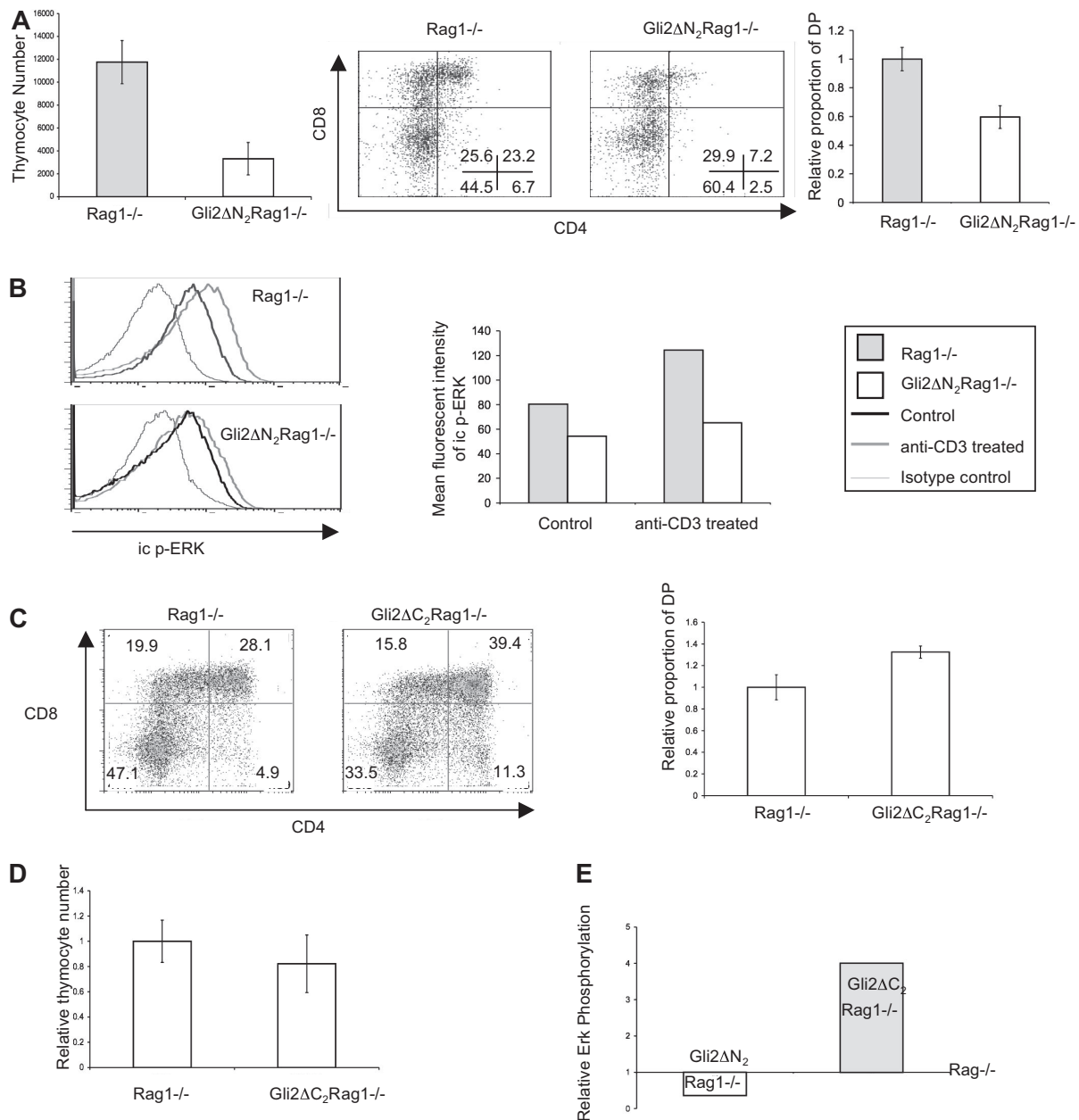
Figure 6. The effect of Gli2ΔN$_2$ expression and Gli2ΔC$_2$ expression on Rag1$^{-/-}$ FTOC stimulated with anti-CD3. (A) Right bar chart shows thymocyte number in E15.5 GliΔN$_2$Rag1$^{-/-}$ FTOC (n = 5) and littermate Rag1$^{-/-}$ FTOC (n = 4), after treatment for 3 days with 1 μg/mL anti-CD3 mab. The difference was statistically significant (P = .008). Dot plots show CD4 and CD8 expression on Gli2ΔN$_2$Rag1$^{-/-}$ FTOC (n = 5) and littermate Rag1$^{-/-}$ FTOC (n = 4), after treatment for 3 days with 1 μg/mL anti-CD3 mab. Left bar chart shows the relative proportion of DP cells in the same experiments. The difference between GliΔN$_2$Rag1$^{-/-}$ and littermate Rag1$^{-/-}$ is significant (P = .009). Error bars show SD. (B) ERK phosphorylation in anti-CD3–treated Rag1$^{-/-}$ and GliΔN$_2$Rag1$^{-/-}$ thymocytes. Histograms of icphospho-ERK levels in control (untreated) and anti-CD3–treated (5 × 10^{6}cells/mL activated with 10 μg/mL anti-CD3 mab cross-linked with biotinylated anti–hamster IgG for 2 minutes) thymocytes from Rag1$^{-/-}$ and GliΔN$_2$Rag1$^{-/-}$. Bar chart shows mean fluorescence intensity (MFI) of icphospho-ERK staining in control and anti-CD3–stimulated Rag1$^{-/-}$ (n = 3) and GliΔN$_2$Rag1$^{-/-}$ (n = 3) thymocytes. (C) Dot plots show CD4 and CD8 expression on E15.5 Rag1$^{-/-}$ (n = 10) and Gli2Δ2C$_2$Rag1$^{-/-}$ (n = 10) FTOC after treatment for 3 days with 1 μg/mL anti-CD3 mab. Bar chart shows the relative proportion of DP cells for anti-CD3–treated Gli2ΔC$_2$Rag1$^{-/-}$ FTOC and littermate Rag1$^{-/-}$ FTOC. The difference is significant (P = .001). Error bars show SD. (D) Relative thymocyte number in Gli2ΔC$_2$Rag1$^{-/-}$ FTOC and littermate Rag1$^{-/-}$ FTOC, 3 days after anti-CD3 treatment. There was no significant difference. Error bars show SD. (E) Relative change in ERK phosphorylation in anti-CD3–treated Gli2ΔC$_2$Rag1$^{-/-}$ (5 × 10^{6}cells/mL activated with 10 μg/mL anti-CD3 mab cross-linked with biotinylated anti–hamster IgG for 2 minutes; and Gli2ΔN$_2$Rag1$^{-/-}$, n = 3) thymocytes, normalized to that of their Rag1$^{-/-}$ littermate controls. Error bars show SE.

Telomerase reverse transcriptase (*Tert*; itself a transcriptional target of Myc), the antiapoptotic factor *Birc1a*, *Cadherin 1*, and *HoxA1*.

Hh signaling maintains FoxA2 expression on pre-TCR signal transduction

We then looked for genes that were differentially regulated by the pre-TCR on repression of Hh signaling compared with WT,

and interestingly identified only one significant transcriptional difference: *FoxA2* (P = .04; Figure 7C). There were no significant differences in gene expression between Rag1$^{-/-}$ and Gli2ΔC$_2$Rag1$^{-/-}$ thymocytes before anti-CD3 treatment, indicating that the difference in transcription of *FoxA2* was a consequence of pre-TCR signal transduction. On anti-CD3 treatment, there was a 6-fold down-regulation of the transcription factor
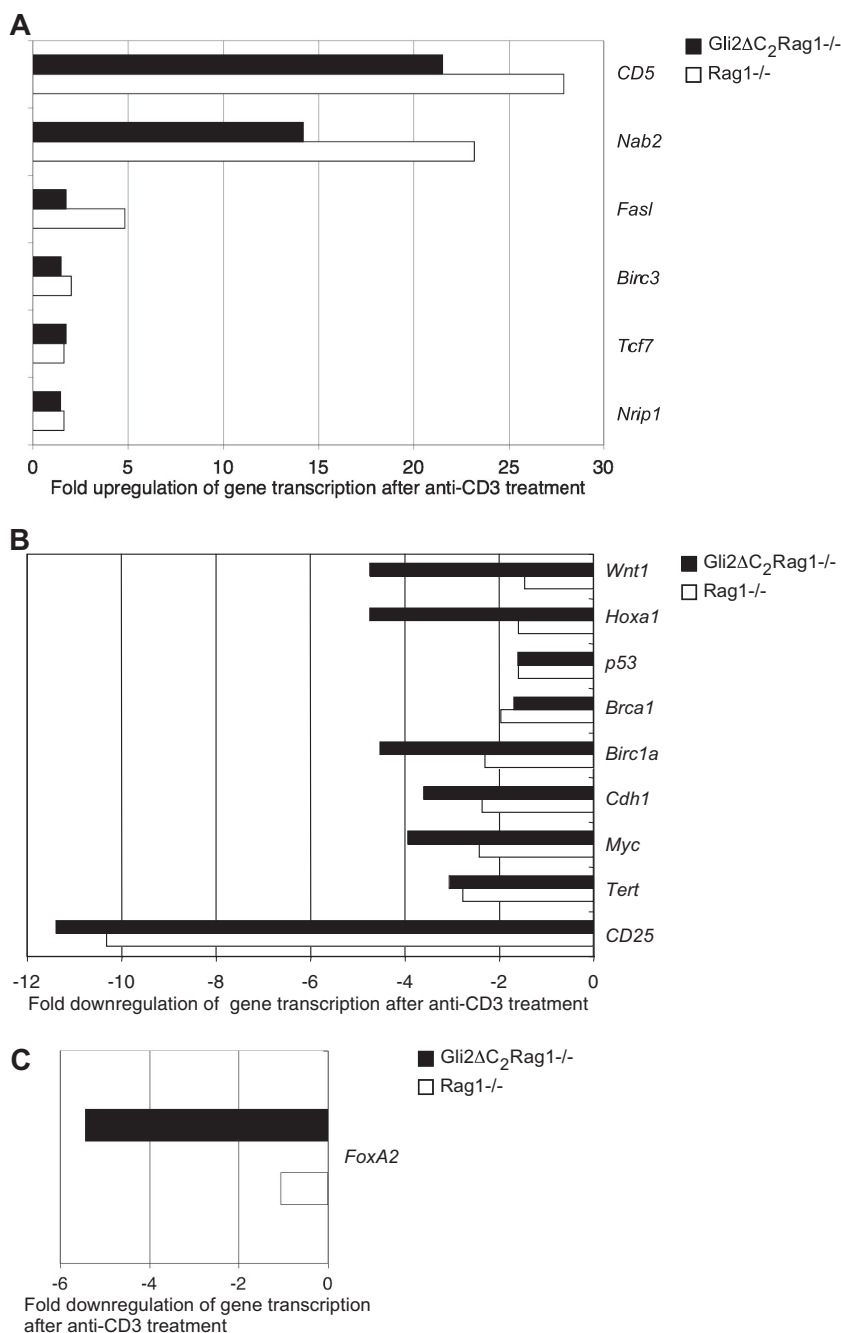
Figure 7. PCR array analysis of gene transcription in control and anti-CD3–treated Rag1⁻/⁻ and Gli2ΔC₂Rag1⁻/⁻ thymocytes. Bars show average fold change in gene transcription on anti-CD3 treatment, relative to transcription in untreated samples. Values were calculated by dividing the average gene transcription in the treated samples (Rag1⁻/⁻ + anti-CD3 or Gli2ΔC₂Rag1⁻/⁻ + anti-CD3) by the corresponding ones from the control samples (Rag1⁻/⁻ or Gli2ΔC₂Rag1⁻/⁻). (A,B) All genes shown exhibited statistically significant change after anti-CD3 treatment in Rag1⁻/⁻ and/or Gli2ΔC₂Rag1⁻/⁻ thymocytes. (A) Genes significantly up-regulated after anti-CD3 treatment. P values for Rag1⁻/⁻: $Nrip1$, $P = .02$; $Tcf7$, $P = .06$; $Birc3$, $P = .02$; $Fasl$, $P = .04$; $Nab2$, $P = .002$; and $CD5$, $P = .004$. P values for Gli2ΔC₂Rag1⁻/⁻: $Nrip1$, $P = .005$; $Tcf7$, $P = .03$; $Birc3$, $P = .5$; $Fasl$, $P = .4$; $Nab2$, $P = .04$; and $CD5$, $P = .02$. (B) Genes significantly down-regulated after anti-CD3 treatment. P values for Rag1⁻/⁻: $CD25$, $P = .002$; $Tert$, $P = .07$; Myc, $P = .01$; $Cdh1$, $P = .04$; $Birc1a$, $P = .05$; $Brca1$, $P = .007$; $p53$, $P = .1$; $Hoxa1$, $P = .005$; and $Wnt1$, $P = .03$. P values for Gli2ΔC₂Rag1⁻/⁻: $CD25$, $P = .01$; $Tert$, $P = .01$; Myc, $P = .06$; $Cdh1$, $P = .08$; $Birc1a$, $P = .2$; $Brca1$, $P = .04$; $p53$, $P = .04$; $Hoxa1$, $P = .2$; and $Wnt1$, $P = .3$. (C) Change in transcription of $FoxA2$ after anti-CD3 treatment. Difference in change in transcription of $FoxA2$ was significant between geno-types, $P = .05$.

$FoxA2$ in the Gli2ΔC₂Rag1⁻/⁻ only. FoxA2 is essential for mouse development and FoxA2⁻/⁻ embryos die at E10 with defects in node, notochord, neural tube, and gut.[43,44] In the mouse floor plate, $FoxA2$ is a Shh target gene and the $FoxA2$ enhancer contains a Gli-binding site.[45] Our experiment indicates that in thymocytes, Hh signaling maintains $FoxA2$ expression during pre-TCR signal transduction, as when Hh pathway activation was inhibited by transgenic Gli2ΔC₂ expression, $FoxA2$ was down-regulated.

Discussion

Here we show that Shh signaling, via Gli2, promotes thymocyte development before pre-TCR signal transduction, but that Shh and Gli2 are negative regulators of pre-TCR signal transduction and thymocyte differentiation at the transition from DN to DP cell. Analysis of Shh⁻/⁻, Gli3⁻/⁻, and conditional Smo-deficient thymi[18,21,22] has previously shown that Hh signaling regulates the differentiation, survival, and proliferation of the earliest DN1 and DN2 thymocyte populations, and this analysis of thymocyte development in Gli2-deficient embryos also demonstrated the nonredundant involvement of Gli2 at this developmental stage. The transition from DN1 to DN2 was reduced in the Gli2⁻/⁻ thymus, but we did not detect an influence of Gli2 deficiency on DN1 or DN2 survival or proliferation. This suggests that although Gli2 is an essential mediator of Shh-induced differentiation at this developmental stage, it is not necessary for Shh/Smo-induced survival or proliferation, and that there is redundancy between Gli family members for these functions of Hh signaling in DN1 and DN2 thymocytes.

The function of Hh signaling at the transition from DN to DP has been controversial,[1,18,22] but here we showed, using many different mouse models, that Hh signaling negatively regulates the pre-TCR signal for differentiation to DP cell. When Hh pathway activation was reduced, in the Shh$^{-/-}$Rag1$^{-/-}$, Gli2$^{-/-}$Rag1$^{-/-}$, and Gli2$^{-/-}$ thymus, or by T lineage–specific transgenic expression of a transcriptional-repressor form of Gli2 (Gli2ΔC_2), differentiation to DP cell after pre-TCR signal transduction was increased. We observed increased production of DP cells in the E17.5 Gli2$^{-/-}$ thymus, and in anti-CD3–treated Shh$^{-/-}$Rag1$^{-/-}$, Gli2$^{-/-}$Rag1$^{-/-}$, and Gli2ΔC_2Rag1$^{-/-}$ FTOC, and in the adult Gli2ΔC_2 thymus after depletion of the DP pool by HC treatment. In contrast, when Hh signaling was constitutively activated in thymocytes by transgenic expression of a constitutive transcriptional activator form of Gli2 (Gli2ΔN_2), the production of DP cells was decreased, both in anti-CD3–treated Gli2ΔN_2Rag1$^{-/-}$ FTOC, and in adult HC-treated Gli2ΔN_2 thymus.

The fact that Shh$^{-/-}$Rag1$^{-/-}$ and Gli2$^{-/-}$Rag1$^{-/-}$ FTOCs both showed increased differentiation to DP cell on anti-CD3 treatment, whereas the Gli3$^{-/-}$Rag1$^{-/-}$ differentiated less efficiently,[21] indicated that Gli2 functions as a transcriptional activator downstream of Shh at this developmental stage, and therefore that Gli3 functions as a transcriptional repressor.

The experiments presented here seem to conflict with a previous analysis of a conditional T lineage–specific Smo knockout model, which was interpreted as showing that Hh does not influence thymocyte development after the DN2 stage.[22] In that study, Smo deficiency greatly reduced survival, proliferation, and differentiation at the DN1 to DN2 transition, resulting in a smaller thymus with fewer DP cells than WT. However, in vivo anti-CD3–induced differentiation of Rag2$^{-/-}$ thymocytes produced similar numbers of DP cells, in both conditional Smo-deficient mice and littermates.[22] Taken together, it therefore seems that Smo deficiency did in fact increase differentiation from DN to DP stage, as the small Smo-deficient Rag2$^{-/-}$ thymus was able to "catch up" in terms of DP production and number with its control Rag2$^{-/-}$ littermate thymus on transduction of the pre-TCR signal. Given that Smo is a nonredundant component of the Hh pathway, essential for signal transduction,[46] its conditional deletion should completely abrogate Hh pathway activation in T-lineage cells. Thus, in the adult steady-state thymus, it would have been difficult to detect the effect of simultaneous loss of both positive and negative regulation of T-cell development by Smo, as the profound effects of complete loss of the earlier positive Hh signal would mask the effects of loss of the later negative regulation of pre-TCR–induced differentiation. By analysis of mouse mutants in components of the signaling pathway, which are functionally at least partially redundant (eg, redundancy between Gli proteins or between Hh family members), we have been able to detect the influence of Hh signaling at multiple stages of development. This has also been facilitated by following thymocyte development in situations where the transition from DN to DP is more or less synchronized, such as on E16.5 and E17.5 of embryonic development, in anti-CD3–treated Rag1$^{-/-}$ FTOC, and in adult thymus as it recovers from HC depletion.

It might seem surprising that although Shh provides positive signals for survival, differentiation, and proliferation of the early DN thymocyte populations,[18,22] it then "switches" to function as a negative regulator of thymocyte differentiation at later stages of thymocyte development. However, similar opposing functions for Hh signaling have been described in the development of other tissues, such as gut and retina,[47,48] and are consistent with the action of a morphogen, which can specify multiple cell fates. The outcome of Hh signal transduction in a cell will depend on not only strength and duration of the signal (determined in part by the position of the cell relative to the source of Hh), but also the intracellular and extracellular context of signal transduction. In the future, it will be important to understand the context-dependent molecular machinery that accounts for these opposing outcomes of Hh pathway activation at different stages of thymocyte development.

The way in which Hh pathway activation in thymocytes reduced differentiation to DP is not clear, but given that ERK phosphorylation on CD3 ligation was reduced, it seems that Hh pathway activation modulated the pre-TCR signal. The mechanism of modulation remains to be investigated, and it seems likely that it is the result of Hh-induced transcriptional changes in the developing thymocyte.

We have identified novel transcriptional targets of pre-TCR signal transduction: genes up-regulated include the EGR1-binding protein, *Nab2*, the antiapoptotic factor and caspase-inhibitor *Birc3*, and the transcriptional regulator *Nrip1*. In addition, we found down-regulation of genes known to be involved in thymocyte development, such as *Wnt-1*, *Brca-1*, *p53*, and *Myc*, and genes, that to our knowledge, have not previously been studied in thymocytes, such as the antiapoptotic factor *Birc1a*, and the homeobox gene *HoxA1*. Use of the Gli2ΔC_2 transgenic allowed us to look for genes that, on pre-TCR signal transduction, are differentially regulated by inhibition of Hh signaling in developing thymocytes. Interestingly, this approach demonstrated that the physiologic level of Hh signaling in thymocytes maintains expression of *FoxA2*, encoding a forkhead box transcription factor and known Hh target gene in other tissues, on pre-TCR signal transduction. As the *FoxA* gene family members are regulators of many aspects of mammalian development,[49] it will be important in the future to assess their function in thymocyte differentiation and their potential role in differentiation to DP cell, as a negative regulator downstream of Hh.

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Authorship

Contribution: N.J.R. and T.C. designed research and wrote the paper; N.J.R., A.L.H.-T., and T.C. performed research, collected, analyzed, and interpreted data, and performed statistical analysis; and A.L.F., S.E.R., S.V.O., and J.T.D. performed research.

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