Differential Geminin Requirement for Proliferation of Thymocytes and Mature T Cells

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A regulated balance between proliferation and differentiation is fundamental for development, organogenesis, and tissue homeostasis. To achieve this, cells must integrate diverse extrinsic signals, remodel chromatin, and alter their transcriptional profiles. Changes in chromatin organization and transcription must be coordinated with replication to establish or maintain the transcriptional profile of progeny cells.

Geminin has been implicated in proliferation-differentiation decisions through balanced interactions with multiple binding partners. Geminin participates in cell cycle control as a negative regulator of licensing, a process that precedes S-phase initiation and consists of the formation on origins of DNA replication of a multisubunit prereplicative complex that is essential for S-phase onset (1). Geminin binds to the DNA licensing factor Cdt1 and inhibits its function, ensuring that genome replication takes place only once per cell cycle (1), thereby maintaining genomic integrity (2). In addition, it has been shown that Geminin is expressed in neural progenitor cells and is downregulated in fate-restricted precursor cells (3), although it participates in the control of cell fate specification and progenitor cell maintenance during nervous system development through modulation of proliferation, transcription, and modification of chromatin structure (4). Ectopic overexpression of Geminin in Xenopus embryos and Drosophila induces neural specific genes and hypertrophy of neural tissue, whereas Geminin has been shown to regulate the initiation of Sox2 expression during the establishment of the neural plate in vertebrate embryos (5–7). During eye development in Medaka, Geminin acts antagonistically to Six3 homeobox-containing transcription factor, regulating the balance between proliferation and differentiation. Thus, Geminin overexpression leads to the reduction in eye size, exhibiting reduced numbers of proliferating cells and premature induction of neuronal marker expression (8). Furthermore, it inhibits Hox transcription factor function through direct and polycomb-mediated interactions, determining axial patterning during chicken embryoogenesis (9). Interestingly, Geminin directly binds to Brg1, the catalytic subunit of the SWI/SNF complex, and reduces the ability of helix-loop-helix-type transcription factors to activate downstream target genes, suggesting a role of Geminin in the maintenance of neuronal progenitor cells in an undifferentiated state (10). Recently, Geminin has been shown to be involved in sustaining hematopoietic stem cells, expanding its potential role in other systems (11). Consistent with an essential role in proliferation and differentiation, mice lacking Geminin expression are dying during the first days of embryogenesis (9).

We have used T cell differentiation as a model to address the role of Geminin in developmental processes that require a regulated balance between proliferation and differentiation. During immune system development and maturation, undifferentiated hematopoietic precursor...
cells originating in the bone marrow enter the thymus and give rise to lineage-committed cells destined to become T cells (14, 15). Developing thymocytes are classified into four major populations based on the expression of two coreceptor molecules, CD4 and CD8. Double-negative (DN) thymocytes, lacking both CD4 and CD8 expression, are the earlier progenitors and can be further subdivided into four subpopulations, DN1–4, based on the expression of CD44 and CD25 markers (16), with a large proportion of cycling cells identified in DN2 and DN4 T cell populations (17). Successful rearrangement of the TCR-β gene (TCRβ) that takes place at the DN3 (18) stage triggers subsequent proliferation (19, 20). DN cells that have successfully passed β-selection point downregulate CD25 and initiate the expression of CD4 and CD8 (double-positive [DP]) accompanied by rearrangement of the TCR-α gene (TCRα) (18, 21, 22). The expansion in thymocyte numbers that occurs between the late DN stage and the DP stage is the one of the main events that determines the final number of thymocytes (19). It has been estimated that 9 to 10 divisions are required from the CD44CD25+ (DN2) stage to the CD4+CD8+ (DP) stage (23). DP thymocytes are screened by stromal cells for useful TCRs, a process that leads to elimination of harmful or useless specificities (negative selection) and retention of T cells with useful TCRs (positive selection). The positive selected thymocytes bear either CD4 or CD8 corespondents only (single-positive [SP]), and they are exported to the periphery to participate in the immune functions in the body (14).

To investigate the in vivo function of Geminin in the development and homeostasis of the lymphoid system, we generated mice that allow the conditional inactivation of the mouse Geminin gene through Cre-mediated recombination. A transgenic line that mice that allow the conditional inactivation of the mouse Geminin gene has been used to allow the lymphoid specific inactivation of Geminin (24). We acquired from the CD44+CD25+ (DN2) stage to the CD4+CD8+ (DP) stage is the one of the main events that determines the final number of thymocytes (19). It has been estimated that 9 to 10 divisions are required from the CD44CD25+ (DN2) stage to the CD4+CD8+ (DP) stage (23). DP thymocytes are screened by stromal cells for useful TCRs, a process that leads to elimination of harmful or useless specificities (negative selection) and retention of T cells with useful TCRs (positive selection). The positive selected thymocytes bear either CD4 or CD8 corespondents only (single-positive [SP]), and they are exported to the periphery to participate in the immune functions in the body (14).

Materials and Methods

Targeting vector, generation of mice, and genotyping

A Geminin genomic clone was isolated from theRPC21-471M19 PAC library derived from 129S6/SvEvTac mouse strain using Geminin cDNA as a probe (3). The 5′ and 3′ regions of homology in the targeting vector consisted of a 3.5-kb SacII-XhoI fragment including exons 1 and 2 and a 6.0-kb BstXI-Asp718 fragment spanning exons 3–7, respectively (regions of homology are indicated in Fig. 1A as interrupted lines). The Flxed-pGKneo tk cassette (courtesy of Dr. T. Mamanadadiotis, Victorian College of Pharmacy, Monash University, Victoria, Australia) was introduced into the XhoI restriction site, whereas a third loxp site was introduced into the BstXI site. This resulted in a 1.7-kb region encompassing exons 3 and 4 being flanked by loxP sites. The linearized vector was electroporated into embryonic stem (ES) cells derived from the C57BL/6 mouse strain using the protamine Cre (25). Postselection with G418, 480 ES cell clones were isolated, expanded, and frozen according to standard methods. To screen for homologous recombinants, ES cell DNA was digested with BamHI, Asp718, and combined BamHI, Asp718, and hybridized to located sequences 5′ (Fig. 1A, probe AflII-SacII, black colored box) and 3′ external (Fig. 1A, probe BglII-BamHI, gray checked box) to the recombination sites, respectively. Twelve correctly targeted clones were identified by Southern blot analysis and five of them with a euploid karyotype were used for injection into C57BL/6 blastocysts. Successful germline transmission was verified via a PCR that uses primers that anchor the neo gene (5′-ctctgctgaattctgttcgc-3′) and exon 3 of the mouse geminin gene (5′-gcaagaagctgctgctgctgc-3′), and DNA was amplified for 30 cycles (95°C for 45 s, 65°C for 45 s, and 72°C for 120 s). CD2Cre and PC3Cre transgenes were also detected by PCR as previously described (24, 25). To analyze excision of the Geminex-3-4 allele, genomic DNA was prepared from tail, thymus, spleen, or LN and analyzed by Southern blot using BamHI digestion and a 5′ probe (Fig. 1A, probe AflII-SacII, and 1D) or by PCR (Fig. 1B). Experiments were performed withFlkOCD2Cre, whereas Flk/wt served as controls. Similar results were obtained usingFlk0/wtCD2Cre mice as controls (data not shown). The Medical Research Council/National Institute for Medical Research Ethical committee approved all animal experiments.

Isolation of cells, in vitro activation and proliferation

Total splenocytes or LN cells (5 × 10^7) were cultured in 96-well plates in triplicate in complete RPMI 1640 (Sigma-Aldrich, Dorset, U.K.) supplemented with 10% FCS (Life Technologies, Paisley, U.K.), 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM glutamine, and 50 μM 2-ME (Sigma-Aldrich). Cells were stimulated with plate-bound anti-CD3 mAb (145.2C11, eBioscience, San Diego, CA) and anti-CD28 (37.51, eBioscience, San Diego, CA) and anti-CD8 (anti-CD8, BD Pharmingen, Heidelberg, Germany) according to the manufacturer’s instructions. Activated T cells were stained for BrdU and DNA content using 7-aminoactinomycin D (7-AAD). BrdU pulse and chase experiment cells were subjected to double immunostaining for BrdU and Ki67 (anti-Ki67 PE; BD Pharmingen). Naive T cells were activated using anti-CD3/CD28 Abs for 3 h and subsequently pulsed with BrdU for 2 h. BrdU was removed and cells were fixed at 2, 8, and 16 h (time is calculated from the addition of BrdU). T cells were stained with BrdU, Ki67, and 7-AAD. Cells that have exited the cell cycle were calculated as the fraction of BrdU expressing cells that are not expressing Ki67.

CFSE labeling

Splenocytes were suspended in 1×10^7 cells/ml in PBS (Life Technologies). A 10 μM stock solution of CFSE (Molecular Probes, Eugene, OR) was added to a final concentration of 1 μM and was incubated at 37°C for 10 min. Subsequently, cells were washed twice in air-buffered IMDM supplemented with 1% FCS and then resuspended in RPMI 1640 (N sigma-Aldrich). We have used CD4 PE (RM4-5, eBioscience), CD8α PerCP (53-6.7, BD Pharmingen), and CD25 allophycocyanin (PC61.5, eBioscience). The dilution of CFSE used was proper to minimize bleed-through to the FL2 channel, and any remaining bleed-through was properly compensated for.

FACS analysis (flow cytometry, cell sorting, and Abs)

Single-cell suspensions were prepared from thymocytes and splenocytes, and cells were stained with saturating concentrations of FITC-PE, -PerCP, or allophycocyanin-conjugated Abs in PBS with 0.1% BSA and 0.05% NaN3 at 4°C for 30 min, then washed with PBS and subsequently analyzed using BD Biosciences FACScanCalibur and FlowJo 8.7.1 software (Tree Star, Ashland, OR). For cell sorting, thymocytes were stained at a concentration of 1×10^6
cells/ml in air-buffered IMDM supplemented with 5% FCS and sorted using a MoFlo sorter (DakoCytomation, Carpinteria, CA). The following Abs were used: TCR-β–FITC (H57-597, eBioscience), CD4-FITC (H129.19, Santa Cruz Biotechnology, Santa Cruz, CA), CD44 FITC (Caltag Laboratories, Buckingham, U.K.), CD4 PE (RM4-5, eBioscience), CD62L PE (MEL-14, eBioscience), CD69 PE (H1.2F3, eBioscience), CD4-PerCP (RM4-5, eBioscience), CD8α-PerCP (53-6.7, BD Pharmingen), CD4-alkophycocyanin (GK1.5, eBioscience), CD8α-alkophycocyanin (53-6.7, BD Pharmingen), and CD25 alkophycocyanin (PC61.5, eBioscience).

**Western blotting analysis**

Whole-cell lysates from thymocytes, splenocytes, and activated T cells or purified naïve T cells were prepared in SDS-PAGE loading buffer, subjected to electrophoresis in 6%, 10%, 12%, or 15% acrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Watford, U.K.). Immunodetection was performed using affinity-purified polyclonal Abs against Geminin and Cdt1 (28), rabbit polyclonal Abs against Cdc2 pTyr15 (9111, Cell Signaling, Danvers, MA), Rb pSer807/811 (9308, Cell Signaling), cyclin E (M-20, Santa Cruz Biotechnology), cyclin B1 (H-433, Santa Cruz Biotechnology), mouse mAbs against cyclin-D3 (DCS22, Cell Signaling, Danvers, MA), p27 (57/kip1/p27 BD Transduction Laboratories), cyclin A (CY-A1, Sigma, Dorset, U.K.), tubulin (B512, Sigma-Aldrich), and goat polyclonal Ab against actin (clone I-19, Santa Cruz Biotechnology).

**FIGURE 1.** Targeting the mouse Geminin locus. A, The mouse Geminin locus (Gem WT allele), targeted locus (Gem floxed allele), and the deleted Geminin allele lacking exons 3 and 4 (Gem Δex3-4 allele). Boxes indicate the mouse Geminin gene exons and triangles the LoxP sites. SacII, XhoI, BstX1, and Asp718 restriction sites define the 5’ and 3’ arms of homology, respectively (shown in WT allele). 5’ external (probe AflII-SacII) and 3’ external (probe BglII-BamHI) are shown in WT allele. A, B, S, X, and Bs indicate sites that are recognized from Asp718, BamHI, SacII, XhoI, and BstXI restriction enzyme, respectively. Relevant restriction sites are shown. Regions of homology are indicated as interrupted lines. B, DNA isolated from tail, thymus, spleen, and LNs were used to genotype mice using PCR. The Gem WT allele (WT), Gem floxed allele, and Gem Δex3-4 allele (knockout) generate PCR-amplified fragments of 549, 597, and 240 bp, respectively. Primers used are indicated as arrows in A. C, The efficiency of Geminin deletion in different T cell populations was established by PCR. DNA isolated from tail and DN, DP, CD4, and CD8 FACS-sorted thymocytes was analyzed using primers detecting the WT, floxed, and deleted allele. D, Genomic DNA was isolated from tail, thymus, spleen, and lymph nodes, digested with BamHI, and analyzed by Southern blotting using the 5’ external probe (probe AflII-SacII). An 11.4-kb band corresponds to the targeted locus (Gem floxed allele) and 9.8 kb to the deleted allele (Gem Δex3-4 allele). E, Whole-cell protein lysates from thymus, spleen, and T cells activated by anti-CD3/CD28 (10 μg/ml and 2 μg/ml) or PDBU/ionomycin (0.5 μg/ml and 1 μg/ml) were subjected to Western blot analysis using specific Abs against Geminin, tubulin, and actin.

**Annexin V staining**

Activated T cells were stained for Annexin V according to the manufacturer’s instructions (BD Pharmingen).

**Statistical analysis**

Average values ± SEM are given, and p values were calculated by employing the two-sample Student t tests using Microsoft Excel software (Microsoft, Redmond, WA).
Results

Generation of mice lacking Geminin in the lymphoid lineage

We have employed the Cre-LoxP system to generate mice permitting the conditional inactivation of the mouse Geminin gene. Mouse genomic DNA harboring mouse Geminin exons 1–7 was used in the targeting construct (Fig. 1A). LoxP sites were inserted in the introns flanking exons 3 and 4 and a neomycin-thymidine kinase cassette flanked by two LoxP sites in intron 2. Cre-mediated recombination would lead to deletion of exons 3 and 4. If exons 2 and 5 were to be spliced and a stable mRNA be produced, a frame-shift mutation is generated, ensuring no functional protein is produced following Cre recombination. The targeting construct was electroporated into ES cells derived from transgenic mice expressing Cre recombinase under the control of the protamine gene promoter, which directs Cre expression in spermatocytes (25). Neomycin-resistant ES cell clones were isolated and correct integration events confirmed using 5’ and 3’ probes external to the homology regions of the targeting construct (Fig. 1A and data not shown). Chimeric mice were generated and germ line transmission of the targeted allele was achieved. The neomycin-thymidine kinase-resistant cassette was removed through intercrossing mice carrying the targeted allele and the protamine Cre with WT mice and selecting progeny carrying the Geminin floxed allele without the neomycin-thymidine kinase cassette. This breeding scheme also produced mice in which the additional deletion of exons 3 and 4 had taken place in the germ line, thus establishing a line carrying one WT and one Geminin null allele (Geminin+/−). Subsequent breeding with WT mice segregated out the protamine Cre transgene. Geminin+/− and Gemininfloxed/− were viable, fertile, and apparently normal. Inter crossing of Geminin+/− and Gemininfloxed/− did not produce live Geminin−/− offspring, whereas analysis for the presence of embryos at embryonic day 7.5 did not reveal any live Geminin−/−embryo at that age (data not shown). These data are consistent with previous findings showing that Geminin is essential for early mouse embryogenesis (12, 13) and verify effective targeting of the locus.

Geminin is expressed in the developing thymus during mouse embryogenesis and its mRNA is present in all progenitor T cell populations and peripheral CD4 and CD8 cells (Supplemental Fig. 1 and data not shown). To investigate the in vivo role of Geminin during lymphocyte progenitor cell development and homeostasis, Gemininfloxed/+ or Gemininflox/− mice were crossed with Geminin+/− mice carrying a transgene that drives expression of Cre recombinase under the control of the human CD2 promoter and LCR (29). This transgene has been previously shown to be expressed in B and T cell lineages. A large proportion of DN1 cells and the vast majority of DN3 and DN4 are expressing Cre recombinase (24). For simplicity, we refer to the Gemininfloxed/−CD2Cre mice as Fl/koCD2Cre and refer to their Gemininflox/−CD2Cre littermates as Fl/wtCD2Cre. DNA isolated from tail, thymus, spleen, and LNs of Fl/ko, Fl/koCD2Cre, and Fl/wt mice were analyzed by PCR and Southern blotting to assess the efficiency of the recombination (Fig. 1). Using specific primers that detect the three different Geminin alleles (floxed, knockout, WT), we show that in Fl/koCD2Cre mice, the Geminin floxed allele is absent from DNA derived from thymus, whereas it is significantly reduced in DNA derived from spleen and LNs, where a residual band is still detectable (Fig. 1B). Developing thymocytes can be subdivided into several subpopulations: ∼5% express neither CD4 nor CD8 (DN cells); ∼80% express both CD4 and CD8 (DP cells); ∼10% express only CD4 (CD4 SP) and ∼5% express only CD8 (CD8 SP). PCR analysis of DNA samples derived from all four stages of T cells in thymus shows a complete absence of the floxed allele in the presence of Cre recombinase (Fig. 1C). Furthermore, Southern blot analysis of DNA samples derived from tail, thymus, spleen, and LNs using a 5’ external probe has confirmed the efficient deletion in tissues where Cre recombinase is expressed.
Moreover, total cellular extracts from thymus and spleen from Fl/koCD2Cre and control littermates show absence of the Geminin protein in thymus and only a residual amount of protein present in spleen, possibly due to the presence of other hematopoietic cells in this organ in which Cre recombinase is not expressed. Furthermore, although T cells derived from the spleen.

**FIGURE 3.** Mice that lack Geminin expression show reduced numbers of naive, regulatory, and memory T cells. Representative FACS analysis of splenocytes using Abs recognizing naive, memory, and regulatory splenic T cells are shown. Average absolute cell numbers for naive CD4CD4 CD62Lhigh (A) and CD8CD4 CD62Lhigh (B) T cells from Fl/wt (n = 6) and Fl/koCD2Cre (n = 10) mice. Average absolute cell numbers of memory T cells (CD4+CD4 CD62Lhigh) from Fl/wt (n = 8) and Fl/koCD2Cre (n = 8) mice (C) and regulatory T cells (CD4+CD25+FoxP3+) derived from Fl/wtCD2Cre (n = 6) and Fl/koCD2Cre (n = 3) mice were measured (D). Values indicate average number of cells (± SE). *p < 0.05; **p < 0.01.

**FIGURE 4.** Activated T cells that lack Geminin expression show reduced proliferation and are unable to undergo multiple divisions. A, T cells that lack Geminin expression show reduced [3H]thymidine incorporation. Proliferation of T cells in the presence of anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) mAbs, measured by [3H]thymidine. Values indicate average counts (± SE) from a representative experiment of three. B, T cells from control (Fl/wt, Fl/wt CD2Cre) and Fl/koCD2Cre animals were stained with CFSE (1 μM) and cultured in vitro in the presence of plate-bound anti-CD3 (1 μg/ml) plus anti-CD28 (2 μg/ml) for 96 h. CFSE dilution histograms gated on CD4+CD25high (left-hand column) and CD8+CD25high (right-hand column) populations are shown. ***p < 0.001.
of control mice show high expression of Geminin following in vitro activation with CD3/CD28 or PDBU/ionomycin. T cells derived from Fl/koCD2Cre mice show no detectable Geminin expression upon activation (Fig. 1E). We therefore conclude that expression of Cre recombinase under the control of human CD2 regulatory elements leads to Geminin deficiency in progenitor T cell populations and their progeny.

Absence of Geminin does not alter the T cell developmental program, but reduces T cell populations of thymus and spleen

Fl/koCD2Cre mice lacking Geminin expression in the T cell compartment develop and grow apparently normally. The total number of thymocytes is reduced by 21% in Fl/koCD2Cre compared with Fl/wt mice (2.55 ± 3 × 10^8, n = 12; t test, p = 0.009). Quantification of individual thymocyte subsets by FACS using the CD4 and CD8 Abs showed that in mice deficient for Geminin, the DP population that lacks Geminin expression shows a 22% decrease (1.84 × 10^8, n = 17, versus 2.36 × 10^8, n = 12; t test, p = 0.009) (Fig. 2A), whereas DN, CD4 SP, and CD8 SP cells do not show statistically significant differences. Further examination of DN subsets shows that DN1 and DN4 cell populations exhibit a statistically significant reduction of 24% (0.721 ± 3 × 10^6, n = 17, versus 0.951 ± 3 × 10^6, n = 12; t test, p = 0.04) and 19% (2.75 ± 3 × 10^6, n = 17, versus 3.4 ± 3 × 10^6, n = 12; t test, p = 0.03), respectively (Fig. 2A). Our results indicate that T cell development in the thymus is not drastically affected in the absence of Geminin because all T cell subtypes are generated, showing only a moderate, statistically significant reduction in DN1, DN4, and DP cell populations.

FACS analysis of T cell populations in the periphery showed that the CD4 and CD8 compartments of the Fl/koCD2Cre, when compared with control mice, are reduced by ~35% (0.89 ± 3 × 10^7, n = 12; t test, p = 0.004) and 33% (4.73 ± 3 × 10^6, n = 17, versus 7.01 ± 3 × 10^6, n = 12; t test, p = 0.002), respectively (Fig. 2B).

The heterogeneous circulating T cell populations are characterized by differences in life span and turnover rate (30). Looking at specific T cell populations, we examined the effects of the absence of Geminin in naive, memory, and regulatory T cells. Fl/koCD2Cre naive CD4 or CD8 T cells not expressing CD44 and

**FIGURE 5.** T cells that lack Geminin expression show abnormal cell cycle profile and reduced ability of re-entering into cell cycle. A. Cell cycle progression of asynchronous T cells from control and mutant animals stimulated in vitro with anti-CD3 (10 μg/ml) plus anti-CD28 (2 μg/ml) was examined. Cells were pulsed with BrdU (10 μM) for 1 h and then stained with anti-BrdU FITC and 7-AAD followed by FACS analysis. Flow cytometric analysis of BrdU and 7-AAD at 48 h is shown. The percentage of cells in each phase of the cell cycle is shown. B. Purified naïve T cells from Fl/koCD2Cre and Fl/wtCD2Cre control animals were stimulated with plate-bound anti-CD3 (10 μg/ml) and anti-CD28 (2 μg/ml) for 38 h. Cells were subsequently pulsed with BrdU (20 μM) for 2 h. BrdU was then removed, and cells were fixed and stained with anti-BrdU FITC and 7-AAD at the indicated time points (2 h, 8 h, and 16 h following BrdU addition). Histogram distribution of 7-AAD staining in the BrdU+ fraction of cells is shown. C. The percentage of BrdU+ cells that are not expressing Ki67 (Ki67−) is shown at the indicated time points (8 h and 16 h after BrdU addition). Control mice and Fl/koCD2Cre mice are indicated as black and white columns, respectively.
expressing high levels of CD62L, when compared with control mice, show a reduction of 43% (2.83 × 10^6, n = 10, versus 5.03 × 10^6, n = 6; t test, p = 0.002) and 32% (2.11 × 10^6, n = 10, versus 3.11 × 10^6, n = 6; t test, p = 0.008), respectively (Fig. 3A, 3B). We have also examined the CD4^+CD62L^- and CD8^+ CD62L^- compartments. We find that CD4^+CD62L^- cells are reduced by 18% in Fl/koCd2Cre animals compared with controls (2.93 × 10^6, n = 10, versus 3.56 × 10^6, n = 6; p = 0.38), whereas CD8^+CD62L^- cells are reduced by 33% in Fl/koCd2Cre animals (1.26 × 10^6, n = 10, versus 1.88 × 10^6, n = 6; p = 0.04) (data not shown). High levels of CD44 expression are characteristic of activated or memory type T cells. CD4^+ CD44^high cells in Fl/koCd2Cre show a reduction of 52% when compared with the Fl/wt mice (1.42 × 10^6, n = 8, versus 2.96 × 10^6, n = 8; t test, p = 0.009) (Fig. 3C). In normal mice, around 10% of the CD4 T Cells express the IL-2R α-chain (IL-2Ra; CD25). These CD4^+CD25^+ T Cells contain a population of forhead box p3^+ (Foxp3^+) cells known as regulatory T cells that have the capacity to modulate immune responses and inhibit development of autoimmune diseases (31, 32). FACS analysis showed that in the spleen of Fl/koCd2Cre mice, CD4^+CD25^+ Foxp3^+ T cells are reduced by ~35% (0.58 × 10^6, n = 3, versus 0.97 × 10^6, n = 6; t test, p = 0.046) when compared with the control mice (Fig. 3D). Notably, this does not reflect an inability of Fl/koCd2Cre T cells to express CD25 because in these cells, CD25 expression is upregulated as efficiently as in WT T cells in response to various stimuli in vitro (data not shown). We conclude that absence of Geminin leads to a reduction of at least three specific T cell subpopulations in spleen.

**Proliferation defects of T cells that lack Geminin**

As Geminin is involved in cell cycle control, we tested the ability of Geminin negative lymphocytes to proliferate. For this purpose, we measured the incorporation of [H]thymidine into splenic T cells in response to mitogenic stimulation. T cells from Geminin-deficient mice show >60% reduction in thymidine uptake poststimulation for 64 h with anti-CD3 (1 μg/ml) plus anti-CD28 (2 μg/ml) Abs (Fig. 4A). To examine the possibility that TCR signaling might be impaired in the Geminin-deficient T cells, we examined changes in expression of cell surface molecules in Geminin-deficient cells. Following stimulation of T cells with anti-CD3 and anti-CD28 or with PDBU and ionomycin or Con A, the expression of the IL-2R (CD25), the upregulation of the early activation marker CD69, and CD44 levels were measured at different time points by FACS analysis. The induction of these cell surface markers did not show any statistically significant difference when T cells from control and Fl/koCd2Cre animals were compared (Supplemental Fig. 2 and data not shown). These results suggest that in Geminin-deficient T cells, TCR activation signals are efficiently transduced, resulting in appropriate upregulation of immediate response genes.

To distinguish whether the lower incorporation of [H]thymidine was due to impairment of cell division and/or enhanced cell death, we measured cell death by measuring Annexin V binding and caspase 3 activation. Fl/wt and Fl/koCd2Cre T cells showed similar Annexin V and caspase 3 staining after 24 or 48 h of TCR activation (Supplemental Fig. 3 and data not shown), suggesting that Geminin-deficient T cells do not show enhanced cell death. To assess cell division more precisely, LN T cells from Fl/koCd2Cre and Fl/wt were purified, labeled with CFSE and stimulated with plate-bound Abs against CD3 and CD28. At 24 h postactivation, no obvious division was observed for either control or mutant T cells (data not shown). At 96 h following stimulation, the majority of CD4-expressing cells derived from control animals had undergone up to four divisions, whereas only a small fraction of CD4^+ cells lacking Geminin had divided up to three times (Fig. 4B). Moreover, a significant percentage of CD8^+ T cells from WT mice performed four divisions, whereas CD8 T cells derived from Fl/koCd2Cre mice remained undivided (Fig. 4B). These data show that Geminin-deficient T cells exhibit severe proliferation defects following TCR activation.

To delineate defects in specific phases of the cell cycle for the Geminin-deficient cells, T cells from spleen were activated with soluble anti-CD3 and anti-CD28 for 48 h and then pulse-labeled for 1 h with BrdU, fixed, permeabilized, and stained with 7-AAD and anti-BrdU FITC. Flow cytometry showed reduced BrdU incorporation in Geminin-deficient cells (27% of Fl/koCd2Cre compared with 39% of Fl/wt T cells) and a greater percentage of the Fl/ko cells remaining in G0/G1 phases (43.7% versus 39.6%). Moreover, Geminin-deficient T cells showed an increased percentage of cells at the G2/M phase (3.33% compared with 1.83% for control cells) (Fig. 5A). The presence of apoptotic or sub-G0 cells was similar in T cell populations from both groups of mice, in agreement with our results for the Annexin V staining. Moreover, we do not observe any statistically significant difference in cells with DNA content >4 N, suggesting that extended over-replication does not take place in activated T cells derived from Fl/koCd2Cre mice (data not shown). To follow a T cell population during the cell cycle, naïve T cells were stimulated using Abs against CD3 and CD28 for 38 h and pulse-labeled with 20 μM

**FIGURE 6.** T cells that lack Geminin expression fail to undergo efficient homeostatic proliferation and to repopulate the spleen. T cells from control (Fl/wt, Fl/koCd2Cre) and Fl/koCd2Cre mice were labeled with CFSE (5 μM) and transferred into Rag2KO^−/−^ mice. Splenocytes were recovered after 3 (first column) and 5 (second column) days, and CD8^+ T cells were analyzed by flow cytometry for CFSE dilution (n = 3). Two weeks postinjections, CD4 and CD8 T cells that have repopulated spleens from control and mutant animals were measured (n = 4). Control mice and Fl/koCd2Cre mice are indicated as black and white columns, respectively. Values indicate average number of cells (± SE). ***p < 0.01.
BrdU for 2 h. BrdU was then removed and cells were fixed at different time points (2 h, 8 h, 16 h). Immediately after BrdU removal, 7-AAD staining of BrdU+ T cells that derived from control and mutant mice show similar numbers in different cell cycle phases (Fig. 5B, 2 h), suggesting that naïve T cells that lack Geminin are entering into S phase as efficiently as the WT T cells. At 8 h after BrdU addition, >49% of the BrdU+ T cells derived from Fl/koCD2Cre mice remain in S/G2/M phase compared with 23% of T cells derived from control animals (Fig. 5B, 8 h), suggesting a delay of T cells that lack Geminin to progress to G1, whereas similar distribution was also observed at 16 h (Fig. 5B, 16 h). Furthermore, at the same time point, >18% of the BrdU-labeled T cells derived from Fl/koCD2Cre mice do not express Ki67 compared with 7.0% in the control T cells, indicating an increased percentage of cells that withdraw from the cell cycle (Fig. 5C). Our data suggest that activated T cells lacking Geminin expression show no defect in entry into the S phase, but they exhibit a partial impairment in exiting from the S/G2/M phase and entering into a second division, without a significant increase in ploidy or apoptosis.

**Defective homeostatic mechanisms in Geminin-deficient T cells**

The overall size and composition of the peripheral T cell pool are controlled by homeostatic mechanisms that regulate cell survival and basal steady-state proliferation (33, 34), resulting in relatively constant size. Thus, when the T cell pool is severely depleted, the remaining T cells undergo spontaneous proliferation (35, 36). To assess the ability of T cells that lack Geminin to proliferate under such conditions, we adoptively transferred into lymphopenic Rag2KOytc−/− recipients an equal number of CFSE-labeled T cells derived from Fl/koCD2Cre or control LNs. Tracking CFSE dilution in adoptive transfer experiments allows us to study the ability of WT and defective for Geminin T cells to undergo homeostatic proliferation. The CFSE dilution profile of CD8+ cells was measured by FACS at 3 and 5 d posttransfer. Control CD8+ T cells had divided at least five times by 3 d, whereas the majority of T cells that lack Geminin expression had undergone a single division with only a small percentage of cells undergoing two or more. At day 5, control CD8+ T cells had undergone >11 divisions, with the vast majority of the Fl/koCD2Cre T cells having undergone less than four divisions (Fig. 6). Spleens were isolated from Rag2KOytc−/− host mice 2 wk posttransfer of T cells, and the CD4 and CD8 T cell populations that repopulated the recipient mice were assessed by FACS. The numbers of CD4 and CD8 T cells that were recovered from Rag2KOytc−/− hosts and derived from Fl/koCD2Cre are reduced by 51% (0.48 × 10⁶, n = 4, versus 0.99 × 10⁶, n = 4; t test, p = 0.06) and 72% (0.43 × 10⁶, n = 4, versus 1.61 × 10⁶, n = 4; t test, p = 0.007), respectively, when compared with the control mice (Fig. 6). These results suggest that in lymphopenic environments, T cells that lack Geminin are not able to divide as efficiently as WT T cells, confirming the significant defect in T cell proliferation kinetics seen during homeostatic T cell proliferation.

**Stimulated T cells that lack Geminin show increased Cdt1 expression levels and accumulate in G2 phase**

Geminin exerts its role during the cell cycle by binding to and inhibiting Cdt1 (1), which is in addition negatively regulated by proteolysis through the Skp2/SCF and Cul4/DDB1 ubiquitin ligases (37–39). To investigate whether thymocytes and stimulated T cells may differ in their ability to regulate Cdt1 levels in the absence of Geminin, protein extracts derived from WT and Geminin-deficient thymocytes and stimulated T cells were subjected to Western blotting analysis using Abs against Cdt1 (Fig. 7A). Cdt1 protein expression is increased in Geminin-deficient peripheral T cells activated with anti-CD3/CD28 for 16 h when compared with stimulated T cells derived from control animals (Fig. 7A). However, Cdt1 protein expression in WT and mutant thymocytes is similar (Fig. 7A). These results suggest that differences in Cdt1 protein levels may, at least partially, account for the defects that we observed in activated T cells for Geminin.

Our analysis shows that developing thymocytes and peripheral T cells exhibit a differential requirement for Geminin for efficient proliferation. Activated T cells that lack Geminin expression accumulate in S/G2/M phase of the cell cycle. To further investigate this cell cycle defect and address the underlying mechanism, we have examined the expression levels of molecules controlling the G1 to S and G2 to M transition. Naïve T cells derived from control and Fl/koCD2Cre mice were stimulated with anti-CD3/CD28 Abs, and total protein extracts were isolated at 0, 16, and 44 h post-stimulation and analyzed by Western blotting. At 44 h, expression of cyclin D3, cyclin E, phosphorylated Rb, and p27 expression appear similar, whereas expression of cyclin B1, cyclin A, and tyrosine 15 phosphorylated cdc2 are increased in activated T cells that lack Geminin expression (Fig. 7B). This suggests that stimulated T cells lacking Geminin accumulate in G2 phase before G2/M transition.

**Discussion**

Geminin has been implicated in the control of DNA replication and neural cell fate acquisition and is believed to work as a molecular link between proliferation and differentiation (4). Inactivation and overexpression experiments in cell lines and model organisms have suggested an essential role for Geminin in establishing neural fate determination and maintaining genomic integrity. The complete...
inactivation of the mouse Gemini gene leads to early lethality before the blastocyst stage and, therefore, cannot address the in vivo function of Gemini in regulating maintenance and differentiation of progenitor cells, organ development, and homeostasis (12, 13, and data in the current study). To study the in vivo role of Gemini, we have specifically deleted Gemini from T cells using Cre recombinase under the regulation of the human CD2 LCR and promoter sequences. Contrary to what has been suggested for the role of Gemini in other experimental systems, conditional inactivation of Gemini in the lymphoid system appears not to be essential for the maintenance and differentiation processes of the T cell lineage in the thymus, because all progenitor populations are present. DN1, DN4, and DP T cells show a small reduction in cell number, suggesting that Gemini does not have a major role in cellular divisions of T cell progenitors in the thymus. It is unlikely that the lack of phenotype during T cell differentiation can be attributed to small amounts of protein remaining after Gemini deletion, because previous studies in tumorigenic cell lines have shown that Gemini is an unstable protein; it is rapidly degraded following ubiquitination from the anaphase promoting complex during mitosis and G1 (40), whereas even during S and G2 phases, Gemini has a short t1/2 (41, 42). Therefore, our data indicate that in vivo, Gemini may not be essential for the proliferation and differentiation of T cell progenitor populations. Our data do not exclude that Gemini may be critical at an earlier stage of hematopoietic system development, as previously suggested for Gemini-Polycomb complex interactions (11).

In contrast to progenitor T cells in the thymus, we show that peripheral T cells lacking Gemini expression present a dramatic defect in proliferation both in vitro, following TCR activation, and in vivo, following adoptive transfer into lymphopenic recipient mice. Furthermore, we show that activated T cells lacking Gemini are able to enter S phase; however, they show reduced ability to perform multiple rounds of division. T cells lacking Gemini show abnormal cell cycle profiles, with increased number of cells in G2/M following activation. Increased expression of cyclin B1 and the Y15 phosphorylated form of cdc2 suggests that activated T cells are accumulated in G2 phase of the cell cycle in the absence of Gemini. G2 cell cycle arrest due to lack of Gemini has been linked with aberrant replication triggered by uncontrolled Cdt1 activity and induction of DNA damage response. This finding is reminiscent of Gemini depletion experiments by antisense oligonucleotides in Xenopus laevis oocytes, which also resulted in G2 arrest (43). Defects during mitosis were also described following RNA interference depletion of Gemini in U2OS, HCT116, and TIG3 human cell lines, suggesting a role of Gemini for censome duplication and chromosome segregation (44). In Drosophila (45), several human cell lines (46, 47), and early embryonic divisions in the mouse (12, 13), absence of Gemini led to over-replication of the genome, in accordance with Gemini’s role as a negative regulator of the licensing factor Cdt1 (1), accompanied in some systems by DNA damage and signs of apoptosis (46, 47). We did not observe a detectable increase in ploidy, apoptosis in T cells lacking Gemini, though partial rereplication cannot be excluded. Interestingly, a similar lack of obvious rereplication in the absence of Gemini was noted in HeL.a cells (48, 49).

Activated T cells labeled for BrdU exhibited a decrease in Ki67 expression in the absence of Gemini, suggestive of cell cycle withdrawal. Our results suggest that peripheral T cells undergoing rapid divisions upon TCR activation or homeostatic proliferation require Gemini for efficient proliferation and repeated divisions, and its absence causes cell cycle delay and promotes cell cycle exit. In contrast, common lymphoid progenitors that migrate into the thymus from the bone marrow (50) appear to a large extent able to undergo a progressive commitment, maturation, and proliferation in the absence of Gemini. Increased protein expression levels of Cdt1 detected in stimulated T cells could partly account for the sensitivity of activated T cells to the lack of Gemini compared with thymocytes. An alternative possibility could be that in vivo proteolytic regulation of Cdt1 by two distinct E3 ubiquitin ligase systems (39) is able to restrict Cdt1 activity efficiently, and therefore Gemini in T cell development operates as a redundant control (45–47). Furthermore, the relative contribution of multiple redundant pathways that regulate licensing in parallel to Gemini (51) could also differ between cells at different stages of development, thus rendering different cells differentially affected by the absence of Gemini.

In this report, we provide in vivo evidence that during T cell development, Gemini is not essential for progenitor T cell proliferation, commitment, and specification. Our results suggest a differentiation stage-specific role for Gemini similar to other central cell cycle regulators, such as cdk2 and cyclin E, that have been previously shown to have cell type-specific effects in vivo (52). Differences in Gemini requirements by developing thymocytes could reflect the existence of alternative mechanisms that operate redundantly or compensatory and control genomic stability, progenitor proliferation, and differentiation in these cells (47, 53, 54). The mice we report in this study, which specifically lack Gemini in T cells, may prove a valuable system to probe these various pathways, but also assess the effects of Gemini absence in the immune response and tumorigenesis. Moreover, these mice might be a useful model to study immune system activation in the absence of efficient T cell expansion.

Disclosures
The authors have no financial conflicts of interest.

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Figure S1

Expression of Geminin mRNA in progenitor and mature T cells.
Total mRNA was isolated from FACS-sorted double negative cells (DN1, DN2, DN3, DN4), double positive cells (DP) and single positive cells from thymus (CD4, CD8) and spleen (splCD4, splCD8) and expression of Geminin mRNA was measured by real time. Values represent Geminin mRNA expression (±standard error) normalised for Hprt expression. Representative data of three independent experiments are shown.
Activated T cells from Control (Fl/ko, Fl/wtCD2Cre, wt/ko) and Fl/koCD2Cre mice show similar expression levels of activation markers. T cells were activated using anti-CD3 (10\(\mu\)g/ml), anti-CD3/CD28 (10\(\mu\)g/ml and 2\(\mu\)g/ml respectively), Concanavalin A (ConA, 5\(\mu\)g/ml) and phorbol 12,13-dibutyrate/Ionomycin (PdbU/Ion 0.5 and 1\(\mu\)g/ml) for 6 and 24 hours. The percentages of CD4 and CD8 T cells expressing CD62L (shown in A) and CD44 (shown in B) were quantified at each time point by flow cytometry. Control mice and Fl/koCD2Cre mice are indicated as black and white columns respectively.
Figure S3
Stimulated T cells that lack Geminin expression do not show increased apoptosis.
T cells derived from Fl/wt and Fl/KoCD2Cre were activated using anti-CD3 (1μg/ml) and anti-
CD28 (2μg/ml) mAbs. Flow cytometric analysis for AnnexinV and PI is shown on CD4^+CD69_{high}
and CD4^+CD25_{high} (24 and 48 hours after stimulation respectively).