HHS Public Access

Author manuscript

Exp Hematol. Author manuscript; available in PMC 2022 June 01.

Published in final edited form as:

Exp Hematol. 2021 June ; 98: 25–35. doi:10.1016/j.exphem.2021.04.001.

The transcriptional regulator GON4L is required for viability and hematopoiesis in mice

Diana F. Colgan[a], **Renee X. Goodfellow**[a], **John D. Colgan**[a,b,c]

[a]Department of Internal Medicine, University of Iowa, 375 Newton Road, Iowa City, IA 52242, USA

[b]Department of Anatomy and Cell Biology, University of Iowa, 375 Newton Road, Iowa City, IA 52242, USA

[c]Interdisciplinary Graduate Program in Immunology, Carver College of Medicine, University of Iowa, 375 Newton Road, Iowa City, IA 52242, USA

Abstract

The *Gon4l* gene encodes a putative transcriptional regulator implicated in the control of both cell differentiation and proliferation. Previously, we described a mutant mouse strain called *Justy* in which splicing of pre-mRNA generated from *Gon4l* is disrupted. This defect severely reduces, but does not abolish, GON4L protein expression, and blocks the formation of early B-lineage progenitors, suggesting *Gon4l* is required for B cell development in vertebrates. Yet, mutations that disable *Gon4l* in zebrafish impair several facets of embryogenesis that include the initiation of primitive hematopoiesis, arguing this gene is needed for multiple vertebrate developmental pathways. To better understand the importance of *Gon4l* in mammals, we created mice carrying an engineered version of *Gon4l* that can be completely inactivated by Cre-mediated recombination. Breeding mice heterozygous for the inactivated *Gon4l* allele failed to yield any homozygous-null offspring, demonstrating *Gon4l* is an essential gene in mammals. Consistent with this finding, as well previously published results, cell culture studies showed loss of *Gon4l* blocks cell proliferation and compromises viability, suggesting a fundamental role in the control of cell division and survival. Studies using mixed bone marrow chimeras confirmed *Gon4l* is required for B cell development, but also showed it is needed to maintain definitive hematopoietic stem/progenitor cells that are the source of all hematopoietic cell lineages. Our findings reveal *Gon4l* is an essential gene in mammals that is required to form the entire hematopoietic system.

Keywords

Gon4l; Mice; Essential Gene; Hematopoiesis; B Cell Development

Corresponding Author: Dr. John D. Colgan, Ph.D., Phone: +1 (319) 335-9561, john-colgan@uiowa.edu, Address: 25 South Grand Ave, Iowa City, IA 52242, USA.

Competing Interests Statement

The authors have no competing interests.

Introduction

Hematopoiesis generates all components of the blood [1]. In vertebrates, bone marrow is the main site of hematopoiesis after birth. Here, rare, self-renewing hematopoietic stem cells (HSCs) respond to environmental cues and produce multipotent progenitors (MPPs). These cell populations differentiate and commit to a particular fate, leading to the generation of erythroid, myeloid, and lymphoid cells. Hematopoiesis is largely controlled by transcription factor networks that enforce widespread remodeling of gene expression and promote differentiation into specific cell lineages [2]. Progression of hematopoiesis is also coordinated with bursts of cell proliferation [3]. Although much has been learned about the factors that guide hematopoiesis, it is likely that additional proteins important for this process remain to be identified.

GON4L is a 250-kDa nuclear protein predicted to contain domains characteristic of transcriptional regulators; these include a highly acidic region with homology to nucleoplasmin histone-binding domains, 2 paired amphipathic helix repeats and a SANT-L domain [4, 5]. GON4L forms complexes with the transcriptional regulators YY1, SIN3A and HDAC1 [5] and also interacts with NPAT, a transcriptional coactivator important for replication-dependent histone gene expression [6, 7]. GON4L appears to have a key role in regulating the expression of genes that guide specific developmental pathways [8–12] as well as those important for cell proliferation [12, 13]. Further supporting a role in gene regulation, GON4L associates with sites throughout the zebrafish genome via some unknown mechanism, and its loss broadly disrupts gene expression in zebrafish embryos [11].

Genetic studies involving various species have suggested *Gon4l* orthologs are important for multiple developmental pathways. In *Caenorhabditis elegans*, *Gon4l* is needed to generate somatic gonadal tissues [14], while in *Drosophila*, the gene is required for muscle cell differentiation in developing embryos [9]. Mutations affecting *Gon4l* in zebrafish disrupt several embryonic developmental pathways, including anteroposterior axis extension, somite formation, heart development and the onset of primitive hematopoiesis [8, 11, 12, 15, 16]. We've studied a mouse strain named *Justy*, which bears a recessive mutation that severely reduces *Gon4l* mRNA expression in B cell progenitors and blocks B cell development [10, 13]. Although these findings suggest *Gon4l* function differs between species, a common theme among them is that loss of *Gon4l* function impacts gene regulation and cell proliferation.

Gon4l mutations cause distinct defects in 2 vertebrate species, zebrafish and mice. Given this difference, we sought to further characterize the role of *Gon4l* in mice using a model in which the gene can be completely disabled rather than partially as the *Justy* mutation does. We therefore created mice carrying a floxed *Gon4l* allele that can undergo Cre-mediated recombination in a drug-inducible or tissue-specific manner, resulting in complete loss of function. Using this model, we found *Gon4l* is an essential gene in mice. We confirmed *Gon4l* was necessary for B cell development, but also found this gene is needed to sustain definitive hematopoietic stem/progenitor cells that give rise to all hematopoietic cell

lineages. Together, our data demonstrate that, as in zebrafish, *Gon4l* has roles in several developmental pathways in mammals, and is critical for hematopoiesis.

Materials and Methods

Mice

Wild-type (WT) C57BL/6J (B6) mice, WT CD45.1 B6 mice, those bearing the FLPe cDNA inserted into the *ROSA26 (R26)* locus [17]), those carrying the *EIIa-cre* transgene [18], those bearing the *Vav-cre* transgene [19], and those carrying the CreERT2 cDNA inserted into the *R26* locus [20] were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Embryonic stem (ES) cells of the C57BL/6N background bearing an engineered *Gon4l* allele were obtained from the International Mouse Phenotyping Consortium. Mice of both sexes were used for experiments. All mouse procedures were approved by the University of Iowa Institutional Care and Use Committee and adhere to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Generation of pro-B cells

The generation of pro-B cells has been described [13]. Pro-B cells were transduced with a retroviral vector that expresses the BCR-ABL p210 oncoprotein and GFP (Addgene, Watertown, MA, USA). The BCR-ABL vector and the plasmid pCL-Eco [21] were transfected into 293T cells. After 24 hours, culture supernatants were collected. 1×10^6 pro-B cells in RPMI (RPMI 1640 (Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum, 1% L-glutamine, 50 units/mL penicillin, 50 ug/ml streptomycin and 50 μM β–mercaptoethanol) containing IL-7 were added to wells in a 24-well plate coated with OP9 stromal cells. The next day, 1 mL of culture supernatant containing retroviral particles was added and the plates centrifuged at RT for 2 hours at 800 x g. Transduction was confirmed by analysis of GFP expression.

Gon4l inactivation in pro-B cells

1×10^6 pro-B cells in RPMI containing IL-7 were added to wells in a 24-well plate. 4-hydroxytamoxifen (4-OHT; Sigma Aldrich, St. Louis, MO, USA) dissolved in ethanol was added to 2 μM, after which cells were cultured for up to 5 days. DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen; Hilden, Germany). Protein lysates were prepared by resuspending cells in RIPA buffer containing protease inhibitors (Roche Life Sciences, Indianapolis, IN, USA), incubating on ice for 10 min, and then centrifuging to remove insoluble material. Cell proliferation was assayed by harvesting cells on day 3 of culture, staining with trypan blue, and counting live cells. Cell survival was tracked by harvesting cells on days 1 through 5 of culture and identifying live cells by flow cytometry and staining with Hoechst 33258 (Sigma Aldrich). Levels of activated caspases 3 and 7 were assayed as described [22].

Mixed bone marrow chimeras (MBMCs)

WT CD45.1 B6 mice were used as bone marrow transplant recipients; these were irradiated with 1100 rads and injected with donor bone marrow 1 hour later. Donor bone marrow was obtained by flushing bones with PBS containing 3% FBS and depleting red blood cells using

Pharm Lyse (BD Biosciences, San Diego, CA, USA). 1×10^7 donor bone marrow cells were injected intravenously into recipients, which were given drinking water containing ampicillin (2mg/ml) for 2 weeks, and allowed to reconstitute for an additional 8 weeks before use. MBMCs were injected intraperitoneally with 40 mg/kg of tamoxifen dissolved in corn oil (both from Sigma Aldrich) for 5 consecutive days and analyzed 3 weeks later.

Flow cytometric analysis

Flow cytometric analysis was performed as described [10]. Data were collected using an LSR II (BD Biosciences) and analyzed using FlowJo software (BD Biosciences). Antibodies were obtained from BD Biosciences, Biolegend (San Diego, CA, USA), and eBioscience (San Diego, CA, USA). Most antibody clones used have been described [13]. Additional clones used were CD45.1 (A20), CD45.2 (104), c-KIT (2B8), and SCA-1 (E13–161.7). GON4L antibodies were described previously [10]. β-Actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Streptavidin Qdot 605 was obtained from BD Biosciences.

Statistical analysis

Data were analyzed using the unpaired, two-tailed Student's *t* test in Prism software (GraphPad, San Diego, CA, USA).

Results

Creation of mice bearing a floxed *Gon4l* allele

To begin our studies, we generated mice carrying a floxed *Gon4l* allele that can be inactivated due to excision of sequences by the Cre recombinase (Supplementary Figure 1). A gene-targeting vector was constructed in which exon 4 of *Gon4l* was floxed, or flanked by LoxP sites that serve as Cre recognition elements. Cre-mediated recombination removes exon 4 of *Gon4l*, creating an allele that produces mRNA in which exon 5 onward is out of frame.

The gene-targeting vector was electroporated into mouse ES cells, and neomycin-resistant clones that had undergone homologous recombination between the vector and an endogenous copy of *Gon4l* were isolated. These were used to generate chimeric mice, which were mated to produce progeny carrying the engineered *Gon4l* allele. These offspring were mated with mice expressing the FLPe recombinase to delete sequences encoding the *LacZ* and neomycin resistance genes. Removal of these sequences generated mice carrying a floxed *Gon4l* allele. Mice homozygous for this version of *Gon4l* are fertile and live a normal lifespan.

The floxed *Gon4l* allele is inactivated by Cre-mediated recombination

We next sought to confirm the floxed *Gon4l* allele was rendered inactive by Cre-mediated recombination. We first generated $Gon4l^{fl/fl}/R26^{CreERT}$ mice, which are homozygous for both the floxed *Gon4l* allele and a modified *R26* gene that expresses the CreERT fusion protein. In the presence of tamoxifen or its derivatives, CreERT migrates from the cytoplasm to the nucleus and recombines floxed genes.

We used MPPs from *Gon4l*$^{fl/fl}$/*R26*^{CreERT} mice to generate pro-B cells, which were then transduced with a retroviral expressing the BCR-ABL p210 oncoprotein (BCR-ABL) to promote growth and survival. These cells were cultured with either vehicle or vehicle containing the tamoxifen derivative 4-OHT to induce CreERT-mediated recombination of the floxed *Gon4l* allele. On day 3 of culture, pro-B cells were harvested to prepare genomic DNA and protein lysates. PCR amplification of genomic DNA showed both floxed *Gon4l* alleles had undergone recombination in cells treated with to 4-OHT, resulting in excision of exon 4 (Fig. 1A, 1B). Western blot analysis of protein lysates using an antibody raised against the C-terminal 514 amino acids of GON4L [10] confirmed deletion of exon 4 abolished expression of full-length GON4L protein (Fig. 1C). These data demonstrate Cre-mediated recombination inactivates the floxed version of *Gon4l*, creating a null allele.

Gon4l inactivation in pro-B cells arrests growth and causes cell death

The *Justy* mutation in *Gon4l* impairs the proliferation and survival of early B cell progenitors [13]. To test whether *Gon4l* inactivation by CreERT elicits the same effects, *Gon4l*$^{fl/fl}$/*R26*^{CreERT} pro-B cells were cultured in medium containing vehicle or 4-OHT. After 3 days, cells were harvested and viable cells counted (Fig. 2A). As expected, cells treated with vehicle proliferated, increasing in number by ~4-fold. In contrast, cells treated with 4-OHT slightly decreased in number, suggesting their proliferation was blocked. Flow cytometry was used to determine the frequencies of live pro-B cells over the course of 5 days in culture (Fig. 2B, 2C). For cells treated with vehicle, the frequencies of live cells remained relatively constant. In contrast, the frequencies of live cells treated with 4-OHT declined dramatically over time. Staining with the vital dye Hoechst confirmed 4-OHT-treated cells died during the culture period (Fig. 2D). Additionally, cells treated with 4-OHT contained higher levels of the active forms of the proapoptotic caspases 3 and 7 (Fig. 2E, 2F). These data indicate *Gon4l* inactivation blocks pro-B cell proliferation and induces cell death via apoptosis.

Gon4l is essential for mouse viability

Studies by others showed *Gon4l* is required for zebrafish embryogenesis and is thus an essential gene [8, 11, 12, 15, 16]. To determine whether *Gon4l* is essential in mice, *Gon4l*$^{fl/fl}$ mice were mated with those bearing the *EIIa-cre* transgene, which expresses Cre in early mouse embryos and directs recombination in the germ line. The resulting offspring were bred to create mice that lacked *EIIa-cre* and were heterozygous (*Gon4l*$^{+/-}$) for the inactivated *Gon4l* allele. These mice were then intercrossed. Genotyping of 62 progeny showed *Gon4l*$^{+/+}$ and *Gon4l*$^{+/-}$ mice were recovered, but *Gon4l*$^{-/-}$ mice were not. These results demonstrate *Gon4l* is an essential gene in mice.

Gon4l is required to maintain bone marrow

In zebrafish, mutation of *Gon4l* blocks the initiation of hematopoiesis [8], so we sought to determine whether *Gon4l* is needed for hematopoiesis in mice. For this purpose, we first attempted to generate *Gon4l*$^{fl/fl}$ mice bearing the *Vav-cre* transgene, which is active in hematopoietic stem/progenitor cells [19]. Despite extensive breeding, we were unable to obtain any viable *Gon4l*$^{fl/fl}$/*Vav-cre* mice (data not shown). These findings suggested that *Gon4l* is required for primitive and/or definitive hematopoiesis in mice.

To circumvent the problems encountered with the *Vav-cre* mouse model described above, mixed bone marrow chimeras (MBMCs) were made and analyzed as outlined in Figure 3A. This strategy allowed us to maintain hematopoietic development from one population of transplanted bone marrow cells (the CD45.1$^+$ fraction) while testing how deletion of *Gon4l* affected hematopoiesis from the other (the CD45.2$^+$ fraction). Further, this strategy allowed us to rule out effects of *Gon4l* deletion on other cell types, such as stromal cells that support hematopoiesis within bone marrow.

To generate control MBMCs, bone marrow from WT CD45.1 mice was mixed 1:1 with that from CD45.2 mice that were *Gon4l*$^{+/+}$/*R26*$^{CreERT+/+}$ (i.e., homozygous WT for *Gon4l*). To create experimental MBMCs, bone marrow from CD45.1 WT mice was mixed 1:1 with that from CD45.2 mice that were *Gon4l*$^{f/f}$/*R26*$^{CreERT+/+}$ (i.e., homozygous for the floxed *Gon4l* allele). Flow cytometric analysis confirmed bone marrow from reconstituted MBMCs contained roughly a 1:1 mix of cells from each donor (data not shown).

To confirm tamoxifen-induced CreERT activity did not affect bone marrow that was WT for *Gon4l*, control MBMCs were generated, injected with either vehicle or vehicle containing tamoxifen, and their bone marrow analyzed 3 weeks later. Similar numbers of total bone marrow cells were recovered from control MBMCs treated with vehicle or vehicle containing tamoxifen (Supplementary Figure 2A). Flow cytometric analysis showed neither the frequencies nor the numbers of *Gon4l*$^{+/+}$/*R26*$^{CreERT+/+}$ CD45.2$^+$ cells were altered by tamoxifen treatment relative to injection of vehicle (Supplementary Figure 2B, 2C). These data confirm that tamoxifen-induced activation of CreERT has no effect on *Gon4l*$^{+/+}$ bone marrow.

To test how *Gon4l* inactivation affected bone marrow, control and experimental MBMCs were injected with tamoxifen and analyzed 3 weeks later. Similar numbers of total bone marrow cells were recovered from control and experimental MBMCs (Fig. 3B). Strikingly, however, flow cytometric analysis showed that *Gon4l*$^{f/f}$ CD45.2$^+$ cells were almost completely depleted in bone marrow from experimental MBMCs, while *Gon4l*$^{+/+}$ CD45.2$^+$ cells in control MBMCs were unaffected as expected (Fig. 3C). This difference was reflected in dramatically lower yields of total CD45.2$^+$ cells from experimental MBMCs relative to controls (Fig. 3D). These data demonstrate Cre-mediated inactivation of *Gon4l* disrupts the generation and/or maintenance of bone marrow.

Inactivation of *Gon4l* confirms a key role in B cell development

To test how loss of *Gon4l* affects B cell development, control and experimental MBMCs were injected with tamoxifen and analyzed. Flow cytometric analysis of bone marrow from experimental MBMCs indicated *Gon4l* inactivation largely abrogated B cell development from *Gon4l*$^{f/f}$ CD45.2$^+$ cells, but had no effect on that from *Gon4l*$^{+/+}$ CD45.2$^+$ cells in control MBMCs (Fig. 4A). Consistent with these results, the numbers of late-stage (immature and pre-B cells) and early-stage B cell progenitors (pro-B and pre-pro-B cells) derived from *Gon4l*$^{f/f}$ CD45.2$^+$ cells were dramatically reduced (Fig. 4B–D). These results indicate *Gon4l* is required for B cell development.

Gon4l is required to sustain hematopoietic progenitors in bone marrow

Our data showed *Gon4l* inactivation disrupted all stages of B cell development including pre-pro-B cells, which are the most primitive of B-lineage progenitors. To test whether cells that give rise to pre-pro-B cells were also affected by loss of *Gon4l*, we performed flow cytometric analysis to identify and enumerate common lymphoid progenitors (CLPs), the direct precursors for pre-pro-B cells. Our approach also allowed us to identify and count common myeloid progenitors (CMPs), which are the source of erythroid and myeloid cells, and the $Lin^{-}SCA1^{+}c\text{-}KIT^{+}$ (LSK) fraction of cells, which contains MPPs and HSCs that are the source of both CLPs and CMPs.

Bone marrow from control and experimental MBMCs treated with tamoxifen was analyzed to identify the cell populations described above. Among $Gon4l^{f/f}$ $CD45.2^{+}$ cells in experimental MBMCs, the frequencies of CLPs and CMPs were substantially decreased, while those for the LSK fraction were slightly elevated (Fig 5A). However, cell counts revealed the numbers of CLPs, CMPS and LSK cells were all greatly reduced among $Gon4l^{f/f}$ $CD45.2^{+}$ cells from experimental MBMCs compared to $Gon4l^{+/+}$ $CD45.2^{+}$ cells from control MBMCs (Fig. 5B–D). These findings show *Gon4l* is required for the generation and/or maintenance of primitive progenitor cells that give rise to all hematopoietic lineages.

LSK cells are the source of early thymic precursors (ETPs), which migrate from the bone marrow to the thymus and seed T cell development. Given LSK cells were significantly decreased upon *Gon4l* inactivation, we determined how this affected T cell development by analyzing thymii from control and experimental MBMCs treated with tamoxifen. The total yields of thymocytes from experimental MBMCs were slightly but significantly reduced compared to those from controls (Fig. 6A). Flow cytometric analysis revealed $Gon4l^{f/f}$ $CD45.2^{+}$ cells were almost completely absent among thymocytes from experimental MBMCs, while $Gon4l^{+/+}$ $CD45.2^{+}$ were plentiful in those from control MBMCs (Fig. 6B, 6C). These data indicate thymic T cell development is abrogated by the loss of *Gon4l*, an effect likely due in part to a decrease in the output of ETPs by LSK cells.

Discussion

Gon4l encodes a nuclear factor implicated in gene regulation and cell proliferation. Our prior work showed a point mutation that partially disables *Gon4l* causes a block in B cell development. Here, using a mouse model in which *Gon4l* can be completely inactivated, we found this gene is essential for mouse viability. Our results also revealed *Gon4l* is required for sustaining progenitor cells that give rise to all components of the hematopoietic system. Our findings demonstrate *Gon4l* function in mammals extends beyond B cell development to other developmental pathways, including hematopoiesis.

Our previous studies characterized the effect of a recessive point mutation in *Gon4l* called *Justy* [10, 13]. This lesion gives rise to a remarkably precise phenotype: it arrests B cell development at the pro-B cell stage without causing any other obvious abnormalities. These observations suggested *Gon4l* is specifically required for B cell development in mice. The data reported here support a role for *Gon4l* in this process, but also reveal the gene is needed

for additional developmental pathways. The different phenotypes caused by the *Justy* mutation and complete *Gon4l* inactivation are likely due to variation in the penetrance of the *Justy* mutation between tissues [10]. For example, in brain the *Justy* mutation modestly decreases *Gon4l* expression, while in B cell progenitors it is greatly reduced. This scenario argues the *Justy* mutation results in only partial loss of *Gon4l* function, and that, with the exception of B cell progenitors, the levels *Gon4l* expression in most tissues in *Justy* mice are sufficient to prevent the abnormalities that arise when the gene is fully disabled.

The defects caused by mutation of *Gon4l* orthologs in different species show a somewhat puzzling degree of variability. For invertebrate animal species and plants, loss of *Gon4l* function seems to disrupt singular and unrelated developmental processes [9, 14, 23] as opposed to the several pathways affected by the zebrafish or mouse gene is inactivated. One possible explanation could be that *Gon4l* in lower organisms has a dedicated role in one (or at relatively few) developmental pathways, whereas in vertebrates, the gene and its encoded protein evolved to take on broader importance. This idea would be in line with the relatively poor conservation between GON4L proteins in lower organisms compared to vertebrates, where the conservation is higher (see [23]).

The data presented here support the conclusion that *Gon4l* gene function is similar between vertebrate species, which was refuted by the disparate phenotypes of mutant zebrafish and *Justy* mice. In the zebrafish, mutation of *Gon4l* disrupts several distinct developmental pathways necessary for embryogenesis, showing it is required for viability [8, 11, 12, 15, 16]. As reported here, *Gon4l* is also necessary for the viability of mice. Notably, we have not characterized when in development mice lacking *Gon4l* die, leaving open the possibility that the cause of lethality in mice is different than that for zebrafish. Thus, it would be of interest to characterize how loss of *Gon4l* affects embryogenesis in mice. Nevertheless, that *Gon4l* is required for hematopoiesis in both zebrafish and mice argues the gene has similar roles in these 2 organisms, and perhaps among vertebrates in general.

In the case of zebrafish, it has been demonstrated that mutation of the *Gon4l* gene orthologue disrupts the initiation of primitive hematopoiesis. From our studies, we cannot firmly conclude that this process is also affected by loss of *Gon4l* during mouse embryogenesis. However, our results with the $Gon4l^{fl/fl}$/*Vav-cre* mice provide some support for the idea that *Gon4l* is needed to initiate primitive hematopoiesis in mouse, given that we were unable to recover any mice of this genotype. Additionally, given the high degree of conservation between the zebrafish and mouse GON4L proteins, it seems unlikely that GON4L would have a central role in primitive hematopoiesis in zebrafish, but not in mice. Alternatively, it remains possible that GON4L is only required for definitive hematopoiesis in mice as our data demonstrate. Again, given its conservation among vertebrates, we favor the idea that GON4L is required for both primitive and definitive hematopoiesis in these species.

Our results demonstrate that, as in zebrafish [8], *Gon4l* has a key role in hematopoiesis; this extends to the earliest definitive hematopoietic progenitors, HSCs and MPPs. What remains to be determined are the molecular pathways involving *Gon4l* that are required for hematopoiesis. The cell culture experiments presented here, as well as other studies [8, 12,

13, 16] have implicated *Gon4l* in the control of cell proliferation and survival, and also showed this gene promotes neoplastic cell growth [24]. Additionally, *Gon4l* is important for the expression of genes required for cell-cycle progression [12, 13]. Further, the GON4L-interacting proteins YY1, SIN3A and HDAC1 are all needed for cell proliferation [25–27]. Another related process that may require *Gon4l* is the control of replication-dependent histone gene expression. Such a role is suggested by data showing the *Drosophila Gon4l* ortholog encodes a component of histone locus bodies, and that mutation of the gene disrupts histone gene expression [9, 28]. Additionally, the GON4L protein interacts with NPAT, another component of histone locus bodies [7].

Other data are consistent with the idea that *Gon4l* regulates expression of genes crucial for specific developmental pathways. Such a role, perhaps coupled to one in the control of cell proliferation, would explain why specific developmental pathways are aborted when *Gon4l* is absent. Another function attributed to the GON4L protein is the maintenance of heterochromatin [9], which could also help explain why gene expression is disrupted when *Gon4l* function is lost.

As described above, GON4L appears to have central roles in the control of cell division and the induction of developmentally important genes. In considering GON4L's specific role in hematopoiesis, the 2 main processes that would most likely be affected are the regulation of cell division and the induction, and perhaps sustained expression, of genes important for hematopoietic development. Cell division is a critical feature of hematopoiesis, but must be coordinated with the initiation and establishment of developmental programs. Such are role for GON4L, i.e., orchestrating cell division with expression of genes that drive cellular differentiation, has been suggested by studies of species ranging from worms to plants to zebrafish [8, 14, 16, 23]. In the case of zebrafish, mutation of *Gon4l* disrupts expression of specific genes important for the initiation of primitive hematopoiesis[8]. Thus, there is precedent for proposing that GON4L is a coordinator of cell division and gene expression in the context of hematopoietic development, which could explain the dramatic effects caused by loss of Gon4l in mouse bone marrow cells. For future work, it will be important to identify specific molecular pathways that are disabled in hematopoietic progenitors lacking *Gon4l*, which should provide new and valuable insights regarding mechanisms that control hematopoiesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank the University of Iowa Genome Editing and Flow Cytometry Facilities for technical assistance and advice. This work was supported by the National Institutes of Health (NIH) Grant AI093737 (to J.D.C.). Some data presented herein were collected in the Flow Cytometry Facility, a Carver College of Medicine and Holden Comprehensive Cancer Center core research facility at the University of Iowa, which is funded through user fees and financial support from the Carver College of Medicine, the Holden Comprehensive Cancer Center, the Iowa City Veteran's Administration Medical Center and grant RR027219 from the National Center for Research Resources of the NIH.

References

[1]. Jagannathan-Bogdan M, Zon LI. Hematopoiesis. Development. 2013;140:2463–2467. [PubMed: 23715539]

[2]. Menegatti S, de Kruijf M, Garcia-Alegria E, Lacaud G, Kouskoff V. Transcriptional control of blood cell emergence. FEBS Lett. 2019;593:3304–3315. [PubMed: 31432499]

[3]. Hao S, Chen C, Cheng T. Cell cycle regulation of hematopoietic stem or progenitor cells. Int J Hematol. 2016;103:487–497. [PubMed: 27084253]

[4]. Ohtomo T, Horii T, Nomizu M, Suga T, Yamada J. Molecular cloning of a structural homolog of YY1AP, a coactivator of the multifunctional transcription factor YY1. Amino Acids. 2007;33:645–652. [PubMed: 17297563]

[5]. Lu P, Hankel IL, Hostager BS, et al. The developmental regulator protein Gon4l associates with protein YY1, co-repressor Sin3a, and histone deacetylase 1 and mediates transcriptional repression. J Biol Chem. 2011;286:18311–18319. [PubMed: 21454521]

[6]. Zhao J, Kennedy BK, Lawrence BD, et al. NPAT links cyclin E-Cdk2 to the regulation of replication-dependent histone gene transcription. Genes Dev. 2000;14:2283–2297. [PubMed: 10995386]

[7]. Yang XC, Sabath I, Kunduru L, van Wijnen AJ, Marzluff WF, Dominski Z. A conserved interaction that is essential for the biogenesis of histone locus bodies. J Biol Chem. 2014;289:33767–33782. [PubMed: 25339177]

[8]. Liu Y, Du L, Osato M, et al. The zebrafish udu gene encodes a novel nuclear factor and is essential for primitive erythroid cell development. Blood. 2007;110:99–106. [PubMed: 17369489]

[9]. Bulchand S, Menon SD, George SE, Chia W. Muscle wasted: a novel component of the Drosophila histone locus body required for muscle integrity. J Cell Sci. 2010;123:2697–2707. [PubMed: 20647374]

[10]. Lu P, Hankel IL, Knisz J, et al. The *Justy* mutation identifies Gon4-like as a gene that is essential for B lymphopoiesis. J Exp Med. 2010;207:1359–1367. [PubMed: 20530203]

[11]. Williams MLK, Sawada A, Budine T, Yin C, Gontarz P, Solnica-Krezel L. Gon4l regulates notochord boundary formation and cell polarity underlying axis extension by repressing adhesion genes. Nat Comm. 2018;9:1319.

[12]. Budine TE, de Sena-Tomas C, Williams MLK, Sepich DS, Targoff KL, Solnica-Krezel L. Gon4l/Udu regulates cardiomyocyte proliferation and maintenance of ventricular chamber identity during zebrafish development. Dev Biol. 2020;262:223–234.

[13]. Barr JY, Goodfellow RX, Colgan DF, Colgan JD. Early B Cell Progenitors Deficient for GON4L Fail To Differentiate Due to a Block in Mitotic Cell Division. J Immunol. 2017;198:3978–3988. [PubMed: 28381640]

[14]. Friedman L, Santa Anna-Arriola S, Hodgkin J, Kimble J. gon-4, a cell lineage regulator required for gonadogenesis in Caenorhabditis elegans. Dev Biol. 2000;228:350–362. [PubMed: 11112335]

[15]. Hammerschmidt M, Pelegri F, Mullins MC, et al. Mutations affecting morphogenesis during gastrulation and tail formation in the zebrafish, Danio rerio. Development. 1996;123:143–151. [PubMed: 9007236]

[16]. Lim CH, Chong SW, Jiang YJ. Udu deficiency activates DNA damage checkpoint. Mol Biol Cell. 2009;20:4183–4193. [PubMed: 19656853]

[17]. Farley FW, Soriano P, Steffen LS, Dymecki SM. Widespread recombinase expression using FLPeR (flipper) mice. Genesis. 2000;28:106–110. [PubMed: 11105051]

[18]. Lakso M, Pichel JG, Gorman JR, et al. Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. Proc Natl Acad Sci, USA. 1996;93:5860–5865. [PubMed: 8650183]

[19]. Georgiades P, Ogilvy S, Duval H, et al. VavCre transgenic mice: a tool for mutagenesis in hematopoietic and endothelial lineages. Genesis. 2002;34:251–256. [PubMed: 12434335]

[20]. Ventura A, Kirsch DG, McLaughlin ME, et al. Restoration of p53 function leads to tumour regression in vivo. Nature. 2007;445:661–665. [PubMed: 17251932]

[21]. Naviaux RK, Costanzi E, Haas M, Verma IM. The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. J Virol. 1996;70:5701–5705. [PubMed: 8764092]

[22]. Gorman JV, Starbeck-Miller G, Pham NL, et al. Tim-3 directly enhances CD8 T cell responses to acute Listeria monocytogenes infection. J Immunol. 2014;192:3133–3142. [PubMed: 24567532]

[23]. Brownfield L, Hafidh S, Durbarry A, et al. Arabidopsis DUO POLLEN3 is a key regulator of male germline development and embryogenesis. Plant Cell. 2009;21:1940–1956. [PubMed: 19638475]

[24]. Agarwal N, Dancik GM, Goodspeed A, et al. GON4L Drives Cancer Growth through a YY1-Androgen Receptor-CD24 Axis. Cancer Res. 2016;76:5175–5185. [PubMed: 27312530]

[25]. Dannenberg JH, David G, Zhong S, van der Torre J, Wong WH, Depinho RA. mSin3A corepressor regulates diverse transcriptional networks governing normal and neoplastic growth and survival. Genes Dev. 2005;19:1581–1595. [PubMed: 15998811]

[26]. Yamaguchi T, Cubizolles F, Zhang Y, et al. Histone deacetylases 1 and 2 act in concert to promote the G1-to-S progression. Genes Dev. 2010;24:455–469. [PubMed: 20194438]

[27]. Kleiman E, Jia H, Loguercio S, Su AI, Feeney AJ. YY1 plays an essential role at all stages of B-cell differentiation. Proc Natl Acad Sci, USA. 2016;113:E3911–3920. [PubMed: 27335461]

[28]. White AE, Burch BD, Yang XC, et al. Drosophila histone locus bodies form by hierarchical recruitment of components. J Cell Biol. 2011;193:677–694. [PubMed: 21576393]

Highlights

1. Previous study of a recessive, hypomorphic point mutation in the gene *Gon4l* suggested a role in the early stages of B cell development. However, the point mutation does not completely abolish *Gon4l* gene function.
2. Unlike the point mutation, conventional knockout of *Gon4l* revealed the gene is required for mouse viability.
3. Conventional knockout of *Gon4l* also demonstrated that *Gon4l* is required not only for B cell development, by for the generation and maintenance of the entire hematopoietic system.
4. The defects observed due to conventional knockout of *Gon4l* extended to definitive hematopoietic stem/progenitor cells, which are the source of all components of the hematopoietic system.
5. Mechanistic studies using transformed pro-B cells showed that loss of *Gon4l* induced growth arrest and apoptosis, suggesting the GON4L protein is essential for cell viability.

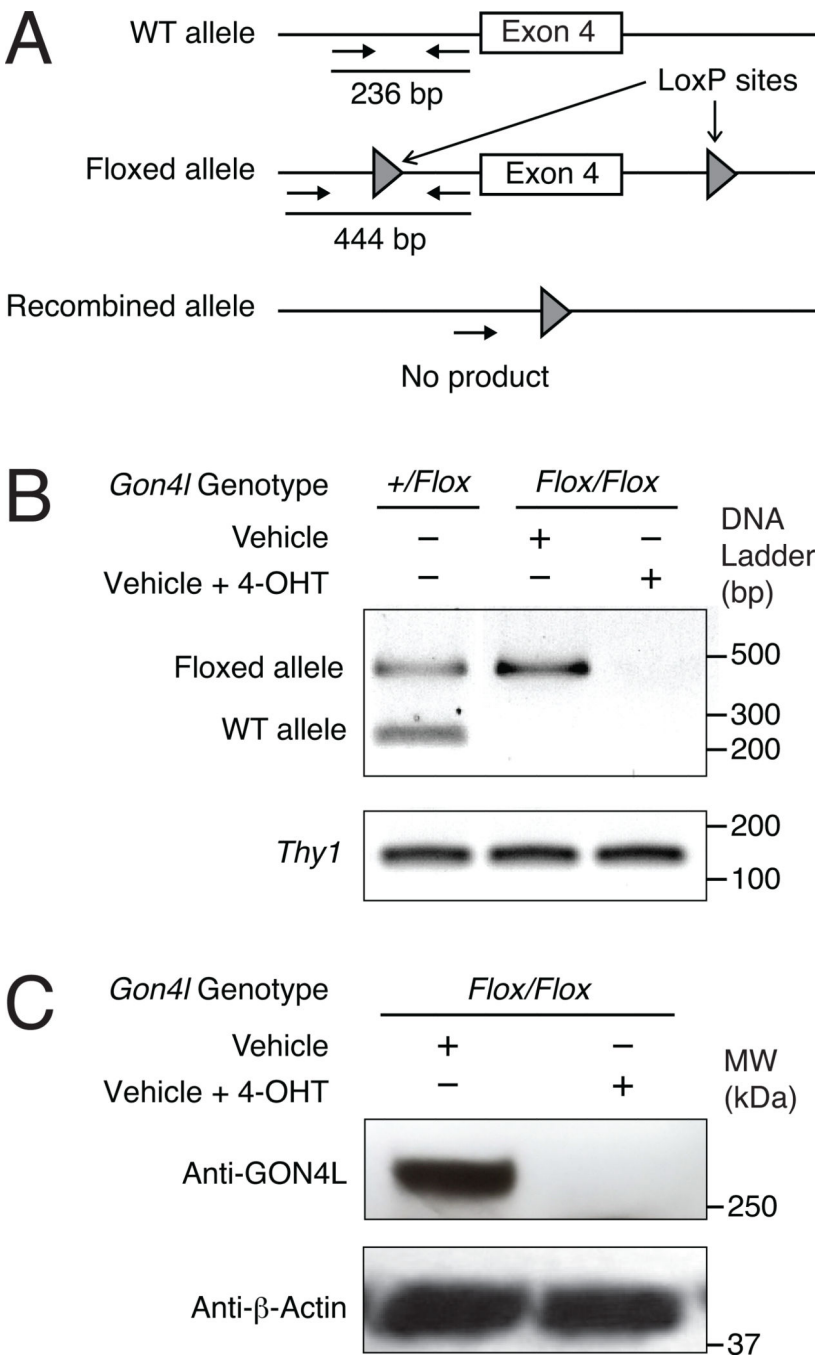

Figure 1. Cre-mediated recombination prevents protein expression from the floxed *Gon4l* allele.

BCR-ABL-expressing pro-B cells homozygous for both the floxed *Gon4l* allele and the *R26*^{CreERT} gene were cultured in medium containing either vehicle or vehicle plus 4-OHT. Cells were harvested on day 3 of culture to isolate genomic DNA and make protein lysates. (**A**) Schematic diagram of the WT, floxed and Cre-recombined *Gon4l* alleles. The position of polymerase chain reaction (PCR) primers (denoted by the arrows) and the PCR products generated from each allele are shown. Diagram is not to scale. (**B**) Top shows products generated by PCR amplification of genomic DNA isolated from mice heterozygous for the

floxed *Gon4l* allele (lane 1) or from BCR-ABL-expressing *Gon4l*$^{f/f}$/*R26*^{CreERT} pro-B cells treated with vehicle (lane 2) or vehicle containing 4-OHT (lane 3). The identities of the PCR products are shown at the left. Bottom shows the PCR products generated using primers specific for the *Thy1* gene, which confirmed all samples contained similar amounts of template DNA. **(C)** Western blot analysis of protein lysates prepared from BCR-ABL-expressing *Gon4l*$^{f/f}$/*R26*^{CreERT} pro-B cells treated with vehicle (lane 1) or vehicle plus 4-OHT (lane 2). Primary antibodies used are shown at the left. All data are representative of 3 independent experiments.

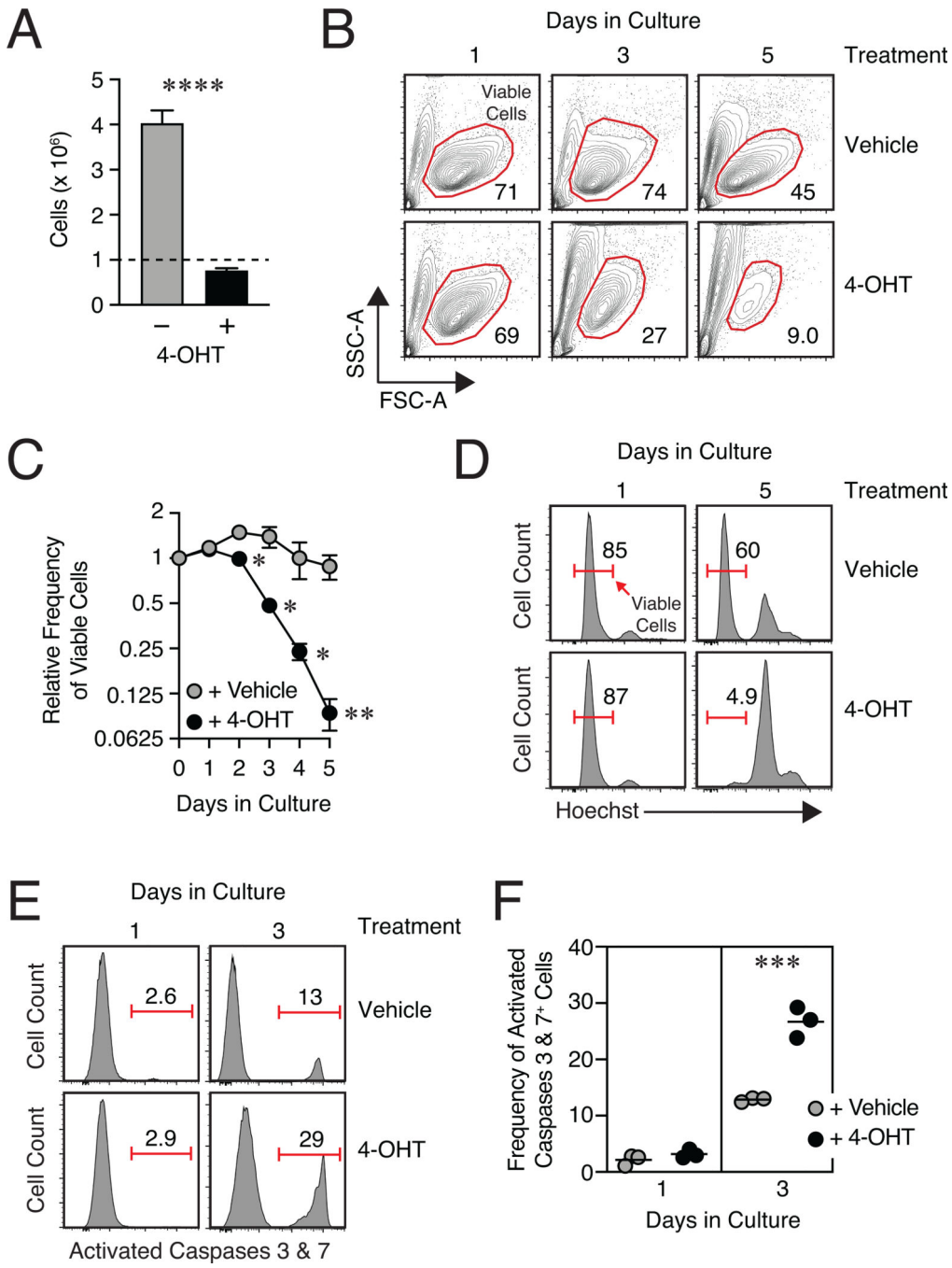

Figure 2. *Gon4l* inactivation greatly impairs pro-B cell growth and survival.

BCR-ABL-expressing *Gon4l*$^{f/f}$/*R26*^{CreERT} pro-B cells were cultured in medium containing either vehicle or vehicle plus 4-OHT and harvested for analysis. (**A**) Yields of live (trypan blue negative) pro-B cells harvested on day 3 of culture. The dotted line represents the number of cells (1×10^6) added to start the cultures. (**B**) Flow cytometric analysis of cell viability based on forward (FSC-A) and side scatter (SSC-A). Numbers are the frequencies of cells in the gate. (**C**) Graph of the frequencies of viable cells detected when harvested on the indicated days of culture. Data were generated by the analysis shown in panel B. Values

are relative to those obtained on day 0, which were set at 1. The y-axis represents a Log 2 scale. (**D**) Histograms showing uptake of the vital dye Hoechst by cells treated with vehicle or vehicle plus 4-OHT. Numbers are the frequencies of cells in the gate, which are those that excluded Hoechst and are thus viable. (**E**) Analysis of the levels of activated Caspases 3 and 7 in cells treated with vehicle or vehicle plus 4-OHT. (**F**) Graph of the frequencies of cells staining positive for activated Caspases 3 and 7. Data were generated by the analysis shown in panel E. Histograms shown in panels B, D and E are representative of 3 independent experiments. The bar graphs in panels A, C and F show the average and SEM of values obtained from 3 independent experiments. * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$.

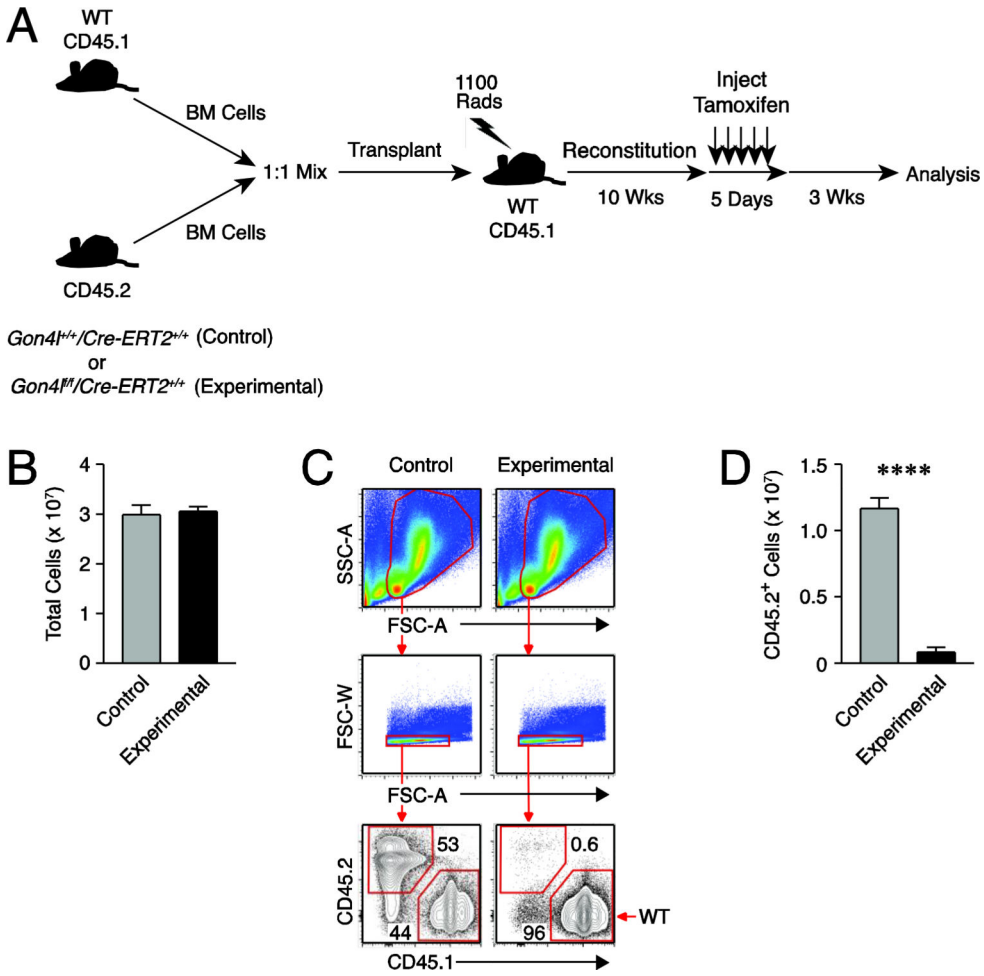

Figure 3. Inactivation of *Gon4l* gene results in loss of bone marrow cells.

MBMCs were created and injected with tamoxifen. Bone marrow was analyzed 3 weeks later. (**A**) Schematic diagram depicting how control and experimental MBMCs were generated and the time course for hematopoietic reconstitution, tamoxifen administration, and analysis of bone marrow. (**B**) Total bone marrow cell yields from the indicated MBMCs. (**C**) Representative flow cytometric analysis of bone marrow from the indicated MBMCs. (**D**) Yields of $CD45.2^+$ bone marrow cells from the indicated MBMCs. Bar graphs in panels B and D show the mean and SEM of values obtained from 3 independent experiments in which 3 control and 3 experimental MBMCs were analyzed (n = 9 MBMCs for each group). **** $P < .0001$.

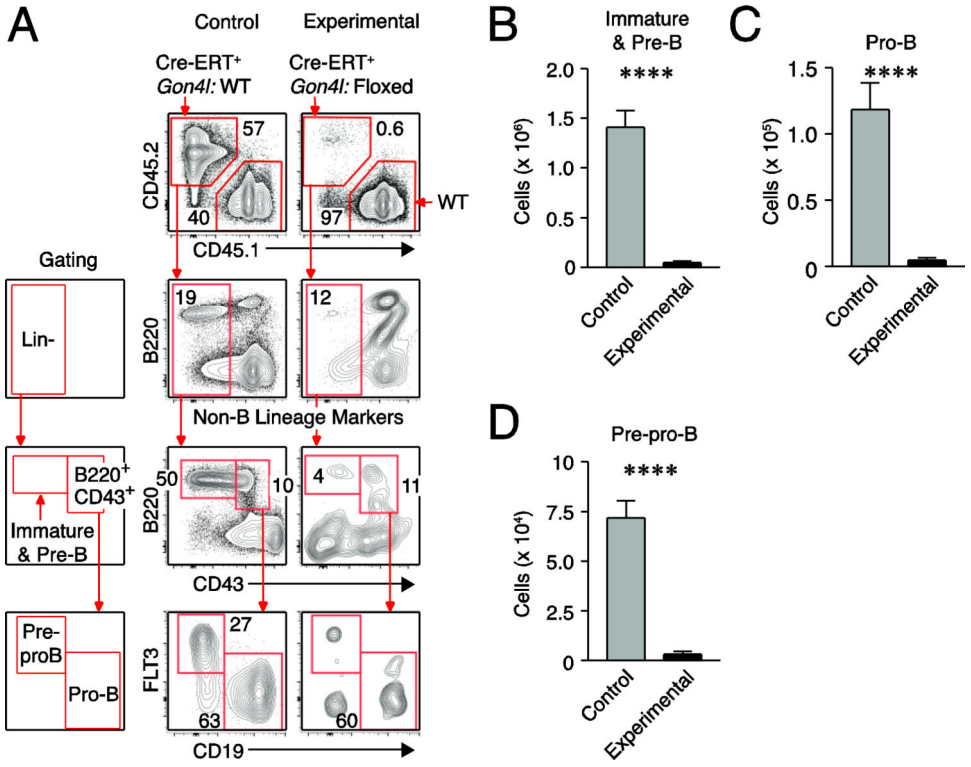

Figure 4. B cell development is abolished by inactivation of *Gon4l*.

MBMCs were created and injected with tamoxifen. B cell progenitors in bone marrow were analyzed 3 weeks later. (**A**) Representative flow cytometric analysis of B cell progenitors in bone marrow from the indicated MBMCs. The gating strategy used is shown at the left. Non-B lineage markers signifies a mixture of antibodies specific for CD11b/Mac-1, Gr-1, Ly6C, Ter119 (to label myeloid and erythroid cells), DX5 (to label NK cells), and CD4 and CD8a (to label T cells). (**B-D**) Yields of the indicated B cell progenitors in bone marrow from control and experimental MBMCs. Bar graphs show the mean and SEM of values obtained from 3 independent experiments in which 3 control and 3 experimental MBMCs were analyzed (n = 9 MBMCs for each group). **** $P < .0001$.

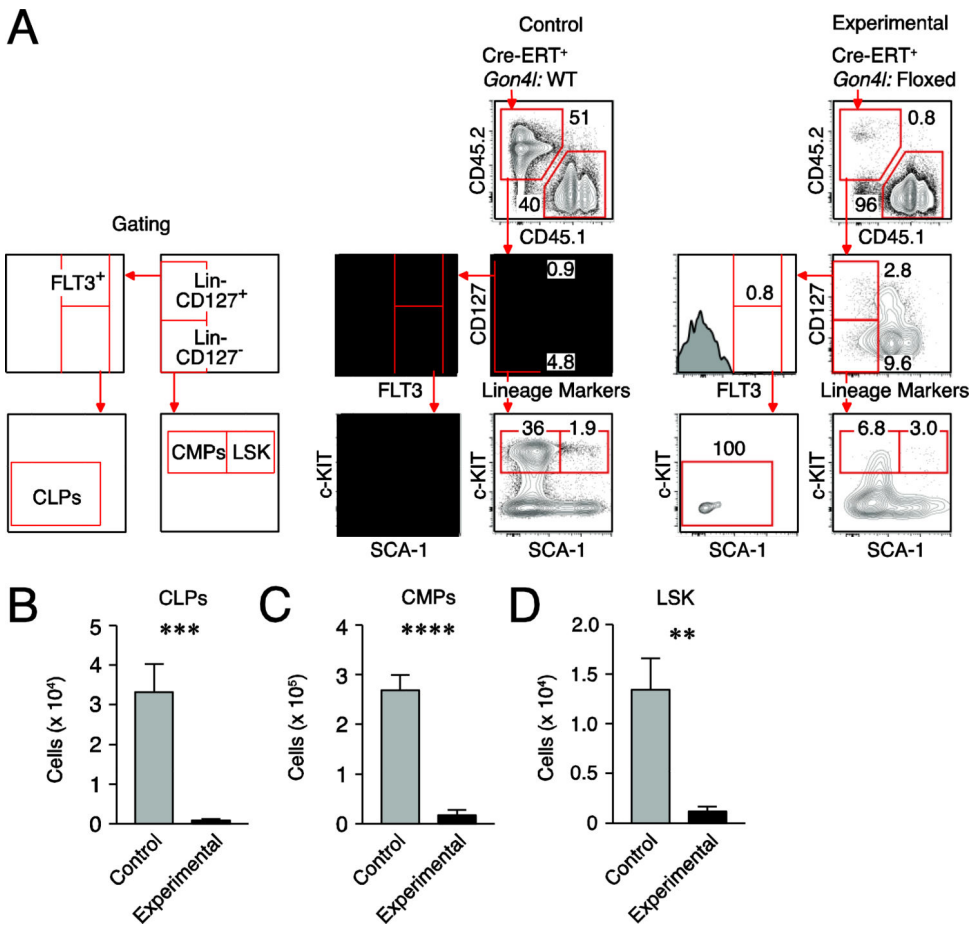

Figure 5. Early hematopoietic progenitors are depleted by inactivation of *Gon4l*.

MBMCs were created and injected with tamoxifen. Bone marrow was analyzed 3 weeks later. (**A**) Representative flow cytometric analysis of hematopoietic progenitors in bone marrow from the indicated MBMCs. The gating strategy used is shown at the left. Lineage markers signifies a mixture of antibodies specific for CD11b/Mac-1, Gr-1, Ly6C, Ter119 (to label myeloid and erythroid cells), B220 and CD19 (to label B-lineage cells), DX5 (to label NK cells), and CD4 and CD8a (to label T cells). (**B-D**) Yields of the indicated hematopoietic progenitors in bone marrow from control and experimental MBMCs. Bar graphs show the mean and SEM of values obtained from 3 independent experiments in which 3 control and 3 experimental MBMCs were analyzed (n = 9 MBMCs for each group). ** $P < .01$; *** $P < .001$; **** $P < .0001$.

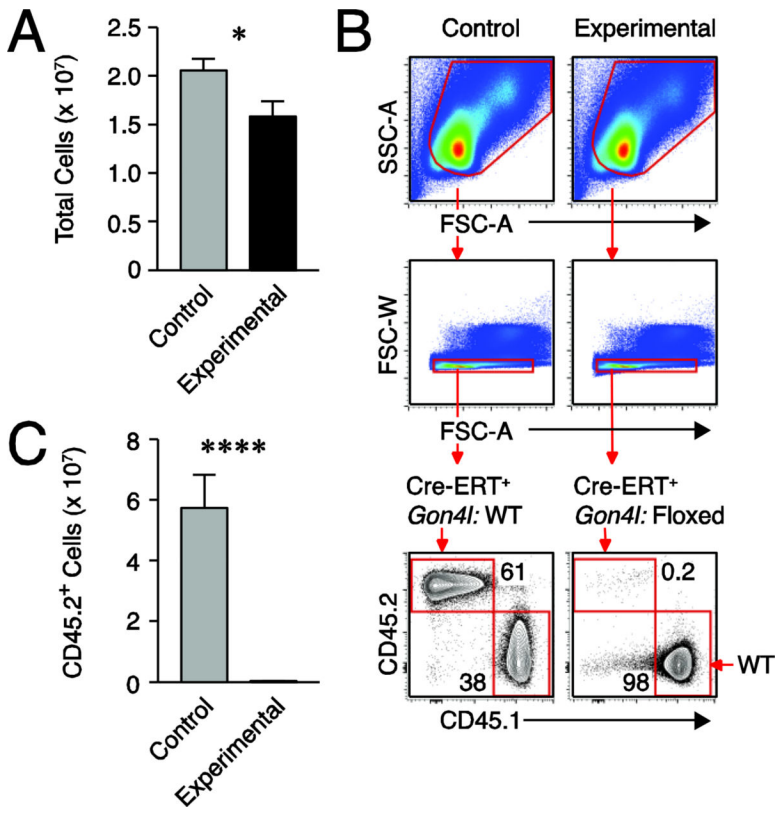

Figure 6. Inactivation of *Gon4l* in bone marrow prevents T cell development.

MBMCs were created and injected with tamoxifen. Thymii were analyzed 3 weeks later. (**A**) Total thymocyte yields from the indicated MBMCs. (**B**) Representative flow cytometric analysis of thymocytes from the indicated MBMCs. (**C**) Yields of $CD45.2^+$ thymocytes from the indicated MBMCs. Bar graphs in panels A and C show the mean and SEM of values obtained from 3 independent experiments in which 3 control and 3 experimental MBMCs were analyzed (n = 9 MBMCs for each group). * $P < .01$; **** $P < .0001$.