The Embryonic Function of Germ Cell Nuclear Factor Is Dependent on the DNA Binding Domain*

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Germ cell nuclear factor (GCNF), an orphan nuclear receptor, is essential for mouse embryogenesis. GCNF specifically binds to a DR0 response element via its DNA binding domain (DBD) in vitro and functions as a repressor of gene transcription. To further study the role of GCNF during embryogenesis, we have employed a Cre/loxP strategy and generated a line of GCNF mutant mice (GCNFlox/lox) in which the 243-base pair DBD-encoding exon has been deleted in the germline. However, the ligand binding domain (LBD) of GCNF is still expressed at the mRNA and protein levels in the ligand binding domain (LBD) of GCNF is still expressed at the mRNA and protein levels in the ligand binding domain (LBD) of GCNF during early mouse embryogenesis, and that the LBD does not mediate any function independent of the DBD at this stage of embryonic development. Our results also suggest that GCNF is indeed a transcriptional factor that represses gene transcription mediated by its DBD.

Germ cell nuclear factor (GCNF,1 NR6A1) is a novel orphan member of the nuclear receptor superfamily as it is more distantly related to other members and forms a sixth and separate subbranch of the family (1, 2). GCNF was initially cloned by our laboratory using low stringency screening with a DNA binding domain (DBD) probe (3) and subsequently cloned by other laboratories and given other names, e.g. RTR (retinoid receptor-related testis-specific receptor (4)) and NCNF (neuronal cell nuclear factor (5)). To date, homologs of GCNF have been cloned from several other species including human, Xenopus, and zebrafish (2, 6–8). The mouse GCNF gene contains 11 exons (9) and is located on chromosome 11, 18, 25–27). This expression pattern indicates that GCNF is required for normal development of mouse GCNF, one located in the DBD including the adjacent TA box and the other in α-helix 3 of the LBD, have been characterized (17). The TA box is critically involved in homodimeric interactions on the DR0 element (16, 17), while both the TA box and the α-helix 3 are required for homodimeric binding of mouse GCNF to the extended DR0 half-site (17). Characterization of the DNA binding motif of GCNF led to the identification of several potential target genes for GCNF including the protamine 1 and 2 genes in the testis (12, 18) and the Oct4 gene in the mouse embryo (19). In the absence of a ligand, GCNF functions to repress the transcription of these target genes in vitro and in vivo (7, 19, 20).

It has been shown that GCNF is not only expressed in mouse and Xenopus embryos after the onset of gastrulation (14, 21–24) but also in the gametogenic cells of adult vertebrates (3, 4, 11, 18, 25–27). This expression pattern indicates that GCNF may play a role in gametogenesis and normal embryonic development during gastrulation. Recently, we have generated a GCNF knockout mouse model (GCNF−/−) using a conventional embryonic stem cell strategy to address the functions of GCNF during mammalian embryogenesis (24). Insertion of PGK-neomycin (PGK-neo) into the GCNF locus causes embryonic lethality at 9.5–10.5 dpc. The most remarkable phenotype of these GCNF mutant embryos is that the posterior tailbud develops outside of the yolk sac (24). These mutant embryos have serious defects in posterior and trunk development and somitogenesis (24). In addition, expression of the Pou domain transcription factor, Oct4, is not repressed in the somatic cells of these GCNF mutant embryos at 8.25–8.75 dpc when it is silenced in normal embryos (19). It appears that GCNF is required for normal anteroposterior development, somitogenesis, and Oct4 expression in mouse embryos. However, recent studies in several other laboratories have shown that the introduction of the PGK-neo cassette into the mouse genome by conventional ES cell targeting can cause unexpected phenotypes in the resulting animal models (28–32). Therefore, generation and phenotypic analysis of a line of GCNF mutant mice, in which the PGK-neo cassette is removed, will definitely clarify whether the pheno-

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1 The abbreviations used are: GCNF, germ cell nuclear factor; DBD, DNA binding domain; LBD, ligand-binding domain; dpc, days postcoitum; ES, embryonic stem; RT, reverse transcription; PGK, phosphoglycerate kinase; CMV, cytomegalovirus.

2 A. J. Cooney, unpublished data.

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types observed in the conventional GCNF knockout mice are due to the ablation of the GCNF gene or to the insertion of the PGK-neo cassette in the GCNF allele, causing misexpression of neighboring genes such as the steroidogenic factor 1 (SF-1) gene, which is located 3' to the GCNF gene.

As a member of the nuclear receptor superfamily, the DBD of GCNF is required to bind to its response elements and regulate gene transcription. Deletion of the 55 amino acid residues containing the DBD zinc finger region in the N terminus of Xenopus GCNF abolishes its DNA binding activity, and overexpression of this truncated GCNF protein in Xenopus embryos causes abnormal head development (14). How much of the function of GCNF is mediated by the DBD in mouse embryonic development remains to be determined. GCNF may have DNA binding-independent activities, similar to other nuclear receptors such as glucocorticoid receptor (33) and TR3 (34).

In this study, we have generated a line of GCNF mutant mice lacking the 243-bp DBD-encoding exon 4 of the GCNF gene (9) using the Cre/loxP system (35). We found that these mutant mice have the same phenotypes as the conventional GCNF knockout mice (19, 24). Unlike the conventional GCNF knockout mice, the LBD of GCNF was still expressed in these mutant embryos at both the mRNA and protein levels. Therefore, the DBD of GCNF is essential for mediating the function of GCNF during mouse embryonic development.

**EXPERIMENTAL PROCEDURES**

**Construction of the Targeting Vector—**Genomic clones of the murine GCNF gene have been isolated from a 129Sv strain mouse genomic library (24). The targeting vector was constructed from a genomic clone containing exon 4 encoding the GCNF DBD. First, oligonucleotides containing an XhoI restriction site and a loxP site were synthesized and then inserted into a neo/tk selection cassette (pNeoTKLOX, generously provided by Dr. Allan Bradley) flanked by two loxP sites to generate the plasmid ZJ-1. A second plasmid, ZJ-2, was constructed when the 3.6-kb BglI/ApaI fragment, downstream of the DBD exon from the genomic clone, was ligated into the BstNI site of the plasmid ZJ-1 in the presence of linkers. Then, the 1.3-kb BstEI/BglII fragment flanking exon 4 from the genomic clone was inserted into the SacII site of the plasmid ZJ-2 to generate the plasmid ZJ-3. Finally, the targeting vector, ZJ-5, was constructed when the 5.1-kb EcoRV/BstEl fragment from the genomic clone 5' of the DBD exon was inserted into the KpnI site of the ZJ-3 plasmid in the presence of linkers. This targeting vector (ZJ-5) contains 6.4 kb of homologous DNA on the 5' long arm and 3.6 kb of homologous sequence on the 3' short arm flanking the neo/tk cassette (Fig. 1A). Generation of GCNFlect mice—GCNFlect mice were generated by homologous recombination in the AB1.2 ES cell line (provided by Dr. Allan Bradley) using the Cre/loxP system. The KpnI-linearized targeting vector ZJ-5 (20 μg) was electroporated into 108 ES cells, and stably transfected clones were isolated after selection with 400 μg/ml G418 for 10 days. Homologously recombined clones were identified by mini-Southern blot analysis using 5'-GCNF and neo probes (24). Subsequently, correctly recombined clones were amplified and then transiently transfected with a Cre expression vector (pOG231, 1 μg) (36). After negative selection in FIAU (1-2'-deoxy-2'-fluoro-1-β-D-arabinofuranosyl-5-iodo-o-uracil), surviving clones were picked and analyzed for recombination of the two loxP sites using mini-Southern blot analysis with the 5' GCNF and neo probes. Correctly recombined clones were expanded and microinjected into mouse C57BL/6 blastocysts to generate chimeric animals. Male chimeric mice (greater than 90% agouti coat color) were generated by mating with heterozygous males, and the embryos were collected at weaning. Male chimeric mice were intercrossed to generate homozygous GCNFlox/lox embryos.

**Genotyping and Identification of GCNFlox/lox Embryos—**Genomic DNA was extracted from either mouse tails or embryos. Genotypes of weaned mice or embryos were determined by Southern blot analysis using a 5'-GCNF probe or by PCR using two separate sets of primers. The primer set (Primer 1, 5'-CTGACACGACCGCTTCTC-3'; Primer 2, 5'-TTCCTGTGTCAGCTCATCTC-3'; Primer 3, 5'-CAAGATGGGTCCAGCA-3', a GCNF DNA product) and its PCR reactions for determining the wild type GCNF allele were described previously (24). The primer set (Primer 3, 5'-CAGTCTGAGGGACCTATAAC-3', a 400-bp DNA product) was used to determine the GCNFlox allele.

PCR reactions for determining the GCNFlox allele were carried out for 35 cycles (94°C, 1 min; 54°C, 2 min; 72°C, 2 min) in a buffer containing 2.5 mM MgCl₂.

**RNA Analysis—**Total RNA from embryos was isolated using Trizol reagent (Invitrogen). For RT-PCR analysis, first strand cDNA synthesis was performed at 42°C for 60 min using 1 μg of total RNA as template and hexanucleotides as primers using the SuperScript™ First-Strand Synthesis System for RT-PCR kit (Invitrogen). To determine the presence of the DBD and LBD regions of the GCNF mRNA and β-actin mRNA in the embryos, PCR reactions with 1/10 of the cDNA were carried out for 40 cycles (94°C, 1 min; 52°C, 1 min; 72°C, 2 min) in a buffer containing 1.5 mM MgCl₂ with the following primer sets: GCNF DBD primers (Primer 5, 5'-CAGCAAGGAGTCTGGTTC-3'; Primer 6, 5'-TGGACACGACCGCTTCTC-3', a 150-bp DNA product); GCNF LBD primers (Primer 7, 5'-CAGTCTGAGGGACCTATAAC-3', a 260-bp DNA product); actin primers (ACTIN-F, 5'-TGGACACGACCGCTTCTC-3'; ACTIN-R, 5'-AGCCAGGACGCTTCTC-3', a 593-bp DNA product). RNAse protection analysis was performed using an Ambion RPA-III system according to the manufacturer’s protocol (Ambion, Inc., Austin, TX). The [α-32P]UTP-labeled cDNA probe for the LBD of the GCNF mRNA was synthesized from a plasmid containing 260 bp GCNF cDNA sequence (nucleotides 1256–1516).

**Western Blot Analysis—**Proteins from mouse embryos at 8 and 9 dpc were extracted in an extraction buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 0.01% Triton P-100, 0.01% Nonidet P-40, 1 mM proteinase inhibitor). CMVGCNF protein and Cos-1 proteins were prepared from transiently transfected Cos-1 cells as described previously (18). Western blot analysis was performed using a specific polyclonal antibody (LBD pAb) raised against a C-terminal polypeptide (amino acid residues from 477 to 495) of the GCNF protein (37).

**RESULTS**

**Targeted Deletion of the DBD-encoding Exon of the GCNF Gene—**A Cre/loxP targeting strategy was used to delete the 243 bp DBD-encoding exon of the GCNF gene. As shown in Fig. 1A, three loxP sites were introduced into a GCNF allele of ES cells by homologous recombination with the targeting vector. Mini-Southern blot analysis was performed to identify the ES clones that had gone under correct homologous recombination using 5'-GCNF and neo probes (Fig. 1B). Nineteen ES cell clones carrying three loxP sites were obtained from the screening of 160 ES cell clones. Subsequently, two independent ES cell clones carrying three loxP sites were amplified and then transiently transfected with a CMV-Cre expression plasmid to delete the GCNF DBD-encoding exon and the neo/tk cassette. ES cell clones carrying the recombined GCNFlox allele were identified by the Southern blot analysis using the 5'-GCNF and neo probes (Fig. 1C). Sixty-seven (of 80) ES cell clones carrying the GCNFlox allele were obtained, two of which were used to generate chimeric mice. Chimeric mice from each clone transmitted the recombined GCNFlox allele to their offspring. Heterozygous (GCNFlox/+) and homozygous (GCNFlox/lox) embryos were identified by Southern blot analysis and PCR (Fig. 1D).

**Embryonic Lethality of GCNFlox/lox Mutants—**At weaning, no homozygous GCNFlox/lox mice were obtained from the intercross of heterozygous mice (Table 1), indicating that deletion of the DBD-encoding exon caused embryonic lethality, similar to the GCNF null mutant mice reported previously (24). To determine when homozygous GCNFlox/lox mutants die, heterozygous females were sacrificed at various days postcoitum after mating with heterozygous males, and the embryos were collected and then genotyped by either PCR or Southern blot analysis (Fig. 1D). At 8.5–9.5 dpc, embryos obtained from the GCNFlox/lox X GCNFlox/lox mating showed the expected 1:2:1

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**Fig. 1.** Generation of GCNF<sup>flox</sup> mice using ES cell homologous recombination and Cre/loxP technologies. 

**A**, targeting strategy for homologous recombination. GCNF<sup>flox</sup> allele represents the structure of the GCNF locus after homologous recombination with the targeting vector. This recombined flox allele contains three loxP sites (filled triangles), one upstream of the DBD-encoding exon (filled box) and the others downstream of the DBD-encoding exon flanking the neo/tk selection cassette. The Cre recombed allele (GCNF<sup>flox<sub>3lox</sub></sup>) marks the structure after subsequent recombination of the two-loxP sites, one upstream of the DBD-encoding exon and the other downstream of the neo/tk cassette. 

**B**, mini-Southern blot analysis to identify ES clones containing the GCNF<sup>flox</sup> allele. Digestion with Acc<sub>65I</sub> and XhoI yielded a 20-kb band for the wild type GCNF allele and a 10-kb band for the targeted GCNF<sup>flox</sup> allele, indicating the correct homologous recombination in the 5'-arm. Digestion with Acc<sub>65I</sub> and SalI yielded a 12.6-kb band for the targeted GCNF<sup>flox</sup> allele, indicating the correct homologous recombination in the 3'-arm. 

**C**, Southern blot analysis to determine ES cell clones containing the GCNF<sup>flox</sup> allele. Digestion with Acc<sub>65I</sub> yielded a 20-kb band for the wild type GCNF allele and an 18.6-kb band for the GCNF<sup>flox</sup> allele. No band detected by the neo probe confirms the complete recombination between the loxP sites in the GCNF<sup>flox</sup> allele by Cre recombinase. 

**D**, genotyping of the offspring resulting from the heterozygous intercross by Southern blot analysis and PCR. Digestion with Acc<sub>65I</sub> and XhoI yielded a 20-kb band for the wild type GCNF allele and a 10-kb band for GCNF<sup>flox</sup> allele. Using Primer 1 and Primer 2, a 264-bp wild type DBD exon DNA fragment was detected in the GCNF<sup>+/+</sup> and GCNF<sup>flox</sup> animals, but not in the GCNF<sup>flox/flox</sup> embryos. Primer 3 and Primer 4 were used to detect a 400-bp DNA fragment for the GCNF<sup>flox</sup> allele in the embryos.
Medelian ratio of GCNF<sup>+/+</sup>, GCNF<sup>+/lox</sup>, and GCNF<sup>lox/lox</sup> genotypes (Table I). By 10.5–11 dpc, the litter size was reduced compared with that at 8.5–9.5 dpc, and no viable GCNF<sup>lox/lox</sup> embryos, except resorbing embryos, were obtained. These results confirm that GCNF<sup>lox/lox</sup> embryos die at 9.5–10.5 dpc, similar to the GCNF null mutant embryos reported previously (24).

Presence of an Intact GCNF LBD in the GCNF<sup>lox/lox</sup> Mutants at Both the mRNA and Protein Levels—Since the size of the DBD-encoding exon of the GCNF gene is 243 bp and the loxP sites were inserted into the intron surrounding the DBD exon, we postulated that deletion of the 243-bp DBD-encoding exon should not cause a reading frameshift in the GCNF downstream LBD in GCNF<sup>lox/lox</sup> mutants (Fig. 2A). To determine whether the DBD of GCNF is deleted and the LBD of GCNF is still expressed in the GCNF<sup>lox/lox</sup> embryos, we performed RT-PCR analysis and RNase protection analysis. As shown in Fig. 2B, the DBD of the GCNF transcript was completely deleted in the GCNF<sup>lox/lox</sup> mutant embryos. However, the LBD of the GCNF transcript was still expressed in the mutant embryos (Fig. 2B). To determine whether the LBD protein is present in the GCNF<sup>lox/lox</sup> embryos, we performed Western blot analysis using antibodies raised against the LBD of GCNF. As shown in Fig. 2C, a 58-kDa GCNF protein band was detected in GCNF<sup>+/+</sup> and GCNF<sup>lox/lox</sup> embryos but not in GCNF<sup>−/−</sup> embryos. A truncated GCNF protein band (50 kDa) was also detected in the heterozygous and homozygous GCNF<sup>lox/lox</sup> embryos, indicating that the LBD is present in GCNF<sup>lox/lox</sup> embryos.

**TABLE I**

| Age          | Genotypes of weaned mice and dissected embryos | Numbers of mice/embryos (% of totals) |
|--------------|-----------------------------------------------|--------------------------------------|
|              | GCNF<sup>+/+</sup> | GCNF<sup>+/lox</sup> | GCNF<sup>lox/lox</sup>           |
| Day 21 (weaned pups) | 34 (33.7%) | 67 (66.3%) | 0 (0%)                          |
| 10.5–11 dpc  | 7 (29.2%)  | 15 (62.5%)  | 2 (8.0%)                         |
| 8.5–9.5 dpc  | 28 (26.7%) | 51 (48.6%)  | 26 (24.8%)                       |

*Resorbing embryos.

**Discussion**

Nuclear receptors such as ER, PR, AR, TR, and RAR are well known transcription factors that bind their cognate DNA-responsive elements through their DNA binding domains and then regulate the expression of their target genes via their transactivation domains, which ultimately elicits their distinct physiological functions in response to their cognate hormones (40, 41). Recently, DNA binding-independent functions of nuclear receptors such as GR (33) and TR3 (34) have been described. As a member of the nuclear receptor superfamily, GCNF has been shown to bind to DR0 elements and repress gene transcription. Similar to other orphan nuclear receptors, such as SF-1 (41) and TR3 (34), the DBD of GCNF is required for its DNA binding activity as deletion of 55 amino acid residues at the N terminus of Xenopus GCNF (14) or of 163 amino acid residues at the N terminus of mouse GCNF<sup>+</sup> abolishes its DNA binding activity. Replacement of the GCNF DBD-encoding exon by a PGK-neo cassette in the mouse germline using a conventional knockout strategy causes embryonic lethality at 9.5–10.5 dpc (24). These mouse embryos have defects in the correct formation of somites and anteroposterior axis postgastrulation. In addition, unsilenced expression of the Oct4 gene in somatic cells is observed in these mutant embryos at 8.25 dpc. Even though the DBD region of the GCNF mRNA is completely deleted in these mutant mice (24), it is still unclear whether the phenotypes observed previously are due to the loss of GCNF or to the insertion of the PGK-neo cassette in the GCNF<sup>+</sup> allele.

Several studies in other laboratories have suggested that the introduction of PGK-neo cassettes can interfere with RNA processing of targeted transcripts (even when inserted in an intron) (28) and cause position effects on neighboring genes, particularly when inserted within gene clusters and locus control regions (29–31) leading to unexpected phenotypes in the resulting animal models. Additionally, bi-directional transcriptional activities of PGK-neo, normal sense PGK promoter activity, and aberrant antisense promoter activity in neomycin and PGK promoter regions, which drive the production of aberrant transcripts, have been reported in cultured cells (42) and in vivo in mice (32). Considering that the SF-1 gene, encoding another nuclear receptor, is immediately adjacent to the GCNF gene in the mouse genome and is known to be involved in adrenal and gonadal development (43), it is possible to speculate that GCNF may also have similar effects in developing embryos.
that insertion of the PGK-neo in the GCNF locus affects SF-1 expression, complicating the interpretation of phenotypes observed in our conventional knockout embryos. Therefore, generation of a mouse line without the PGK-neo cassette in the GCNF locus is necessary to determine the role of GCNF during embryonic development by comparison of the phenotypes of the conventional knockout embryos to those mice without the PGK-neo insertion. In this study, we have successfully generated GCNF<sup>lox/lox</sup> mice that do not contain the DBD-encoding exon of the GCNF gene nor the selection marker gene, PGK-neo, using Cre/loxP technology. Similarly to the conventional GCNF knockout mice, GCNF<sup>lox/lox</sup> mice died in utero at 9.5–10.5 dpc with defects in posterior embryonic development and the formation of somites and the anteroposterior axis. Expression of the Oct4 gene in GCNF<sup>lox/lox</sup> embryos was not silenced in somatic cells at 8.25 dpc. Therefore, GCNF<sup>lox/lox</sup> mice phenocopy the conventional GCNF knockout mice (19, 24), indicating that the phenotypes observed in the conventional knockout embryos result from the ablation of GCNF, not from the insertion of the PGK-neo cassette into the GCNF locus, confirming that GCNF is essential for normal embryonic development.

As no RNA splicing acceptor consensus sequences, which are located upstream of the DBD-encoding exon of the GCNF gene, were deleted in our knockout strategy and no consensus RNA splicing acceptor elements or transcription termination signals were included in the loxP site and its surrounding DNA sequences, it was possible that transcription of the GCNF gene in GCNF<sup>lox/lox</sup> mice could be initiated and that normal RNA splicing can occur between exon 3 and exon 5 forming a truncated GCNF message, which encodes a protein that does not contain DBD of GCNF Is Essential for Mouse Embryonic Development

![Diagram of protein-coding regions of the wild type GCNF mRNA (GCNF<sup>+/+</sup>) and GCNF mutant mRNA (GCNF<sup>lox/lox</sup>). Numbers listed in the figure represent the amino acid residues of the wild type GCNF protein. Primer 5 and primer 6 recognize the DBD cDNA sequences derived from wild type GCNF mRNA, while primer 7 and primer 8 recognize the LBD cDNA sequences derived from the wild type GCNF mRNA or mutant GCNF mRNA. Localization of an antisense LBD cRNA probe and the epitope of an anti-GCNF polyclonal antibody (LBD pAb) are also included.](http://www.jbc.org/)

![Loss of the DBD in the GCNF<sup>lox/lox</sup> embryos at 8.5 dpc by RT-PCR analysis and RNase protection assay. Total RNA from GCNF<sup>+/+</sup>, GCNF<sup>+/lox</sup>, and GCNF<sup>lox/lox</sup> embryos at 8.5 dpc was reverse transcribed and then amplified by PCR using different sets of primers. Primers 5 and 6 were used to determine the presence of the DBD region in the GCNF mRNA, while primers 7 and 8 were used for detecting the LBD region of the wild type and mutant GCNF mRNA. Actin primers were used as positive controls for the RT-PCR assays. For RNase protection assay, 10 μg of total RNA from GCNF<sup>+/+</sup>, GCNF<sup>+/lox</sup>, or GCNF<sup>lox/lox</sup> embryos at 8.5 dpc were hybridized with radiolabeled antisense GCNF LBD and GAPDH cRNA probes. A 260-bp GCNF LBD and a 110-bp glyceraldehyde-3-phosphate dehydrogenase band were observed in mouse embryo RNA samples.](http://www.jbc.org/)

![Western blot analysis showing the presence of a truncated GCNF protein (50 kDa) lacking the DBD in the GCNF<sup>lox/lox</sup> embryo. Protein extracts from GCNF<sup>+/+</sup>, GCNF<sup>+/lox</sup>, and GCNF<sup>lox/lox</sup> embryos at 8.0 dpc or GCNF<sup>−/−</sup> embryos at 9.0 dpc, and from Cos-1 cells transfected with a CMVGCNF expression plasmid (CMVGCNF) or with an empty CMV vector (Cos-1) were immunoblotted with a specific antibody against a 19-amino acid peptide at the C terminus of the GCNF protein. A 58-kDa protein band represents the wild type GCNF protein, while a 50-kDa protein band represents the truncated GCNF protein, which lacks the 81-amino acid residues of the DBD.](http://www.jbc.org/)
the DBD but does have an intact LBD. Indeed, we found that the LBD region, but not the DBD region, of the GCNF mRNA was expressed in the GCNFlox/lox mice detected either by RT-PCR or by RNase protection assay (Fig. 2). Since the size of the DBD exon of the GCNF gene is 243 bp (9), deletion of this exon should not cause a reading frameshift in the downstream LBD.

**Fig. 3.** Gross morphology (A) and histology (B) of GCNFlox/lox embryos. A, GCNFlox/lox embryos at 9.25–9.5 dpc do not turn and remain in a lordotic position. The tailbuds (white arrow) of the mutant embryos protrude outside of the yolk sac, similar to GCNF+/− embryos. B, hematoxylin and eosin staining to analyze histology of GCNFlox/lox embryos. Cross sections of GCNF−/−, GCNFlox/lox, and GCNF+/− embryos at 8.5 dpc showing open neural tubes at the anterior and posterior ends. A red arrow indicates the large invagination formed by posterior neural epithelium in the GCNFlox/lox and GCNF−/− embryos.
**Fig. 4. Expression of the Oct4 gene in the GCNF<sup>lox/lox</sup> embryos at 8.25 dpc.** In situ hybridization with a digoxigenin-labeled riboprobe for Oct4 was performed on whole mount wild type (the top two panels), GCNF<sup>lox/lox</sup> (the middle two panels), or GCNF<sup>−/−</sup> (the bottom two panels) embryos at E7.5 (left panels) and E8.25 (right panels). Primordial germ cells (PGC) are shown by arrowheads. AD, the anterior region of the embryo. PD, the posterior region of the embryo.
Using a specific GCNF polyclonal antibody raised against a 19-amino acid peptide at the C terminus, we found that a 58-kDa GCNF protein band was detected in the GCNF\textsuperscript{lox/lox} and GCNF\textsuperscript{lox/lox} mice but not in the conventional GCNF knockout mice (Fig. 2) and GCNF\textsuperscript{lox/lox} mice (Fig. 2). Instead, a 50-kDa-truncated protein band was detected in the GCNF\textsuperscript{lox/lox} (GCNF\textsuperscript{ΔTBD} and GCNF\textsuperscript{ΔDBD}) embryos (Fig. 2). These studies suggest that the truncated GCNF transcript in the GCNF\textsuperscript{lox/lox} mice is translated into a 50-kDa protein that contains an intact LBD but not a DBD. These results clearly indicate that we have successfully deleted the DBD of GCNF in vivo. Although the truncated GCNF protein lacking only the DBD was observed in the GCNF\textsuperscript{lox/lox} mice (Fig. 2), phenotypes of these GCNF\textsuperscript{lox/lox} mice (Figs. 3 and 4) were the same as those of conventional GCNF knockout mice (19, 24), which did not express GCNF protein (Fig. 2). These results suggest that the DBD of GCNF is essential for mediating the function of GCNF during early embryonic development. The results also suggest that at this stage of embryonic development the LBD of GCNF does not mediate any functions independent of the DBD. It should be emphasized that GCNF can bind to the DR0 element in the promoter of the Oct4 gene and repress the transcription of the Oct4 gene in vivo (19). Loss of GCNF in the conventional GCNF knockout mice causes loss of repression of Oct4 expression in somatic cells (19). Similar results were obtained in the GCNF\textsuperscript{ΔTBD} mice. Considering that the LBD of GCNF does not bind to DR0 element,\textsuperscript{2} we can conclude that GCNF is indeed a transcription factor that represses gene transcription in mouse embryos. In addition, the expressed GCNF LBD does not have a dominant negative effect as the GCNF\textsuperscript{ΔTBD} mice are normal. The lack of a dominant negative effect is probably due to an inability of the wild type GCNF and the GCNF LBD to heterodimerize.

In summary, we have generated a line of mice that expresses a truncated GCNF protein lacking only the DBD. These mice phenocopy the conventional GCNF knockout mice. This study clarifies that the phenotypes observed in the conventional GCNF knockout mice are due to the loss of GCNF, not to the insertion of the PGK-neo cassette into the GCNF locus, and that GCNF is essential for normal mouse embryonic development. More importantly, our results suggest that the DBD of GCNF is essential for the function of GCNF during embryonic development, that GCNF does not have DNA-binding independent activity as reported for other nuclear receptors such as GR and TR3 (33, 34) during mouse embryogenesis, and that GCNF is indeed a transcription factor that represses gene transcription in vivo.

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