All Hormone-Producing Cell Types of the Pituitary Intermediate and Anterior Lobes Derive From *Prop1*-Expressing Progenitors

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Mutations in *PROP1*, the most common known cause of combined pituitary hormone deficiency in humans, can result in the progressive loss of all hormones of the pituitary anterior lobe. In mice, *Prop1* mutations result in the failure to initiate transcription of *Pou1f1* (also known as *Pit1*) and lack somatotropins, lactotropins, and thyrotropins. The basis for this species difference is unknown. We hypothesized that *Prop1* is expressed in a progenitor cell that can develop into all anterior lobe cell types, and not just the somatotropes, thyrotropes, and lactotropes, which are collectively known as the PIT1 lineage. To test this idea, we produced a transgenic *Prop1-cre* mouse line and conducted lineage-tracing experiments of *Prop1*-expressing cells. The results reveal that all hormone-secreting cell types of both the anterior and intermediate lobes are descended from *Prop1*-expressing progenitors. The *Prop1-cre* mice also provide a valuable genetic reagent with a unique spatial and temporal expression for generating tissue-specific gene rearrangements early in pituitary gland development. We also determined that the minimal essential sequences for reliable *Prop1* expression lie within 10 kilobases of the mouse gene and demonstrated that human *PROP1* can substitute functionally for mouse *Prop1*. These studies enhance our understanding of the pathophysiology of disease in patients with *PROP1* mutations. **(*Endocrinology* 157: 1385–1396, 2016)**

The pituitary gland plays a critical role in vertebrate physiology through the secretion of hormones that regulate reproduction, stress management, metabolism, lactation, and water balance. Pituitary dysfunction can result from congenital malformation, pituitary adenoma, or head trauma. Although some cases are managed with pharmacotherapy and/or surgery, not all individuals respond, resulting in impaired quality of life or morbidity. Congenital pituitary hormone deficiency is defined as GH deficiency and loss of at least one other pituitary hormone. It is generally caused by mutations in transcription factors necessary for pituitary organogenesis, and the severity of the corresponding disease is related to the molecular function of the transcription factor during development of the pituitary and other craniofacial tissues (1, 2). For example, patients with mutations in transcription factors that act during the earliest stages of head development, such as *HESX1* and *SOX2*, can have a syndromic presentation, including the lack of multiple pituitary hormones and septo-optic dysplasia (1). People with nonsyndromic pituitary deficiencies tend to have mutations in pituitary lineage-specific transcription factors such as *PROP1* and *PIT1* (*POU1F1*).

Mutations in *PROP1* and *PIT1* typically cause deficiency of GH, prolactin (PRL), and TSH, which are produced by 3 separate cell types known as the PIT1 lineage (3–6). PIT1 stimulates differentiation of progenitor cells to these 3 cell fates by organizing target gene enhancer regions in the nuclear matrix, using a mechanism involving MATRIN-3, β-CATENIN, and the DNA-binding pro-

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Abbreviations: BAC, bacterial artificial chromosome; CNS, conserved noncoding sequence; DAPI, 4′, 6-diamidino-2-phenylindole; e, embryonic day of development; eGFP, enhanced green fluorescent protein; EMT, epithelial to mesenchymal transition; kb, kilobases; POMC, proopiomelanocortin; PRL, prolactin; tdTOMATO, tandem dimeric Tomato; TSA, tyramide signal amplification; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

tein, SATB1 (7). PIT1 also acts as a direct transcriptional activator of the GH, PRL, and TSH genes (8–12). PROP1, in turn, is a direct transcriptional activator of *PIT1* (13). PROP1 partners with β-CATENIN and binds an early-acting enhancer of *PIT1* to initiate its transcription (13). $Prop1^{df/df\ (Ser83Pro)}$ and $Prop1^{-/-}$ mice have a congenital deficiency in the PIT1 lineage cell types, but gonadotropes and corticotropes develop normally (14–17). In contrast, humans with *PROP1* mutations often exhibit progressive loss of the PIT1 lineage, gonadotropin deficiency, and ultimately loss of corticotrope function (18). The basis for the phenotypic differences between $Prop1^{-/-}$ mice and humans are unknown, but it could be caused by temporal or spatial differences in PROP1 expression between the 2 species or differences in downstream targets due to evolutionary changes in the proteins.

PROP1 expression has been detected in SOX2-expressing pituitary progenitors (19, 20), which supports the hypothesis that the progressive loss of the hormone-producing lineages in humans could be due to depletion of progenitors marked by PROP1. An alternative theory is that Rathke's pouch harbors 2 separate populations of progenitor cells, one of which forms corticotropes and gonadotropes, and the other forms the PIT1 lineage of somatotropes, lactotropes, and thyrotropes (21). In this scenario, *Prop1* expression is only required in the PIT1 lineage, and is not necessary for differentiation of corticotropes and gonadotropes. More complex models are also possible because there is evidence that antagonism between lineage-specific transcription factors plays a role in some cell fate decisions. For example, TBX19 (also known as TPIT) is an early determinate of corticotrope formation, and it inhibits the activity of STEROIDOGENIC FACTOR 1 (SF1, gene symbol *Nr5a1*), a key transcription factor for gonadotrope differentiation (21). SF1 also inhibits the activity of TBX19, generating a mechanism where the expression of either TBX19 or SF1 in a common progenitor leads to lineage specification and inhibition of the opposite cell type (21). GATA2 synergizes with PIT1 to promote thyrotrope fate and antagonize the gonadotrope fate (22–24). It is important to determine the lineages that derive from cells expressing PROP1 in order to distinguish between these possibilities.

The discovery of pituitary stem cells has focused much attention on the generation and function of this pituitary cell population (25). SOX2 is expressed in the pituitary progenitors of Rathke's pouch, and it is maintained in adult pituitaries in the stem cells that reside near the residual cleft of Rathke's pouch, a region known as the marginal zone (26). The pituitary progenitors arise during mouse embryogenesis at embryonic day of development 9.5 (e9.5) as Rathke's pouch is induced from the oral ectoderm (27). These progenitors continue to proliferate, and they maintain an epithelial structure. As pituitary progenitors exit the cell cycle between e11.5 and e13.5, they leave the epithelia and adopt a more rounded shape characteristic of hormone secreting cells of the anterior lobe (28–30). This transition is similar in appearance to an epithelial to mesenchymal transition (EMT), where cells down-regulate cell adhesion molecules characteristic of epithelial cells and become migratory in nature. However, the pituitary hormone-expressing cells are not truly mesenchymal; therefore, this transition in the pituitary is EMT like. *Prop1* is expressed in Rathke's pouch between e10.5 and e14.5, a time when the pituitary progenitors proliferate, exit the cell cycle, and migrate into the anterior lobe (5). *Prop1* plays a critical role in this transition, as $Prop1^{-/-}$ and $Prop1^{df/df}$ mice have dysmorphic pituitaries with an increase in epithelial-like cells and few anterior lobe cells (31). In addition, $Prop1^{df/df}$ embryos have persistent expression of N-CADHERIN in the progenitor cells at the ventral aspect of Rathke's pouch, and they fail to express SNAI2 (also known as SLUG), both of which are indicative of failed EMT (32). These results support the idea that *Prop1* is expressed in progenitors of all pituitary cell types, not just in the PIT1 lineage.

We used transgenic mice to determine the minimal essential sequences necessary for mouse *Prop1* expression and to assess whether human *PROP1* is sufficiently conserved to functionally compensate for mouse *Prop1*. We also performed a lineage tracing experiment of the *Prop1*-expressing cell population to determine whether *Prop1* is expressed in all pituitary progenitors or whether its expression is limited to the PIT1 lineage. We generated and characterized a *Prop1-cre* transgenic mouse line for the lineage tracing experiment, and this line provides a valuable tool for tissue-specific gene rearrangements in the pituitary progenitors. These studies provide a greater understanding of PROP1 function in pituitary organogenesis.

Materials and Methods

Transgene construct generation

A 25-kb rescue plasmid was subcloned from mouse bacterial artificial chromosome (BAC) RP23-250I22 using gap repair (33). Regions of homology to mouse chromosome 11 were generated by PCR, amplifying nucleotide 50 943 223 to 50 943 595 (using mouse genome reference assembly GRCm38.p2) at the 3′ end of *Prop1* genomic locus and nucleotide 50 968 513 to 50 968 857 at the 5′ end. The amplified regions were cloned into pBluescript II (Aligent Technologies). The linearized plasmid was transformed into RP23-250I22 containing bacteria, and ampicillin-resistant colonies selected. The rescued plasmid was

completely sequenced to confirm identity to the reference region of mouse chromosome 11. The 10-kb rescue plasmid was generated previously (34).

The *Prop1-cre* construct was generated using materials and methodology similar to that reported in the construction of the *Cga-cre* (35). We used a *cre* cassette (*icre*) with codons optimized for mammalian cells (36). The *cre-FRT-gb2-kan-FRT* containing plasmid was further modified to introduce the bovine GH polyadenylation signal (*bGH-pA*) downstream of the *cre* coding sequence. BAC clone RP23-479M11, obtained from the BACPAC Resource Center, was modified by inserting a *cre* expression cassette into *Prop1* exon 2, in frame. An oligonucleotide with homology to *Prop1* exon 2 (nucleotides 50 952 311 to 50 952 236 and 50 952 155 to 50 952 233) was used in combination with a 100-bp oligonucleotide containing 24 bp of homology to *cre* to amplify the *cre-bGH-pA-FRT-gb2-kan-FRT* cassette (Table 1). The PCR product was inserted into the BAC using homologous recombination, and kanamycin-resistant colonies were selected. The *FRT-gb2-kan-FRT* drug selection cassette was removed using enhanced flippase, leaving behind a single *FRT* site (37). Successfully targeted BACs were identified by pulsed field gel restriction enzyme mapping (38) and sequenced over the 10-kb critical region of *Prop1* (Table 1).

Generation and genotyping of transgenic mice

All experiments using mice were approved by the Institutional Committee on the Use and Care of Animals for the University of Michigan (protocol number PR00004640 to S.A.C.) and the University of South Carolina (protocol number 2106-100665-012213 to S.W.D.). Mice were housed in specific pathogen-free conditions with automatic watering and ventilated cages. Mice were fed ad libitum irradiated 5080 chow with 21.5% of the calories from fat. Weaning was delayed to permit mutants to nurse longer, and after weaning, mutants were housed with normal animals to provide warmth.

Prop1$^{df/df}$ mice were provided by A. Bartke in 1988 and maintained as a stock. *Prop1*$^{-/-}$ mice (officially *Prop1*^{tm1Sac}) were generated from mouse embryonic stem cells engineered to contain a deletion in *Prop1* beginning with the initiator ATG in the first exon and extending through the first intron and a portion of exon 2 (17).

Linearized and purified 25- and 10-kb *Prop1* rescue plasmids were injected separately into the pronuclei of fertilized eggs generated from a cross of *DF/B-Prop1*$^{df/+}$ males of mixed genetic background (15) and (*SJL/J X C57BL/6J*) F_1 females. Injected zygotes were transferred into pseudopregnant foster mothers. *Prop1 plasmid transgenic; Prop1*$^{df/+}$ mice were crossed to *N6-B6-Prop1*$^{+/-}$ mice (17) to generate *Prop1 plasmid transgenic; Prop1*$^{df/-}$ offspring. These mice and all littermates were weighed, photographed, and genotyped at weaning. Genotyping for the *Prop1* rescue plasmids was performed by PCR using the primers listed in Table 1, and the *Prop1*^{df} allele and *Prop1*$^{-}$ allele were genotyped using previously described primers (17, 39). BAC RP11-452O4 containing 175 kb spanning the human *PROP1* genomic locus was obtained from BACPAC Resources, expanded, and purified. Purified BAC (*HsPROP1*) was injected into the pronuclei of fertilized eggs from a cross of *DF/B-Prop1*$^{df/+}$ males and (*SJL/J X C57BL/6J*) F_1 females, as detailed above. *HsPROP1; Prop1*$^{df/+}$ mice were crossed with *Prop1*$^{df/+}$ mice and all offspring were weighed, photographed, and genotyped at weaning. *HsPROP1* transgenic mice were genotyped by PCR using primers listed in Table 1 and primers for the chloramphenicol resistance gene in the BAC backbone as previously described (35).

The purified *Prop1-cre* modified BAC was injected into the pronuclei of fertilized eggs from a cross between *C57BL/6J* and *B6D2F1* mice, and the fertilized eggs were transferred into pseudopregnant foster mothers. Transgenic *cre* positive mice were identified by PCR genotyping using primers listed

Table 1. Primers Used in Generating and Genotyping Transgenic Mice

	***Prop1* Homology Domain**	***cre* Cassette Homology Domain**
Prop1-cre transgene construction		
Exon 2 A	GTGAGGATGGGCTGGTGGTCCCATTAATAAGGGGGTCTC CCTCCGTTTTTCTCCCTCCATAGACAGGAGCTCTGAG	ATGGTGCCCAAGAAGAAGAGGAAA
Exon 2 B	GGCGGCGCCGGGAGTGGGGACGGCCTCTCTGTGGGCA AAGCTTAGGTCTCCCCAGCTCTGTACCAGAGAGCC TCCTATAA	CTATACGAAGTTATAAGCTT
Prop1-cre transgene genotyping		
A	CTGCACACAGACAGGAGCAT	
B	GGTCTCCCTCCGTTTTTCTC	
25-kb *Prop1* BAC transgene genotyping		
A	AATTAACCCTCACTAAAGGG	
B	AAGGGCAGGTAAGATGCTGA	
10-kb *Prop1* BAC transgene genotyping		
A	AAACCCCCAGAGAAGAGGAA	
B	GACCATGATTACGCCAAGCTA	
HsPROP1 BAC transgene genotyping		
A	GGAGGGAACAGGTGTGGAG	
B	GGTGTCTCTCAGGCAACAGG	

Each primer is listed 5′–3′, and A and B indicate forward and reverse primers, respectively.

in Table 1 and primers for the chloramphenicol resistance gene.

C57BL/6J, *B6.129S4-Gt(ROSA)26Sor^{tm1Sor}/J* (*Rosa$^{stop\text{-}Lacz}$*), and B6.*Gt(ROSA)26Sor$^{tm4(ACTB\text{-}tdTomato,\text{-}EGFP)Luo}$*(*Rosa$^{mT/mG}$*) mice were obtained from The Jackson Laboratory. Each line was crossed separately with hemizygous *Prop1-cre* mice to produce experimental embryos and pups.

X-gal staining and immunohistochemistry

Prop1-cre; Rosa$^{stop\text{-}LacZ}$ embryos ranging from e9.5 to e12.5 for whole mount 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining, along with *Prop1-cre* negative control tissues, were isolated in 1× PBS (pH 7.2), fixed for 15 minutes at 4°C in 4% paraformaldehyde in LacZ Fix, washed 3 times for 15 minutes at 4°C in LacZ wash buffer, before immersion in X-gal stain overnight at 37°C (40). *Prop1-cre; Rosa$^{stop\text{-}LacZ}$* e14.5 and e16.5 embryos were also collected in 1× PBS, fixed for 15 minutes at 4°C in LacZ Fix, washed 3 times for 15 minutes at 4°C in LacZ wash buffer, equilibrated in 30% sucrose at 4°C, and frozen for sagittal sections in Tissue-Tek Optimum Cutting Temperature Compound (Sakura) in a dry ice/ethanol bath. Tissues were cryosectioned at 10-μm thickness and mounted on Superfrost Plus slides (VWR). After sectioning, tissues were thawed at room temperature for 5 minutes, briefly fixed in LacZ Fix for 5 minutes at room temperature, washed 3 times in LacZ wash buffer, and incubated in X-gal stain overnight at 37°C. X-gal stained sections were counterstained in 2% neutral red for 2 minutes. *Prop1-cre;Rosa$^{stop\text{-}LacZ}$* 3-week-old pituitaries used for X-gal staining and immunohistochemistry were processed similarly, except that incubation in X-gal stain at 37°C occurred for 2.5 hours, instead of overnight. Immunohistochemistry for pituitary hormones was performed as previously described using antibodies from the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases (41).

Prop1$^{+/+}$, *Prop1$^{+/-}$*, and *Prop1$^{-/-}$* e12.5 embryos were dissected from pregnant mothers and fixed in 3.7% formaldehyde in PBS overnight at 4°C. The embryos were processed for paraffin sectioning, and sectioned at 6-μm thickness (40). Select sections of each genotype were treated with xylene to remove paraffin and hydrated through 100% and 95% ethanol and into PBS. Slides were treated in 1.5% H_2O_2 and 50% methanol for 20 minutes to inactivate endogenous peroxidases, boiled in 10mM citrate (pH 6.2), for 10 minutes to unmask antigens, and blocked in 0.5% blocking reagent provided in the tyramide signal amplification (TSA) fluorescein kit (PerkinElmer) in 0.1M Tris-HCl (pH 7.5), and 0.15M NaCl for 10 minutes. Tissue sections were incubated with guinea pig anti-PROP1 antibody (Aimee Ryan) diluted 1:100 in block overnight at 4°C. After removing unbound primary antibody with PBS wash, sections were incubated with 1:100 biotin-conjugated antiguinea pig antibody (Jackson ImmunoResearch) for 30 minutes at room temperature, followed with 1:100 horseradish peroxidase-conjugated streptavidin (TSA fluorescein kit; PerkinElmer) for 30 minutes at room temperature. Slides were counterstained in 4′, 6-diamidino-2-phenylindole (DAPI) for 5 minutes before mounting in fluorescence mounting medium.

The PROP1 immunostaining protocol was modified for analysis of cryosections of *Prop1-cre; Rosa$^{mT/mG}$* embryos. Embryos were dissected from pregnant mothers and fixed, embedded in Optimum Cutting Temperature, and cryosectioned as above for *Prop1-cre; Rosa$^{stop\text{-}LacZ}$* embryos. Select sections were thawed at room temperature and photographed on a Leica DMI3000 B microscope with fluorescence capabilities, using a Leica DFC345 FX camera, to capture the tandem dimeric Tomato (tdTOMATO) and enhanced green fluorescent protein (eGFP) fluorescence (Leica Microsystems). Images were processed with Leica Application Suite Core software to produce red and green overlay images. The tissue sections were fixed in 4% paraformaldehyde in PBS for 1 hour at room temperature, before incubation in 0.1% Triton X-100 in PBS for 15 minutes. Boiling in 10mM citrate for 10 minutes retrieved antigens and eliminated fluorescence from tdTOMATO and eGFP. Sections were incubated in 1% blocking reagent in 1× PBS (Molecular Probes Tyramide Signal Amplification kits; Thermo Fisher Scientific) and then incubated with guinea pig anti-PROP1 antibody diluted 1:500 in block overnight at 4°C. Biotin-conjugated antiguinea pig secondary antibody was used as above, followed by Dylight 488-conjugated streptavidin (Thermo Fisher Scientific) diluted 1:100 in block for 30 minutes at room temperature. Tissues were counterstained with DAPI before mounting in fluorescence mounting media. The green fluorescent signal was captured with the same microscope and camera, but pseudocolored purple instead of green. Fluorescent emission from DAPI was also photographed, but not used in the final overlay images. The purple image was overlaid on the tdTOMATO and/or eGFP images using Adobe Photoshop CS6 (Adobe Systems).

Pituitaries from 3-week-old *Prop1-cre;Rosa$^{mT/mG}$* mice used for hormone and PECAM1 (also known as CD31) immunohistochemistry were collected and fixed, washed, frozen, and sectioned as above for *Prop1-cre; Rosa$^{stop\text{-}LacZ}$* embryos. Selected sections were thawed at room temperature for 5 minutes, fixed in 4% paraformaldehyde in 1× PBS for 15 minutes at room temperature, and incubated in 0.1% Triton X-100 in 1× PBS for 15 minutes. The sections were treated in 1.5% H_2O_2 in 50% methanol for 20 minutes before blocking in 1% blocking reagent in 1× PBS (Molecular Probes Tyramide Signal Amplification kits; Thermo Fisher Scientific) for 10 minutes. Guinea pig primary antibodies for LHβ (1:500), TSHβ (1:1000), proopiomelanocortin (POMC) (1:1000), and PRL (1:1000) and monkey primary antibody for GH (1:1000) (National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases) (41) were diluted into block and incubated overnight at 4°C. PECAM1 rabbit primary antibody (Thermo Fisher Scientific) was diluted 1:100. Biotin-conjugated species appropriate secondary antibodies were diluted 1:100 and incubated for 30 minutes at room temperature, followed by streptavidin-conjugated horseradish peroxidase (1:100) for 30 minutes at room temperature, and then TSA with Alexa Fluor 350 (Thermo Fisher Scientific) for 3 minutes. Sections were mounted in fluorescence mounting media and then imaged as above for endogenous fluorescence from tdTOMATO, eGFP, and Alexa Fluor 350. Overlay images of eGFP and Alexa Fluor 350 (pseudocolored red) were produced with Leica Application Suite Core software. Under these conditions, background staining in the intermediate lobe prevented analysis of POMC expression in the melanotropes. For intermediate lobe POMC expression, an e18.5 *Prop1-cre; Rosa$^{mT/mG}$* embryo was fixed, embedded in paraffin, and sectioned as above. Select sections were processed as above to remove paraffin and hydrate the tissues, followed by a 5-minute citrate boil to retrieve antigens and blocking in 1% blocking reagent in PBS (Molecular Probes Tyramide Signal Am-

plification kits; Thermo Fisher Scientific). Detection of eGFP was performed with a 1:500 dilution of rabbit anti-eGFP (Abcam), 1:200 dilution of biotin-conjugated antirabbit secondary (Thermo Fisher Scientific), and 1:100 dilution of streptavidin-conjugated Dylight 488 (Thermo Fisher Scientific). POMC detection utilized the guinea pig anti-POMC as above; followed by Dylight 594-conjugated antiguinea pig secondary (Thermo Fisher Scientific) diluted 1:100. Images were processed to match images produced for the other pituitary hormones.

All animals and tissues used for these experiments were chosen without regard to sex.

Results

The order of genes flanking *Prop1* differs in rodents and primates, suggesting that genomic rearrangements probably occurred during mammalian radiation (Figure 1A). This implies that conserved *cis*-regulatory elements necessary for *Prop1* transcription are located in the area of homology between human and mouse. Previously, we demonstrated that a mouse line transgenic for a BAC containing the mouse *Prop1* gene and the surrounding approximately 195-kb genomic region (BAC RP23-250I22) was sufficient to rescue the $Prop1^{df/-}$ dwarf phenotype (42). This result indicates that the BAC contains the *cis*-regulatory elements necessary for *Prop1* transcription. We hypothesized that a considerably smaller region might be sufficient for correction because of the lack of synteny homology surrounding the human and mouse *Prop1* genes (Figure 1A) and the clustering of conserved noncoding sequences (CNSs) within 10 kb of the gene (Figure 1B) (42). To identify the minimal essential sequences for *Prop1* expression, we generated transgenic lines with either 25 or 10 kb surrounding the *Prop1* gene and tested at least 4 lines from each construct for the ability to correct the dwarf phenotype (Table 2 and Figure 1A). Four different lines with the 25-kb BAC were able to correct the dwarf phenotype with 100% penetrance (n = 29 informative progeny total). Four out of 5 transgenic lines carrying the 10-kb *Prop1* genomic region were sufficient for rescuing the $Prop1^{df/-}$ dwarf phenotype, but the penetrance was incomplete in 3 of the 4 lines (Table 2). The 10-kb transgene contains all of the obvious CNSs, but the incomplete penetrance of transgene correction relative to the larger constructs reveals a lack of insulation from insertion site effects in this transgene relative to the 25-kb construct.

To test the functional conservation of the PROP1 protein and the *cis*-regulatory elements that regulate its expression, we tested the ability of the human *PROP1* gene to rescue the dwarf mouse phenotype. We generated 4 transgenic mouse lines carrying an approximately 196-kb BAC containing human *PROP1* (Figure 1A) and performed the necessary crosses to generate mice that were $Prop1^{df/df}$ and harbored the human *PROP1* BAC transgene (*HsPROP1*). All 4 *HsPROP1* BAC transgenic lines rescued the mouse dwarf phenotype, and only one of the 3 lines exhibited incomplete penetrance (Table 2 and Figure 1, C and D). This indicates that human *PROP1* can compensate functionally for the mouse gene, despite the 51.5% divergence in amino acids N-terminal to the homeodomain and an overall amino acid identity of 73%. It also demonstrates that transcriptional regulation is sufficiently conserved between mouse and man for rescue of the mouse genetic defect with mouse transactivating factors recognizing human *cis*-acting sequences and driving *PROP1* expression.

BAC recombineering has simplified the ability to engineer large DNA constructs (33); therefore, we chose to generate a mouse *Prop1-cre* transgene by inserting the coding sequence for the *cre* recombinase into a mouse *Prop1* BAC (36). We used a *Prop1* containing BAC, RP23-479M11, which contains mouse genomic DNA similar to that shown to be sufficient for correcting the mutant phenotype (Figure 1A). This BAC was used for generating all *Prop1-cre* transgenes. The CNS in intron 1 of mouse *Prop1* is known to mediate spatial expression within the pituitary gland and response to Notch signaling (42, 43), and it is more conserved than exon 1 (Figure 1B). Therefore, we attempted to preserve its function in the *Prop1-cre* construct. Using BAC recombineering, the *cre* coding sequence was inserted in-frame into exon 2, thus preserving the integrity of intron 1 completely, as well as the spatial arrangement relative to the promoter (Figure 1B). Three transgenic lines were generated and tested for *cre* activity: $Tg(Prop1\text{-}cre)^{432Sac}$, $Tg(Prop1\text{-}cre)^{506Sac}$, $Tg(Prop1\text{-}cre)^{517Sac}$. All 3 lines exhibit *cre* activity throughout the pituitary anterior lobe at 3 weeks, when combined with *cre* reporter line $Rosa^{mT/mG}$, where a membrane bound red fluorescent tdTOMATO protein is expressed before recombination, and a membrane bound green fluorescent eGFP protein is expressed following recombination (Figure 1E and data not shown) (44).

To determine whether *Prop1-cre* expression is properly restricted to the pituitary gland, we performed whole mount X-gal staining on $Tg(Prop1\text{-}cre)^{517Sac}$; $R26R^{stop\text{-}Lacz}$ embryos at e11.5 (Figure 2, A and B) (45). Robust X-gal staining was observed in the pituitary gland with no detectable ectopic activity in other tissues in 75% of the embryos analyzed (n = 18). A portion of the embryos (25%) displayed variable, ectopic expression in the brain and peripheral tissues in addition to the expression in Rathke's pouch (n = 6, data not shown). Individual mice and fetuses with ectopic *cre* activity were excluded

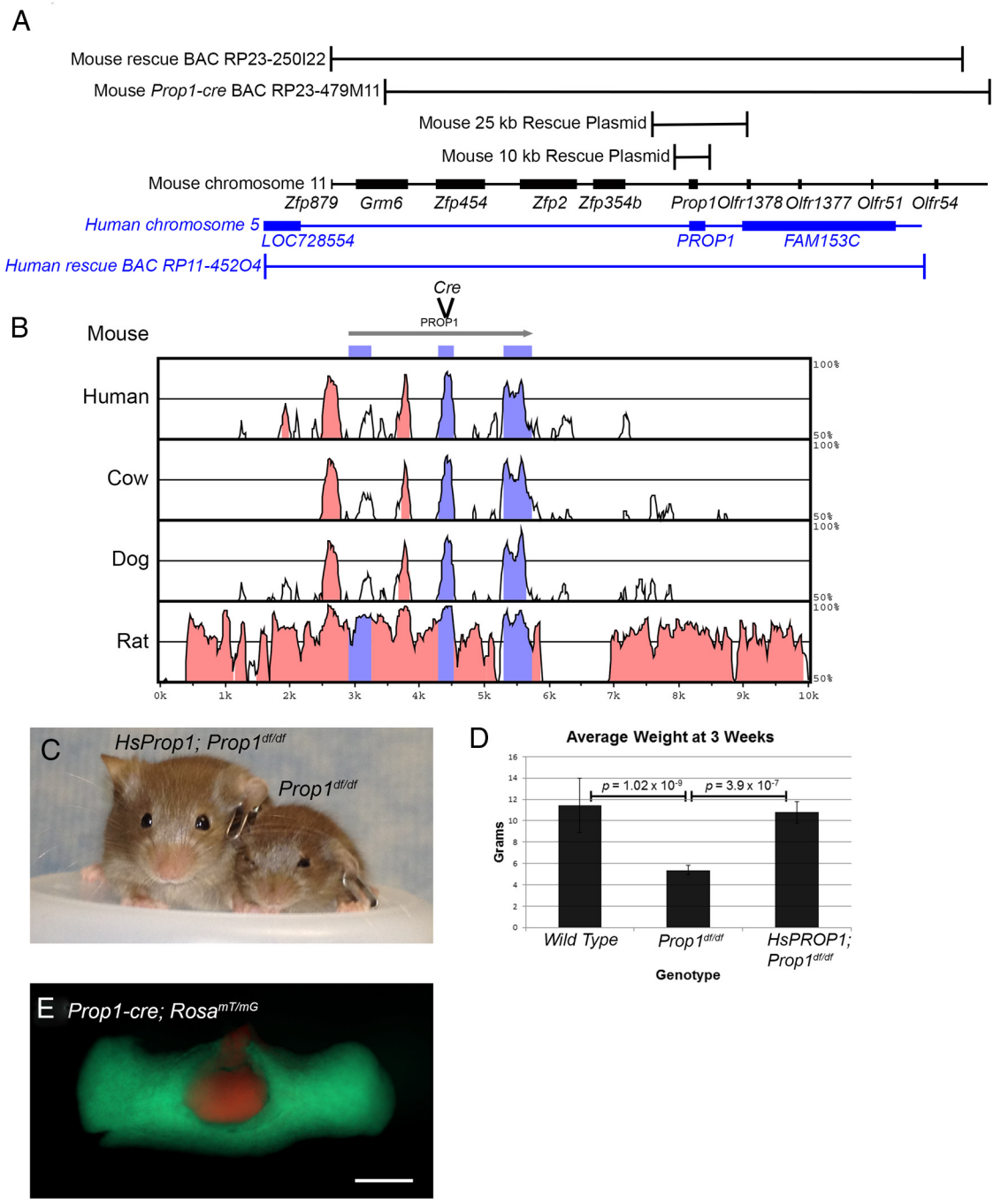

Figure 1. *Prop1* genomic regions and conservation, *Prop1-cre* generation, and *HsPROP1* transgenic rescue. A, Schematic diagram of mouse *Prop1* genomic region on chromosome 11 (black) and human *PROP1* on chromosome 5 (blue) as visualized using the UCSC Genome Browser (http://genome.ucsc.edu). Black brackets above the genomic schematic indicate the portion of mouse chromosome 11 contained in the original rescue BAC, RP23-250I22, the BAC used for generating the *Prop1-cre*, RP23-479M11, and 2 rescue plasmids of 25 and 10 kb. The blue bracket below the genomic schematic indicates the portion of human chromosome 5 contained in *HsPROP1* BAC RP11-452O4. B, VISTA plot (http://genome.lbl.gov/vista/mvista/submit.shtml) comparing conserved genomic regions between mouse, human, cow, dog, and rat genomes in the region of the 10-kb rescue plasmid. Pink peaks indicate CNSs and purple peaks represent conserved coding sequences. Also indicated is the location where the *cre* cassette was inserted into BAC RP23-479M11 to generate *Prop1-cre*. C, Three-week-old *HsPROP1; Prop1*$^{df/df}$ transgenic and *Prop1*$^{df/df}$ mice. D, Graph of average weight at 3 weeks for wild-type, *Prop*$^{df/df}$, and *HsProp1; PROP1*$^{df/df}$ mice. Brackets indicate *P* values for 2-tailed, *t* tests between wild-type (n = 37) and *Prop1*$^{df/df}$ (n = 11) and between *Prop1*$^{df/df}$ and *HsPROP1; Prop1*$^{df/df}$ (n = 18) mice. E, Pituitary from a 3-week-old *Prop1-cre; Rosa*$^{mT/mG}$ mouse. Green fluorescence indicates recombination from *Prop1-cre*, and red fluorescence indicates no recombination. Scale bar, 500 μm.

from further analyses. We selected Tg(Prop1-cre)^{517Sac} for our studies because it best reflected endogenous *Prop1* expression. Tg(Prop1-cre)^{432Sac} displayed a similar degree of ectopic cre activity, and cre reporter expression was detectable before endogenous *Prop1* expression. Tg(Prop1-cre)^{506Sac} displayed a higher rate of ectopic expression

Table 2. Transgenic Rescue of *Prop1* Mutant Mice

Transgenic Construct	Line	Full Sized *Tg; Prop1*$^{df/-}$ Mice[a]	Dwarf *Tg; Prop1*$^{df/-}$ Mice[a]	Penetrant Correction
25-kb *Prop1 Tg*	103	5	0	100
	114	6	0	100
	450	12	0	100
	453	6	0	100
	231	2	5	29
	241	1	0	100
10-kb *Prop1 Tg*	244	0	2	0
	248	6	1	86
	463	11	0	100
	853	5	0	100
HsPROP1 BAC Tg	862	4	1	80
	868	4	0	100
	884	4	0	100

Each line number represents the ear tag of the founder transgenic mouse.

[a] The number represents the total number of dwarf mice or transgenic dwarf mice collected over multiple litters. Only 1/8 of the mice are expected to be transgenic and compound heterozygotes for *Prop1* loss of function mutations (*Prop1*$^{df/-}$).

(data not shown). All data for the exon 2 *Prop1-cre* presented here was generated using Tg(Prop1-cre)^{517Sac} and will be referred to from here on as *Prop1-cre*.

PROP1 antibodies have been used to detect mouse and rat PROP1 in pituitary sections (20, 46). We characterized the specificity of the PROP1 antibody B directed against the N-terminus by performing immunofluorescence for PROP1 on e12.5 wild-type and *Prop1*$^{-/-}$ tissues. As expected, strong, nuclear PROP1 immunoreactivity is detected throughout the middle zone of Rathke's pouch in wild-type embryos at e12.5, with lighter staining in the dorsal aspect of the pouch, and no staining in the rostral tip (Figure 2C). In contrast, no PROP1 staining was detected in the pituitaries of *Prop1*$^{-/-}$ embryos at e12.5, confirming the specificity of the antibody (Figure 2D). There is background staining in nonpituitary tissues, but it is not nuclear.

To determine whether the *Prop1-cre* transgene accurately reflects the endogenous PROP1 expression pattern, we used the *Rosa*$^{stop\text{-}LacZ}$ and *Rosa*$^{mT/mG}$ reporter lines to characterize the timing of *Prop1-cre* activity in comparison with PROP1 expression by immunostaining. At e10.5, no *cre* activity is observed in Rathke's pouch of *Prop1-cre; Rosa*$^{stop\text{-}LacZ}$ or *Prop1-cre; Rosa*$^{mT/mG}$ embryos (Figure 3, A and B). After imaging *Prop1-cre; Rosa*$^{mT/mG}$ cryosections for fluorescence generated from the *Rosa*$^{mT/mG}$ allele, the sections were processed to quench the endogenous fluorescence and then subjected to PROP1 immunostaining. Overlaying the initial *Prop1-cre; Rosa*$^{mT/mG}$ fluorescence image with the PROP1 immunostaining allows for direct comparison of *Prop1-cre* activity and PROP1 expression. Nuclear PROP1 is not detected in Rathke's pouch at e10.5 (Figure 3, C and D).

As expected, *Prop1-cre* mediated recombination is detected in Rathke's pouch at e11.5, using both reporter lines (Figure 3, E–G). Some cells in Rathke's pouch are negative for PROP1 immunoreactivity at e11.5, demonstrating that the initial expression of PROP1 does not occur simultaneously in all pituitary progenitor cells. The overlay of PROP1 immunostaining and *Rosa*$^{mT/mG}$ fluorescence indicates that PROP1 expression is detectable before *Prop1-cre* activity (Figure 3H). At e12.5, *Prop1-cre* activity detected by *Rosa*$^{stop\text{-}LacZ}$ and *Rosa*$^{mT/mG}$ appears uniform throughout Rathke's pouch and extends to the rostral tip (Figure 3, I and J). In contrast, PROP1 protein is not expressed in the forming rostral tip (Figure 3K). Therefore, cells in the rostral tip must have expressed PROP1 before their migration into the forming anterior lobe.

By e14.5, *Prop1-cre* activity as assayed by both *Rosa*$^{stop\text{-}LacZ}$ and *Rosa*$^{mT/mG}$ is uniform throughout the luminal area and the forming anterior lobe (Figure 3, M and N). PROP1 expression is still observed in the luminal area, but is down-regulated in the differentiating cells of the anterior lobe (Figure 3O). At e14.5, mesenchymal cells, which are *Prop1-cre* and PROP1 negative (Figure 3, M–P) appear to be entering the developing anterior lobe. Both the intermediate (Figure 3, Q and R) and anterior lobes (Figure 3, Q and S) are almost completely comprised of *LacZ* positive cells at e16.5. Those cells that do not express *LacZ* at e16.5 are likely part of the forming pituitary vasculature (Figure 3, Q and S). Immunostaining for PECAM1 on *Prop1-cre; Rosa*$^{mT/mG}$ pituitaries at 3 weeks of age confirm that the nonrecombined, red fluorescent cells in the anterior lobe are endothelial cells (Figure 3, T–V).

The descendants of the *Prop1-cre*-expressing progenitor cells are permanently marked by the recombination event, as evidenced by the green fluorescence in the anterior lobe of a postnatal day 21 *Prop1-cre; Rosa*$^{mT/mG}$ pituitary (Figure 1E). Similar results were obtained with the 2 other *Prop1-cre* transgenic lines. To confirm that the lineage-tracing experiments indicate that all hormone-expressing cell types are descended from a *Prop1* progenitor, we assessed the colocalization of eGFP and individual hormones in *Prop1-cre; Rosa*$^{mT/mG}$ mice. As expected, we observed PIT1 lineage cells that were eGFP positive and expressed the hormones GH, TSH, or PRL (Figure 4, A–I). We also observed eGFP positive cells that expressed POMC in the anterior lobe (Figure 4, J–L) and intermediate lobe (Figure 4, M–O), indicating that *Prop1*-expressing progenitors gave rise to both corticotropes and melanotropes. LHβ immunostaining was also observed in

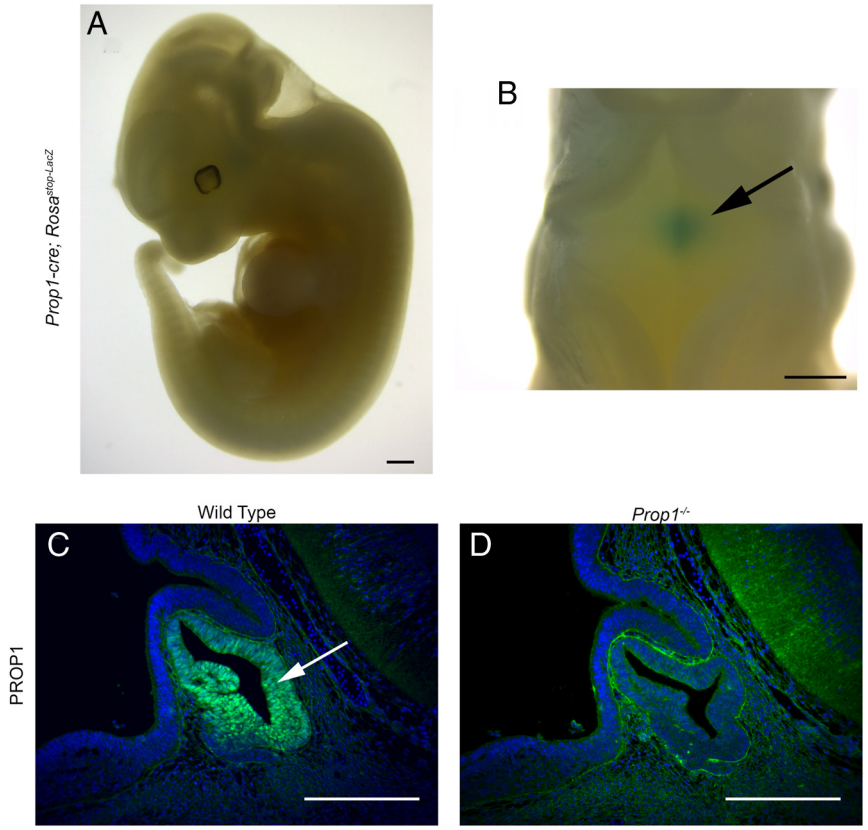

Figure 2. Pituitary-specific *Prop1-cre* activity and PROP1 immunostaining. A and B, Whole mount X-gal-stained e11.5 *Prop1-cre; Rosa*$^{stop\text{-}LacZ}$ embryo. Blue indicates *LacZ* activity and *cre* expression. A, Lateral view. B, Dorsal view. Arrow indicates the developing pituitary gland as seen through the hindbrain. C and D, PROP1 expression (green) assayed by immunostaining, and counterstained with DAPI (blue), on e12.5 sagittal sections. C, Wild type. Arrow indicates PROP1 expression in Rathke's pouch. D, *Prop1*$^{-/-}$. Scale bars, 500 μm (A and B) and 100 μm (C and D).

eGFP positive anterior lobe cells (Figure 4, P–R). Therefore, all hormone-expressing cell types of the pituitary anterior and intermediates lobe arise from *Prop1*-expressing progenitors.

Discussion

We have generated *Prop1-cre* mouse strains that deliver efficient and highly penetrant *cre* activity early in pituitary gland development, in Rathke's pouch. Our lineage tracing experiments demonstrate that *Prop1-cre* marked progenitors give rise to all hormone secreting cell types of the anterior and intermediate lobes of the pituitary. This result suggests that there is a common *Prop1*-expressing progenitor pool in Rathke's pouch that gives rise to all hormone-producing cells of the anterior and intermediate lobes. *Prop1* mutant mice are able to produce corticotropes, melanotropes, and gonadotropes, indicating that *Prop1* is not necessary for the formation of these cell types in mice, despite being expressed in a common progenitor (14–16).

Prop1 mutant mice have an increase in the epithelial-like cells that surround Rathke's cleft, and the developing mutant pituitaries fail to express *Slug*, a transcription factor involved in EMTs (32, 47). The essential function of *Prop1* is likely to be in regulating the release of progenitor cells from the marginal zone and promoting differentiation via an EMT-like mechanism.

PROP1 forms a complex with β-CATENIN to activate *Pou1f1* transcription (13). However, *Prop1* may not be absolutely required to activate *Pou1f1*. Small clonal populations of *Pou1f1*-expressing thyrotropes, somatotropes, and lactotropes form in the anterior lobe of *Prop1*$^{df/df}$ mice (48). This observation is in alignment with the fact that patients with *PROP1* loss of function mutations typically present with short stature and low but detectable GH production, suggesting the initial presence of functional somatotropes (18, 49). Patients tend to subsequently lose production of other hormones, including reproductive hormones and ACTH. For example, patients may not present with short stature and begin GH replacement therapy until age 6, sex steroid replacement at age 15, and hydrocortisone replacement at age 18 or even later (18). This contrasts with the typical presentation of patients with *PIT1* loss of function mutations, with onset of short stature and other hormone deficiencies before the age of 2 and no progressive features (49). The progressive loss of hormonal production by all anterior pituitary cell types may occur because *PROP1* regulates a common pool of pituitary progenitors, not just the PIT1 lineage, and the progenitor pool may be exhausted over the life of the patient. It is also possible that activation of *PIT1* is less dependent upon *PROP1* in humans than in mice, resulting in an early expansion of the PIT1 lineage which cannot be sustained.

The expression of *Prop1* in a common pituitary progenitor pool helps to explain the progressive loss of all hormone cell types in humans, but does not adequately explain why mice do not evolve ACTH deficiency. Both *Prop1*$^{df/df}$ and *Prop1*$^{-/-}$ mice on mixed genetic backgrounds or backcrossed onto a C57BL/6 background for 4 generations maintained a functional pituitary adrenal

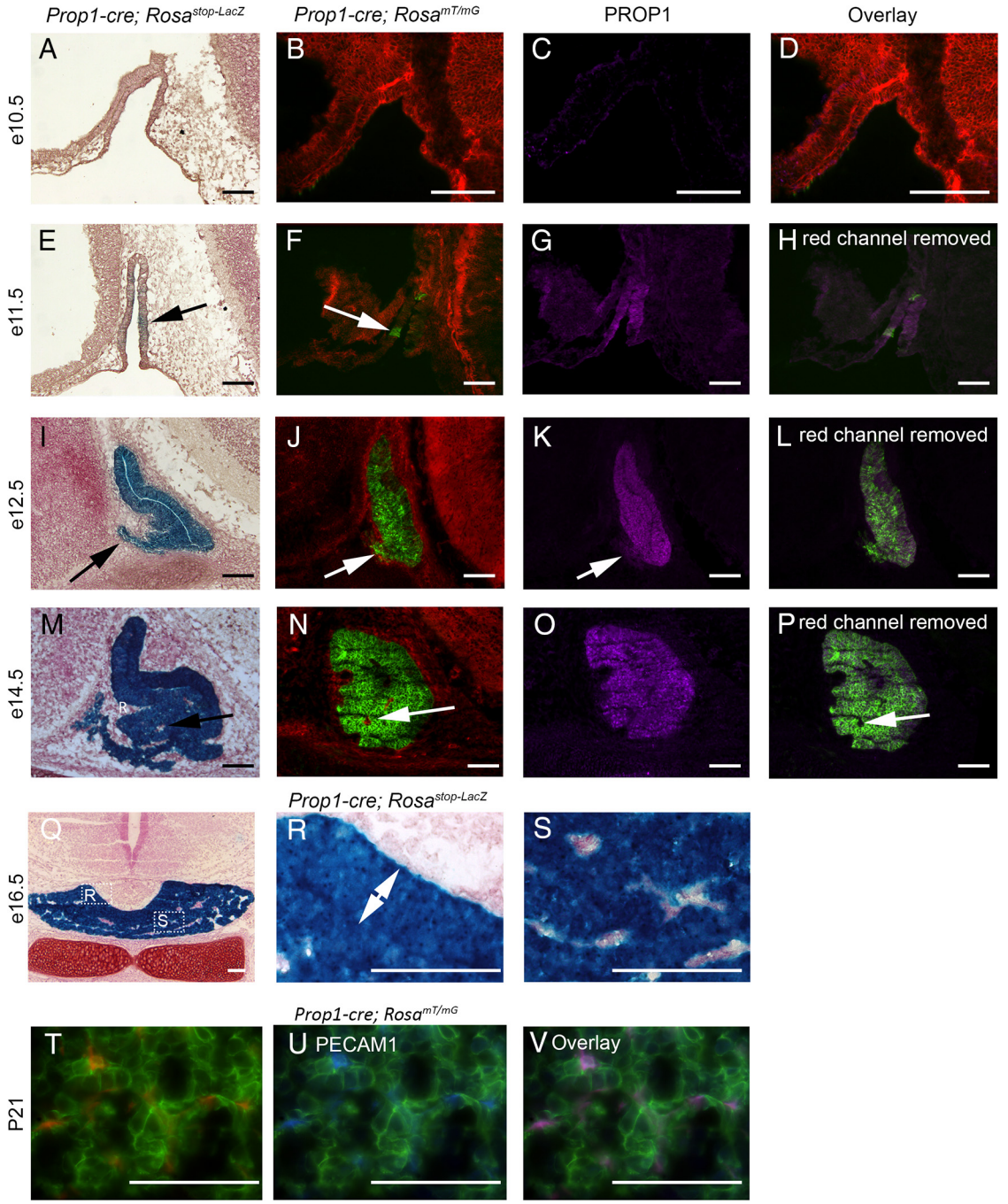

Figure 3. *Prop1-cre* activity compared with PROP1 expression. A–D, e10.5 sagittal sections. E–H, e11.5 sagittal sections. I–L, e12.5 sagittal sections. M–P, e14.5 sagittal sections. Q–S, e16.5 coronal sections. T–V, Postnatal day 21 (P21) pituitary coronal sections. A, E, I, M, Q, R, and S, *Prop1-cre; Rosa*$^{stop\text{-}LacZ}$ embryos X-gal stained (blue) to indicate *LacZ* activity caused by *cre* recombination, and counterstained with neutral red. Arrow in E indicates small region of *LacZ* activity. Arrow in I indicates *LacZ* activity in the rostral tip. B, F, J, and N, *Prop1-cre; Rosa*$^{mT/mG}$ embryos where red fluorescence indicates no *cre* activity and green fluorescence indicates *cre* recombination. Arrow in F indicates a small region of *cre* activity. Arrow in J indicates *cre* activity in the rostral tip. Arrow in N indicates a region with no cre activity (red). C, G, K, and O, PROP1 immunostaining (purple) on the same sections as B, F, J, and N. Arrow in K indicates no expression of PROP1 in the rostral tip. D, Overlay of *Prop1-cre; Rosa*$^{mT/mG}$ and PROP1 immunostaining images. H, L, and P, Overlay of eGFP fluorescence and PROP1 immunostaining. Arrow in P indicates a small region with no *cre* activity and no PROP1 expression in the pituitary anterior lobe. R and S, Boxed regions in Q, presented at higher magnification. Double arrow in R indicates the intermediate lobe. T, Fluorescence from *Prop1-cre; Rosa*$^{mT/mG}$ tdTOMATO (red) and eGFP (green). U, Same image as T, with the red channel removed and immunostained for PECAM1 (blue). V, Same image as T with all 3 channels, demonstrating colocalization of PECAM1 with tdTOMATO. Sale bars, 100 μm.

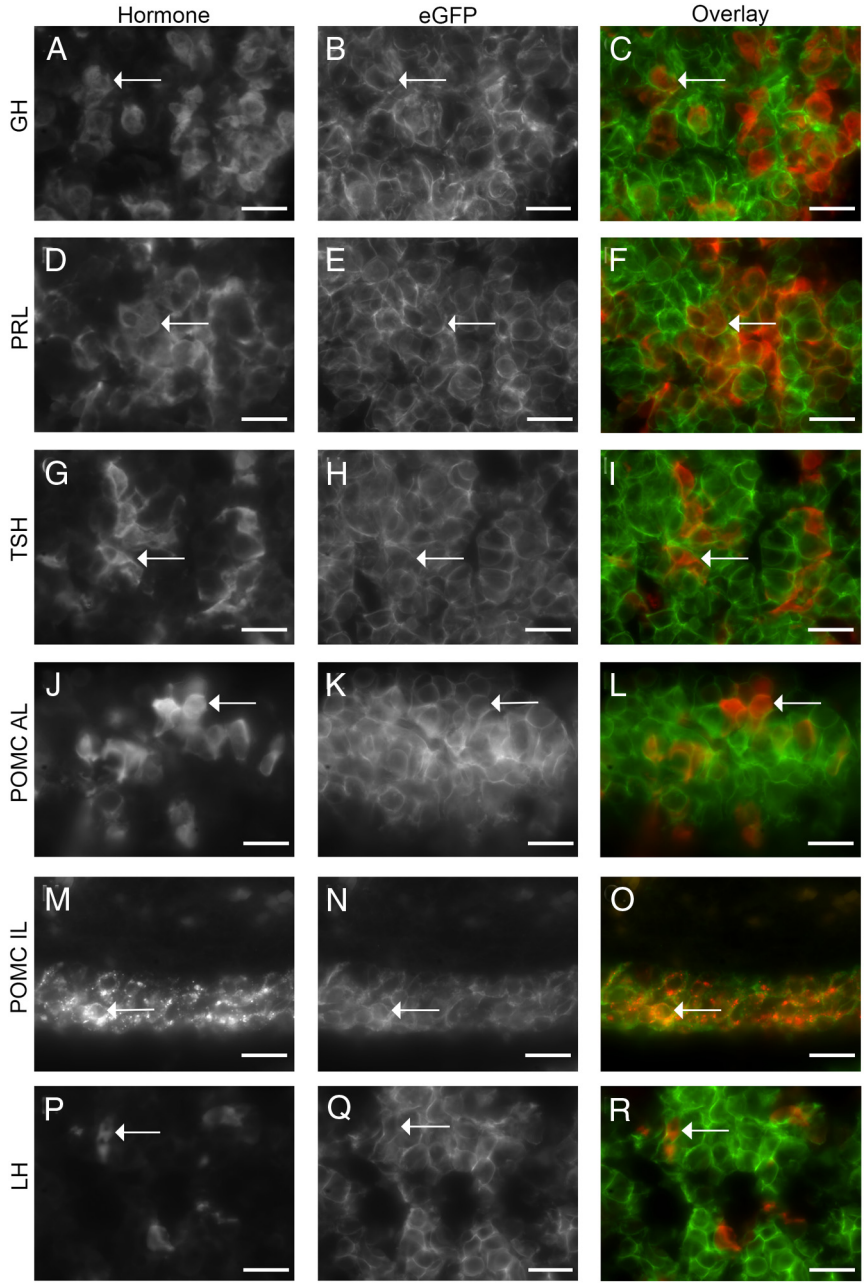

Figure 4. All anterior and intermediate lobe hormone-expressing cell types are derived from a *Prop1-cre* progenitor. A, D, G, J, M, and P, Gray scale images of immunostaining for hormones from *Prop1-cre; Rosa*$^{mT/mG}$ pituitaries. GH (A), PRL (D), TSHβ (G), POMC in the anterior lobe (J), POMC in the intermediate lobe (M), and LHβ (P). (B, E, H, K, N, and Q) Gray scale images of eGFP detection of same images as A, D, G, J, M, and P. C, F, I, L, O, and R, Overlay of hormone (red) and eGFP (green) images. Arrows indicate the same cell in each image. Immunostainings for A–L and P–R were performed on P21 pituitaries, while immunostainings for M–O were performed on e18.5 pituitaries. Scale bars, 20 μm.

axis throughout life, with no loss of corticotropes in aged mice (50). Although we cannot rule out the possibility that some genetic background might provoke ACTH deficiency in mice, it seems that there are species differences in the requirement for PROP1 to initiate and maintain pituitary function. Longitudinal studies in humans and analysis of very large human pedigrees with *PROP1* mutations reveals that the age of onset and degree of hormone insufficiency can vary even in individuals with the same mutation (18, 51). Mouse *Prop1* and *Hes1*, a mediator of the Notch signaling pathway, regulate the pool of common pituitary progenitor cells such that loss of both *Prop1* and *Hes1* leads to premature differentiation of corticotropes and a smaller pituitary gland (32). Perhaps mice maintain sufficient Notch signaling in the absence of *Prop1* to maintain a progenitor pool sufficient for continual corticotrope production, whereas humans do not.

PROP1 patients sometimes present with a "waxing and waning" pituitary phenotype, where sequential magnetic resonance imaging scans reveal that the pituitary undergoes periods characterized by hyperplasia and hypoplasia (52, 53). This phenotype has not been observed in mice. Instead, the mouse embryonic pituitary is dysmorphic, but has a normal volume, and the postnatal pituitary is hypoplastic due to reduced proliferation and increased apoptosis (31). The rescue of the mouse *Prop1* deficiency with an *HsPROP1* BAC demonstrates that the *cis*-regulatory elements and trans acting factors are sufficiently conserved for *HsPROP1* to be expressed in mice and for it to activate the necessary downstream targets in mice. These experiments do not address the possibility of a temporal difference in PROP1 expression between mice and humans, which could contribute to the differential phenotypes.

The generation of a well-characterized *Prop1-cre* strain expands the available repertoire of genetic reagents available for tissue-specific conditional inactivation in the pituitary gland. There are several existing lines that target the oral ectoderm before Rathke's pouch formation, including *Pitx1-cre*, *Hesx1-cre*, and *Foxg1-cre,* but these promoters also drive *cre* expression in other tissues besides the pituitary gland (54–56). The *Prop1-cre* strains reported here offer pan-pituitary deletion from

e11.5-e12.5 onward in most embryos. This complements other *cre* strains that are available for cell-specific deletion, such as the improved *Cga-cre* that is activated at e11.5 and promotes deletion in the thyrotropes and gonadotropes (35). The *Pou1f1-cre*, *Sf1-cre,* and *GnRHR-cre* (*GRIC*) mediate recombination during the differentiation process, beginning at e14.5, in a subset of cell types in the anterior lobe (13, 57, 58). The *Gh-cre*, *Tshb-cre*, *Lhb-cre*, *Prl-cre*, and *Pomc-cre* all mediate recombination in the specific pituitary hormone cell types, after terminal differentiation (59–64). The *Prop1-cre* complements these genetic resources by expressing *cre* after progenitor cell formation from e9.5 to e10.5, but before cell specification at e13.5–e14.5, and it targets all hormone secreting cell types of the pituitary gland.

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