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***Enpp2/Autotaxin* in Dermal Papilla Precursors is Dispensable for Hair Follicle Morphogenesis**

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Abstract

Systematic ablation of previously identified dermal papilla (DP) signature genes in embryonic DP precursors will reveal their functional roles during hair follicle morphogenesis. In this study we validate *Enpp2/Autotaxin* as one of the highest expressed signature genes in postnatal DP, and demonstrate specific expression of this lysophosphatidic acid (LPA) generating enzyme in embryonic dermal condensates. We further identify dermal and epidermal expression of several LPA receptors suggesting that LPA signaling could contribute to follicle morphogenesis in both mesenchymal and epithelial compartments. We then utilize the recently characterized Cre-expressing *Tbx18* knock-in line to conditionally ablate *Enpp2* in embryonic DP precursors. Despite efficient gene knockout in E14.5 dermal condensates, morphogenesis proceeds regularly with normal numbers, lengths and sizes of all hair follicle types, suggesting that *Enpp2* is not required for hair follicle formation. To interrogate DP signature gene expression, we finally isolate control and *Enpp2* null DP precursors and identify the expression and upregulation of *LIPH*, an alternative LPA producing enzyme, suggesting that this gene could functionally compensate for the absence of *Enpp2*. We conclude that future co-ablation of both LPA producing enzymes or of several LPA receptors may reveal the functional role of LPA signaling during hair follicle morphogenesis.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

Keywords

Dermal papilla; *Enpp2*; *Atx*; Hair follicle formation

INTRODUCTION

Dermal papilla (DP) precursors in dermal condensates are thought to exchange signals with hair placodes to regulate embryonic hair follicle formation (Millar, 2002; Sennett and Rendl, 2012). Likewise, mature DP cells interact with matrix progenitors and bulge/germ stem cells to coordinate postnatal hair growth and adult follicle regeneration in the hair cycle, respectively (Hsu and Fuchs, 2012; Lee and Tumber, 2012). Despite the importance of these interactions, a comprehensive knowledge of DP genes was missing for many years due to the long-standing absence of genetic tools for specific labeling, isolation and characterization of DP cells. In the last few years, we and others have established protocols to specifically isolate DP cells from growing and cycling follicles with the help of fluorescent genetic drivers, and we defined DP molecular gene signatures (Driskell *et al.*, 2009; Greco *et al.*, 2009; Rendl *et al.*, 2005). To date, a complete knowledge of gene expression in embryonic DP precursor cells is still missing in the field.

Similarly, the systematic functional analysis of newly identified DP genes in genetic studies was unavailable, due to the lack of DP-specific gene targeting tools. Recent genetic advances with a *Corin^{Cre}* mouse line allowed specific targeting of DP niche cells for gene ablation, but these tools were limited in that they could only be used to study mature DP cells in growing hair follicles starting at 3–7 days after birth (Enshell-Seijffers *et al.*, 2010). For embryonic skin, widespread dermal targeting with *Prx1* transgenic drivers was applied in recent gene ablation studies, which is limited to less well-characterized ventral and limb skin in a non-DP-specific fashion (Logan *et al.*, 2002; Woo *et al.*, 2012). *Engrailed1* is another driver that has broad expression in the upper dermis of the head and central back regions early during embryonic dermis specification (Atit *et al.*, 2006). When used to ablate *β -catenin* it compromises dermal development (Atit *et al.*, 2006) or widely blocks dermal Wnt signaling before hair follicle formation (Chen *et al.*, 2012), precluding the specific analysis of its role in developing dermal condensates. Most recently, we introduced *Tbx18* genetic drivers for labeling, isolating and inducible targeting of embryonic DP precursors (Grisanti *et al.*, 2012). *Tbx18* specifically marks dermal condensates at embryonic day (E)14.5 of the first hair follicle wave, but becomes more broadly expressed in the dermis starting at E16.5. Using this tool we subsequently ablated the transcription factor *Sox2* and demonstrated an important role in controlling epithelial hair growth (Clavel *et al.*, 2012).

Enpp2, also known as *Autotaxin*, is a member of the ectonucleotide pyrophosphatase/phosphodiesterase family and is the main lysophospholipase-D enzyme responsible for conversion of lysophosphatidyl choline (LPC) into lysophosphatidic acid (LPA) (van Meeteren *et al.*, 2006). Active LPA binds to LPA receptors (*Lpar*) in target cells directly affecting cell growth, survival, differentiation and migration (Luquain *et al.*, 2003). *Enpp2*-LPA signaling plays important roles during embryonic development, wound healing and tumor growth and metastasis (Houben and Moolenaar, 2011). In skin, *Enpp2* is one of the

highest expressed signature genes in the DP of growing hair follicles (Rendl *et al.*, 2005), but its physiological function during embryonic follicle formation and growth is currently unknown. *Enpp2* gene ablation in full knockout mice results in early embryonic lethality at E9.5, due to impaired vascular development (van Meeteren *et al.*, 2006), precluding the analysis of its role during follicle morphogenesis.

In this study, we directly test the role of *Enpp2* in embryonic DP precursors for hair follicle formation by *Tbx18^{Cre}*-mediated conditional gene ablation. Despite the specific expression of *Enpp2* in embryonic dermal condensates, the presence of dermal and epidermal LPA receptors, and our demonstration of efficient *Enpp2* ablation, we find normal hair follicle morphogenesis. Since follicle numbers, lengths and sizes are unaffected we conclude that *Enpp2* is dispensable for normal hair follicle formation. Our molecular analysis of *Enpp2* conditional knockout DP precursor cells reveals the expression of another LPA producing enzyme, *LIPH*, which could functionally compensate the lack of *Enpp2*.

RESULTS AND DISCUSSION

***Enpp2* is specifically expressed in embryonic DP precursor cells of dermal condensates**

Among all previously identified postnatal DP signature genes (Rendl *et al.*, 2005), *Enpp2* was the highest expressed gene in microarrays of isolated DP cells compared to fibroblasts (Supplementary Figure S1). Real-time PCR of DPs, fibroblasts, melanocytes and hair follicle epithelial cells (isolated as previously described (Rendl *et al.*, 2005)) confirmed robust *Enpp2* enrichment in DP cells (Figure 1a). *In situ* hybridization and immunofluorescence stainings validated *Enpp2* transcript and ENPP2 protein expression specifically in postnatal DP cells (Figure 1b,c).

To test whether *Enpp2* is expressed in DP precursors during embryonic hair follicle formation, we performed whole-mount *in situ* hybridization, real-time PCR and immunofluorescence stainings. At E13.5, before hair follicle formation, *Enpp2* mRNA was absent in skin (Figure 1d). At E14.5, when placodes and dermal condensates are forming, *Enpp2* transcripts were detectable in a hair follicle pattern (Figure 1d). Sections from whole-mount embryos at E14.5, and later at E15.5, revealed specific *Enpp2* mRNA expression in dermal condensates (Figure 1e). Real-time PCR of DP precursors, isolated by fluorescence activated cell sorting (FACS) from E14.5 *Tbx18^{H2BGFP}* embryos (Grisanti *et al.*, 2012), confirmed *Enpp2* expression in dermal condensates, along with other known DP genes (Figure 1f). Whole-mount immunofluorescence staining of E14.5 skin confirmed presence of ENPP2 protein in DP precursors as identified by co-localization with the dermal condensate marker SOX2 (Figure 1g). Starting at E15.5 ENPP2 protein was readily detectable in sagittal back skin sections as well (Figure 1h). Taken together, these data demonstrate that *Enpp2* is present in dermal condensates and DPs to participate in the regulation of hair follicle formation and growth.

Analysis of LPA receptor expression revealed that several *Lpar* family members were present in both epidermal and dermal cells isolated by FACS at E14.5 (Supplementary Figure 2a) and in hair follicle epithelial cells and DP cells at P5 (Supplementary Figure 2b). Among these receptors, *Lpar1* and *Lpar4* are preferentially expressed in the dermal

compartment and DP, while *Lpar2* and *Lpar3* are present in dermis and epidermis. Since LPA can signal through each receptor and LPA receptors are expressed in both epithelium and mesenchyme, this suggests that locally produced LPA by *Enpp2* in DP precursors could signal to either compartment during hair follicle formation and growth.

Efficient *Tbx18^{Cre}*-mediated ablation of *Enpp2* in embryonic dermal condensates

To investigate whether *Enpp2* in DP precursors plays a role during hair follicle formation, we ablated *Enpp2* in DP precursors at E14.5 by crossing *Tbx18^{Cre}* with gene targeted *Enpp2* floxed lines (Figure 2a). *Tbx18*-driven cre activity is specific in DP precursors at E14.5 in most parts of the back skin (Grisanti et al., 2012). *Enpp2* floxed mice (*Enpp2^{fl/fl}*) harbor loxP sites flanking *Enpp2* exons 6 and 7 and their Cre-mediated excision generates an early stop codon that abolishes ENPP2 protein translation (van Meeteren et al., 2006). Whole-mount in situ hybridization of E14.5 *Tbx18^{Cre};Enpp2^{fl/fl}* conditional knockout (cKO) embryos demonstrated efficient *Enpp2* ablation as no transcript was detectable (Figure 2b, right), while wild-type (WT, *Enpp2^{fl/fl}*) embryos displayed normal mRNA levels (Figure 2b, left).

Whole-mount immunofluorescence staining for ENPP2 and SOX2 proteins confirmed robust ENPP2 deletion at the protein level in dermal condensates of E14.5 cKO skin (Figure 2c, bottom). 3D-reconstruction of the z-dimension of confocal laser scans also confirmed the absence of ENPP2 in cKO condensates at E14.5 (Figure 2d). Quantification of ENPP2 expression in E15.5 DP precursors (Figure 2e), as identified by SDC1 (Syndecan-1) double-staining (Richardson et al., 2009), further established robust ablation in cKO skin (Figure 2f). This is reminiscent of the efficient activation with the same *Tbx18^{Cre}* line of two independent Cre reporters (Grisanti et al., 2012) and the robust ablation of the dermal condensate gene *Sox2* (Clavel et al., 2012). Taken together, these data confirm that *Tbx18^{Cre}* efficiently ablates *Enpp2* and prevents the expression of *Enpp2* mRNA and ENPP2 protein.

Enpp2 in embryonic DP precursors is dispensable for hair follicle morphogenesis

Surprisingly, efficient *Enpp2* ablation during the first wave of hair follicle formation and during later waves at E18.5 (Supplementary Figure 3) did not cause any perturbation of hair follicle development. At E18.5, regular hair follicle morphologies with apparently normal sizes, lengths and distribution were observed in *Enpp2* cKO skin in hematoxylin & eosin staining (Figure 3a). Quantification revealed normal hair follicle numbers of all hair follicle types (Figure 3b). Biochemical marker analysis in *Enpp2* cKO and control E18.5 embryos revealed unchanged expression of DP marker SOX2 and normal Alkaline Phosphatase (AP) activity (Figure 3c). Proliferation of hair follicle epithelial cells, as detected by KI67 immunofluorescence, was comparable in E18.5 cKO and controls (Figure 3d).

By postnatal day P8, we observed normal appearing hair shafts protruding from the skin (Figure 3e) and their lengths were comparable between WT and cKO pups (Figure 3f). Microscopically, hair follicles from all three waves were similar in morphology (Figure 3g) and in length (Figure 3h), and epithelial matrix cell proliferation, assessed by KI67 immunofluorescence, was comparable (Figure 3i). Finally, expression of DP markers SOX2, HHIP and GFRA1 was unaffected in both control and cKO (Figure 3j–l). From these data

we conclude that *Enpp2* is not required in DP precursors and mature DP cells for hair follicle formation and growth, respectively.

Upregulated *LIPH* and *LPA* receptors suggest compensation of *Enpp2* loss

We next wondered whether DP gene expression is affected in dermal condensates in the absence of *Enpp2*, despite the absence of an overt follicle formation phenotype. To be able to isolate and analyze both control and *Enpp2* null DP precursor cells we crossed *Tbx18^{Cre};Enpp2^{fl/fl}* mice with the *R26^{ACTB-mT/mG}* Cre reporter line (Figure 4a). In this line, cells ubiquitously express the cell membrane-bound red fluorescent protein tdTomato (mT) under the control of the actin B promoter (ACTB), but in Cre recombinase expressing cells, mT is replaced by cell membrane-bound GFP (mG) (Grisanti *et al.*, 2012; Muzumdar *et al.*, 2007). Single cells were prepared from E14.5 back skins of Het (*Tbx18^{Cre};Enpp2^{fl/+};R26^{ACTB-mT/mG}*) and cKO (*Tbx18^{Cre};Enpp2^{fl/fl};R26^{ACTB-mT/mG}*) embryos, and mG⁺ cells were isolated by FACS (Figure 4b). Real-time PCR for dermal condensate markers *Sox2* and *Tbx18* confirmed enrichment of DP precursors (Figure 4c, “mG”). Analysis of *Enpp2* mRNA expression levels in control and *Enpp2* cKO cells further validated efficient ablation in cKO DP precursors (Figure 4d). Next we analyzed expression levels of several dermal condensate genes, such as *Sox2*, *Tbx18*, *Sox18*, *Fgf10*, *Bmp4*, *p75* and *Noggin*. As shown in Figure 4e, none of these genes was dramatically altered after ablation of *Enpp2*, which is not surprising given the absence of a hair follicle formation phenotype, although *Fgf10* was significantly increased (1.6 fold), and *Bmp4* and *Noggin* were decreased (3 fold). These data suggest that *Enpp2* expression in dermal condensates is inessential for hair follicle formation, and as such *Enpp2*/LPA signaling may not play a role in this process.

In an alternative possibility, functional compensation through genetic redundancy at the individual gene level or the systems level could potentially explain the absence of a phenotype (Zhang, 2012). Indeed, recent studies suggested that *LIPH*, a Phospholipase A1 (*mPA-PLA1a*), can generate LPA in an alternative pathway (Aoki *et al.*, 2008). Interestingly, mutations in the human *LIPH* gene cause hair loss and hair growth defects (Ali *et al.*, 2007; Kazantseva *et al.*, 2006) and genetic ablation of mouse *LIPH* causes aberrant postnatal hair growth by affecting normal inner root sheath function (Inoue *et al.*, 2011). In either case initial follicle morphogenesis appears unaffected. To test whether *LIPH* is present in embryonic skin, and could bypass the need of *Enpp2* for LPA generation, we analyzed *LIPH* expression by real-time PCR. As shown in Figure 4f, *LIPH* was detectable in both embryonic epidermal and dermal compartments (“Epi”, “Der”). Within the dermis, *LIPH* expression was even higher in E14.5 DP precursors (“GFP^{hi}”) compared to a dermal fraction (“Neg”), both isolated from *Tbx18^{H2BGFP}* embryos as described above (Grisanti *et al.*, 2012). Interestingly, *LIPH* expression was further increased in isolated *Enpp2* cKO dermal condensates cells (Figure 4g, “mG”) suggesting compensatory upregulation, while epidermal *LIPH* was not significantly changed (Figure 4g, “Epi”). These data suggest that *LIPH* is expressed at the right time and the right place to potentially compensate for the absence of *Enpp2* during hair follicle formation. We finally analyzed the expression of LPA receptors in control and *Enpp2* cKO DP precursors and epidermis (Figure 4h). While *Lpar3* expression is not affected in cKO condensates, several receptors were upregulated after

Enpp2 ablation. Only one receptor, *Lpar4*, was upregulated in the epidermis, although *Lpar2* and *Lpar3* expression is already higher by default compared to DP precursors. Among all increased receptor expression in cKO condensates, the most dramatically upregulated receptor was *P2Y5*, also known as *Lpar6* (Figure 4h). Interestingly, homozygous mutations in the human *P2Y5* gene are found in individuals with another congenital hair disorder that is clinically indistinguishable from the one caused by *LIPH* mutations (Pasternack *et al.*, 2008; Shimomura *et al.*, 2008). This suggests that *P2Y5* is the main receptor for LPA produced by *LIPH* and is likely upregulated in dermal condensates in a compensatory effort.

In summary, our data demonstrate that *Enpp2* is specifically expressed in embryonic DP precursors joining the short list of the few known dermal condensate markers. Our robust ablation experiments show that DP-specific loss of *Enpp2* does not affect hair follicle formation. They also suggest that future co-ablation of both LPA producing enzymes or simultaneous ablation of several LPA receptors may be required to reveal the functional role of LPA signaling during hair follicle morphogenesis.

MATERIALS AND METHODS

Mice

Tbx18^{H2BGFP} and *Tbx18^{Cre}* mice were described previously (Cai *et al.*, 2008; Grisanti *et al.*, 2012). *Enpp2* floxed mice (*Enpp2^{fl/fl}*) were kindly provided by Dr. Moolenaar (van Meeteren *et al.*, 2006). *R26^{ACTB-mT/mG}* (Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)Luo/J; (Muzumdar *et al.*, 2007) reporter mice were obtained from Jackson Laboratories (Bar Harbor, ME). Knockout embryos for *Enpp2* were generated by crossing *Tbx18^{Cre};Enpp2^{fl/+}* males with female *Enpp2^{fl/fl}* mice and harvested at E14.5 or later stages as indicated. All animal experiments were performed in accordance with the guidelines and approval of the Institutional Animal Care and Use Committee at Icahn School of Medicine at Mount Sinai.

FACS sorting

Back skins of E14.5 Het (*Tbx18^{Cre};Enpp2^{fl/+};R26^{ACTB-mT/mG}*) and cKO (*Tbx18^{Cre};Enpp2^{fl/fl};R26^{ACTB-mT/mG}*) embryos were processed as described previously (Grisanti *et al.*, 2012). Single cell suspensions were labeled with antibodies against E-Cadherin (Invitrogen) followed by detection with donkey anti-rat APC-conjugated secondary antibodies (Jackson ImmunoResearch). Cell purifications were performed on a FACSAria system equipped with FACS DiVa software (BD Biosciences).

RT-PCR

Total RNA obtained from FACS sorted cells was purified by Absolutely RNA Nanoprep kit (Stratagene), quantified with the NanoDrop spectrophotometer (Thermo Scientific) and reverse transcribed using oligo(dT) primers (Superscript III First-Strand Synthesis System, Invitrogen). Real-time PCR was performed with a LightCycler 480 (Roche) instrument with Lightcycler DNA master SYBR Green I reagents. Differences between samples and controls were calculated based on the $2^{-\text{CP}}$ method and normalized to *Gapdh*. Measurements were performed in duplicate.

***In situ* hybridization and immunofluorescence staining**

The mouse *Enpp2* probe (2,589 bp) for in situ hybridizations was generated from cDNA obtained from Open Biosystems (clone ID 3499038) using the DIG RNA labeling kit (SP6/T7) (Roche, Indianapolis, IN). The *Enpp2* probe specific for the exons 6–7, was generated by PCR amplification of foaling *Enpp2* cDNA using the following primers: Forward, GTAGTCGACGTAGTCCGCCCTCCGTTAATCATCTTCTCTGTG (SalI-*Enpp2_6*), Reverse, TAGCGGCCGCCAGTGGCCAGCGTATACAGATTA (*Enpp2_7*-NotI). The amplified 174bp fragment was subcloned into the pCMV-sport6 vector. In situ hybridization in whole-mount embryos and in sections were performed according to standard protocols as described (Clavel *et al.*, 2012; Olson and Soriano, 2009). Digoxigenin was detected with the substrate 4-Nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate 4-toluidine (NBT/BCIP, Roche). For immunofluorescence, whole-mounted embryos or sections were incubated with antibodies against ENPP2 (goat, Invitrogen), Integrin β 4, Syndecan-1 (rat, BD Pharmingen, San Jose, CA), SOX2 (D-17 goat, Santa Cruz, CA; rabbit, Millipore), KI67 (rabbit, Leica-Microsystems, Novacastra), GFRA1 (goat, Neuromics, Edina, MN), HHIP (goat, R&D, Minneapolis, MN) followed by Rhodamine Red-X conjugated donkey anti-goat or anti-rat and 488 conjugated donkey anti-goat or anti-rat secondary antibodies (Jackson ImmunoResearch). Nuclei were counterstained with DAPI. For detection of Alkaline Phosphatase (AP) activity, NBT/BCIP was used as recommended by the manufacturer's instructions, and nuclei were identified with Fast Red (Roche). Slides were analyzed using a Leica DM5500 (Leica, Wetzlar, Germany) and imaged captured using digital cameras (Leica DFC360FX and DFC340FX) driven by Leica LASAF software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DP dermal papilla

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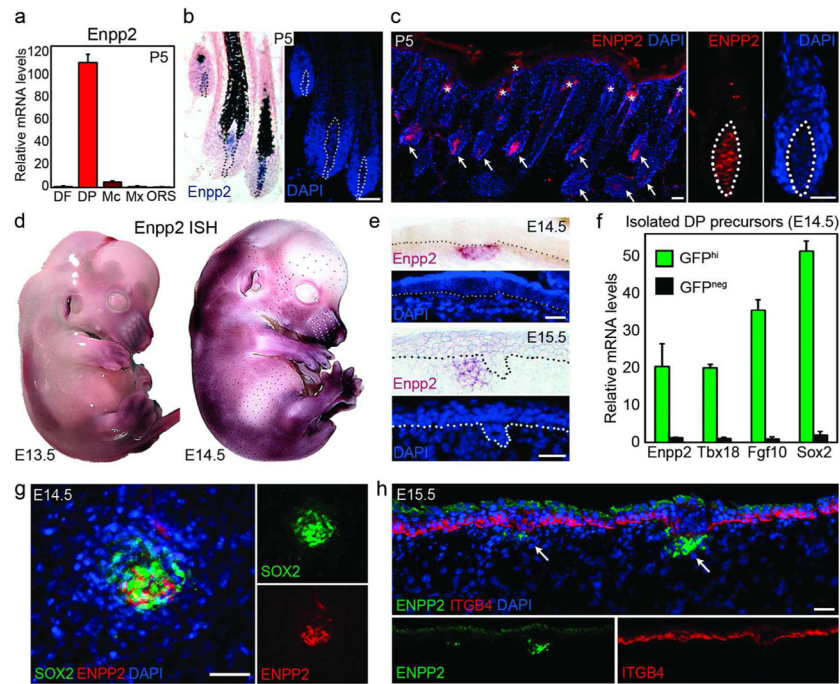


Figure 1. Expression of *Enpp2* in postnatal dermal papilla (DP) and embryonic DP precursors (a) Real-time PCR analysis of *Enpp2* expression in DP and other hair follicle cells isolated by fluorescence activated cell sorting (FACS) from P5 *Lef1-RFP/K14-H2BGFP* skin (n=2). (b) In situ hybridization for *Enpp2* mRNA in P5 back skin section. (c) Immunofluorescence for ENPP2 in P5 back skin section. ENPP2 is expressed in DP cells (arrows). Asterisks mark autofluorescence in hair shafts. (d) Whole-mount *in situ* hybridization for *Enpp2* in E13.5 and E14.5 embryos. (e) Back skin of sectioned embryos at E14.5 and E15.5 show specific *Enpp2* expression in dermal condensates. (f) Real-time PCR analysis of *Enpp2* and other DP genes in FACS isolated DP precursors from E14.5 *Tbx18^{H2BGFP}* embryos (n=2). (g) Whole-mount immunofluorescence for ENPP2 and SOX2 in E14.5 embryo. (h) ENPP2 immunofluorescence staining on back skin section at E15.5. ITGB4 marked the basement membrane. ENPP2 is expressed in DP precursors in dermal condensates (arrows). Scale bars, 25 μ m. Data are mean \pm SD.

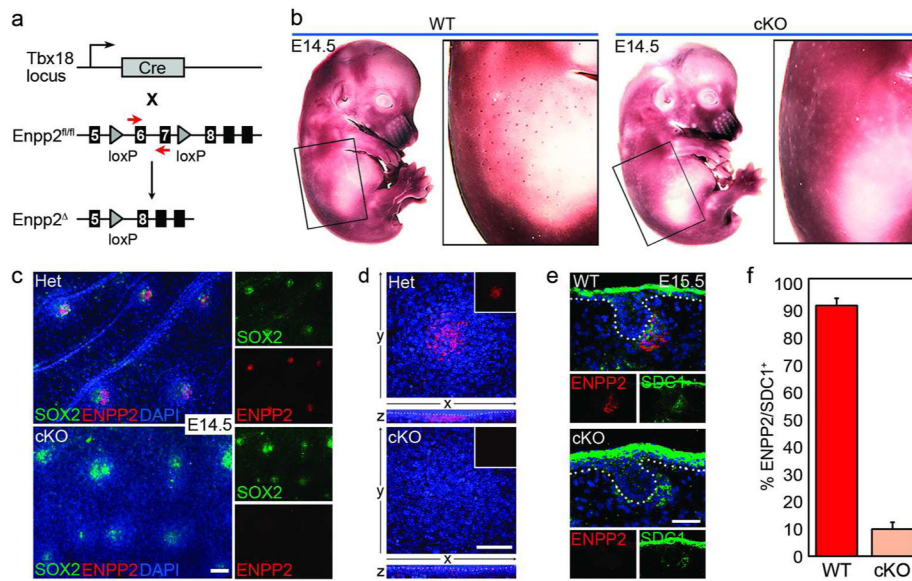


Figure 2. Efficient *Tbx18^{Cre}*-mediated ablation of *Enpp2* in embryonic dermal condensates
(a) Schematic of *Enpp2* ablation in embryonic DP precursors. Red arrows indicate primers for generating riboprobes. **(b)** Whole-mount in situ hybridization of WT (*Enpp2^{fl/fl}*) and cKO (*Tbx18^{Cre};Enpp2^{fl/fl}*) embryos at E14.5. Note absence of *Enpp2* mRNA in cKO. **(c)** Whole-mount immunofluorescence for ENPP2 and SOX2 in Het (*Tbx18^{Cre};Enpp2^{fl/+}*) and cKO (*Tbx18^{Cre};Enpp2^{fl/fl}*) back skin at E14.5 confirms robust ENPP2 protein ablation. **(d)** Confocal imaging and 3D-reconstruction of z-dimension of whole-mount immunofluorescence at E14.5. Note absence of ENPP2 in cKO (bottom). **(e)** ENPP2 immunofluorescence in WT and cKO sections at E15.5. SDC1 marks DP precursors. **(f)** Quantification of ENPP2 and SDC1 double-labeled dermal condensates. Note that the few positive cKO condensates only had a single cell labeled (n=2). Scale bars, 25 μ m. Data are mean \pm SD.

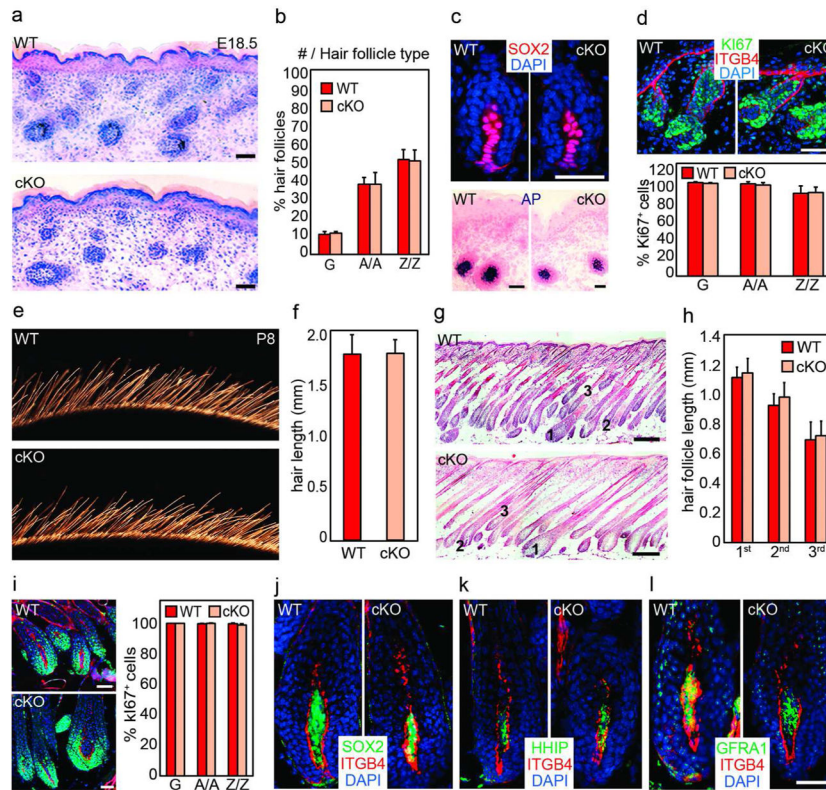


Figure 3. *Enpp2* ablation in embryonic DP precursors is dispensable for hair follicle formation (a) Hematoxylin and eosin staining of WT and cKO back skin at E18.5. (b) Quantification of hair follicle types in *Enpp2* WT and cKO back skin at E18.5 (n=2). (c) Immunofluorescence for DP marker SOX2 and Alkaline Phosphatase (AP) activity at E18.5. (d) Immunofluorescence for proliferation marker KI67 in WT and cKO embryos at E18.5 and quantification of positive cells in all three hair types. (e) Side view of WT and cKO back skin with outgrowing hair shafts at postnatal day P8. (f) Quantification of hair shaft lengths (n=2). (g) Hematoxylin and eosin staining of WT and cKO back skin at P8. (h) Quantification of hair follicle lengths (n=2). (i) KI67 immunofluorescence of back skin at P8 and quantification of proliferating cells (n=2). (j–l) Immunofluorescence for DP markers SOX2 (j), HHIP (k) and GFRA1 (l). Scale bars, 25 μ m (b,c), 50 μ m (i), 200 μ m (g). Data are mean \pm SD.

