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Fzd2 regulates murine hair follicle function and maintenance

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SUMMARY

Fzd2 is required for hair follicle function and maintenance in mice.

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CONFLICT OF INTEREST STATEMENT
The authors have no conflicts of interest to declare.

VERTEBRATE ANIMALS
All mice were crossed and maintained on mixed backgrounds. Mice were housed in AAALAC accredited barrier facilities under standard light-dark cycles, with free access to food and water. Experiments involving mice were reviewed and approved by the Institutional Animal Care and Use Committee of University of Pennsylvania.

Keywords

Hair Biology; Stem Cells; Alopecia; Wnt Signaling

To the Editor:

Canonical Wnt signaling regulates hair follicle development and function in mice and humans (Millar 2002; Andl et al., 2002; Choi et al., 2013; Lien et al., 2014; Heilmann et al., 2013; Xu et al., 2017). Secreted Wnt proteins bind to Frizzled (Fzd) family of G-protein coupled receptors (GPCR), which in turn stabilizes and permits nuclear translocation of β-catenin with subsequent activation of target genes. While several Wnt ligands critical to hair follicle function have been identified, the Fzd receptors involved remain unknown (Reddy et al., 2001; Heilmann et al., 2013; Kandyba & Kobielak, 2014; Xu et al., 2017; Wu et al., 2019). As GPCRs, Fzd receptors represent attractive druggable targets to modulate hair follicle function. Here, we report that Fzd2 regulates function and maintenance of murine hair follicles.

In mice, Fzd2 is the most highly expressed Fzd receptor family member in bulge stem cells and outer root sheath (Joost et al., 2020; Reddy et al, 2004; Sennett et al., 2015). We generated a skin-specific Fzd2 knockout (*Fzd2*-EpKO) by crossing the *Fzd2* floxed allele (Kadzik et al., 2014) with the K5-rtTA tetO-Cre inducible deletion system (Choi et al., 2013). We administered doxycycline chow to induce deletion in mice at postnatal day 1 (P1) for morphogenic deletion (Fig 1a), at P15 to delete during the first hair cycle (Fig 1b), and at ~P50 to delete during second telogen (Fig 1c). Skin samples were collected when mice were between 2–4 months of age. We confirmed deletion of Fzd2 by RNAscope *in situ* hybridization (Fig 1e). Mutant mice exhibited gross hair loss. Histological analysis revealed loss of lower follicular structures (arrowheads indicate remnant hair follicles) and loss of hair follicle stem cells (K15+ cells) (Fig 1a–c). Additional time course analysis of the first hair cycle deletion showed that mutants fail to enter anagen (Supplementary Figure S1). Fzd2 is not highly expressed in the interfollicular epidermis, and we observed minimal epidermal changes in the long-term second telogen deletion model (Fig. 1c) (Joost et al., 2020; Reddy et al, 2004; Sennett et al., 2015). We noted instances of epidermal hyperplasia and skin inflammation during our morphogenic and first hair cycle deletions (Fig. 1a, 1b), but these changes were not evident prior to signs of hair follicle degradation (Supplementary Figure S1, left panels; Supplementary Figure S2, lower panels). Thus, mice lacking epidermal Fzd2 phenocopies the epidermal ß-catenin loss-of-function mutant mouse model (Choi et al., 2013).

We observed that hair loss took longer to develop with second telogen deletion compared to morphogenic and first hair cycle deletion. As follicles can remain in second telogen for weeks and their entry into the next hair cycle is asynchronous, we assessed whether anagen onset accelerated follicular degeneration. Waxing dorsal back skin during second telogen induced anagen-onset and accelerated hair follicle degeneration (Fig 1d, Supplementary Figure S2). Three days post-waxing, control and mutant mice demonstrated similar histological findings. However, while control mouse hair follicles continued into anagen

by 7 days post-waxing, *Fzd2*-EpKO mouse hair follicles did not progress. *Fzd2*-EpKO hair follicles were disorganized by 14 days post-waxing and showed pronounced degeneration by 28 days post-waxing. In contrast, unwaxed skin of 14 days post-waxing *Fzd2*-EpKO mice is comparable to control mice (Supplementary Figure S2; Fig 1f left panel). Whole mount Oil Red O lipid staining also revealed widespread disorganization of the pilosebaceous units as early as 7 days post-waxing in *Fzd2*-EpKO mice compared to control mice (Fig 1f). Disorganized melanocytes (arrowheads) were also observed in *Fzd2*-EpKO skin (Fig 1d, f). Taken together, we concluded that anagen onset promoted hair follicle degeneration in *Fzd2*-EpKO mice.

Next, we identified functional changes in Fzd2-deficient hair follicles that contribute to degeneration. We confirmed loss of K15+ hair follicle stem cells over time (Fig 2a). TUNEL analysis revealed a ~5-fold increase in cell death of hair follicle cells at 3 days post-waxing in *Fzd2*-EpKO compared to control mice, and this increased rate of cell death persisted over time, with some nominal variation between timepoints (Fig 2b). Fzd2-EpKO mice also showed a significant reduction in hair germ cell proliferation by EdU incorporation compared to control mice (Fig 2c, 2d). Bulge cell proliferation showed no difference, possibly due to compensation by *Fzd1* and *Fzd7* which are also expressed in the bulge compartment specifically. *Fzd2*-EpKO follicles failed to differentiate properly, indicated by lack of trichohyalin and cortex cytokeratin expression at 7 days post-waxing (Fig 2e–f). Dermal papilla (DP)-mediated signals are required for hair follicle maintenance. Alkaline phosphatase demonstrated strong DP staining at 14 days post wounding (DPW) even after the follicles have started to degenerate (Fig 2g, middle panel). Widespread DP loss was not observed until 42 DPW (Fig 2g, right panel). Thus, we concluded that Fzd2 is required for hair follicle stem cell proliferation, maintenance, and subsequent differentiation of the hair shaft.

One limitation of our study is that the Fzd2 floxed allele may result in inefficient deletion of the gene (Michalski et al., 2021, pre-print). However, this mouse line has been used to assess Fzd2 function in different organs, and loss of expression was confirmed by multiple methods (Kadzik et al.). We accounted for this possibility by only including mice with reduced Fzd2 transcript expression or the associated phenotype in our analysis, and a more detailed characterization of this allele will be provided in a forthcoming publication.

Our results revealed that the epidermal expression of Fzd2 is required for hair follicle cycling and maintenance. We propose that Fzd2 is the critical receptor of canonical Wnt signaling in murine hair and its deletion largely phenocopies ß-catenin loss-of-function mouse models and supports the finding that canonical Wnt signaling is required for proper hair follicle differentiation (Choi et al., 2013). Thus, we demonstrated that deleting a single Fzd receptor in mice modulated hair follicle cycling and maintenance. Recent analysis in human skin identified *FZD7* as the most highly expressed Fzd receptor in bulge stem cells (Takahashi et al., 2020). Future studies assessing Fzd7 activity in human hair follicle stem cells may provide a novel therapeutic approach to treat human alopecia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Abbreviations:

Fzd	Frizzled
DP	Dermal Papilla
DPW	days past wounding
EpKO	skin-specific knockout

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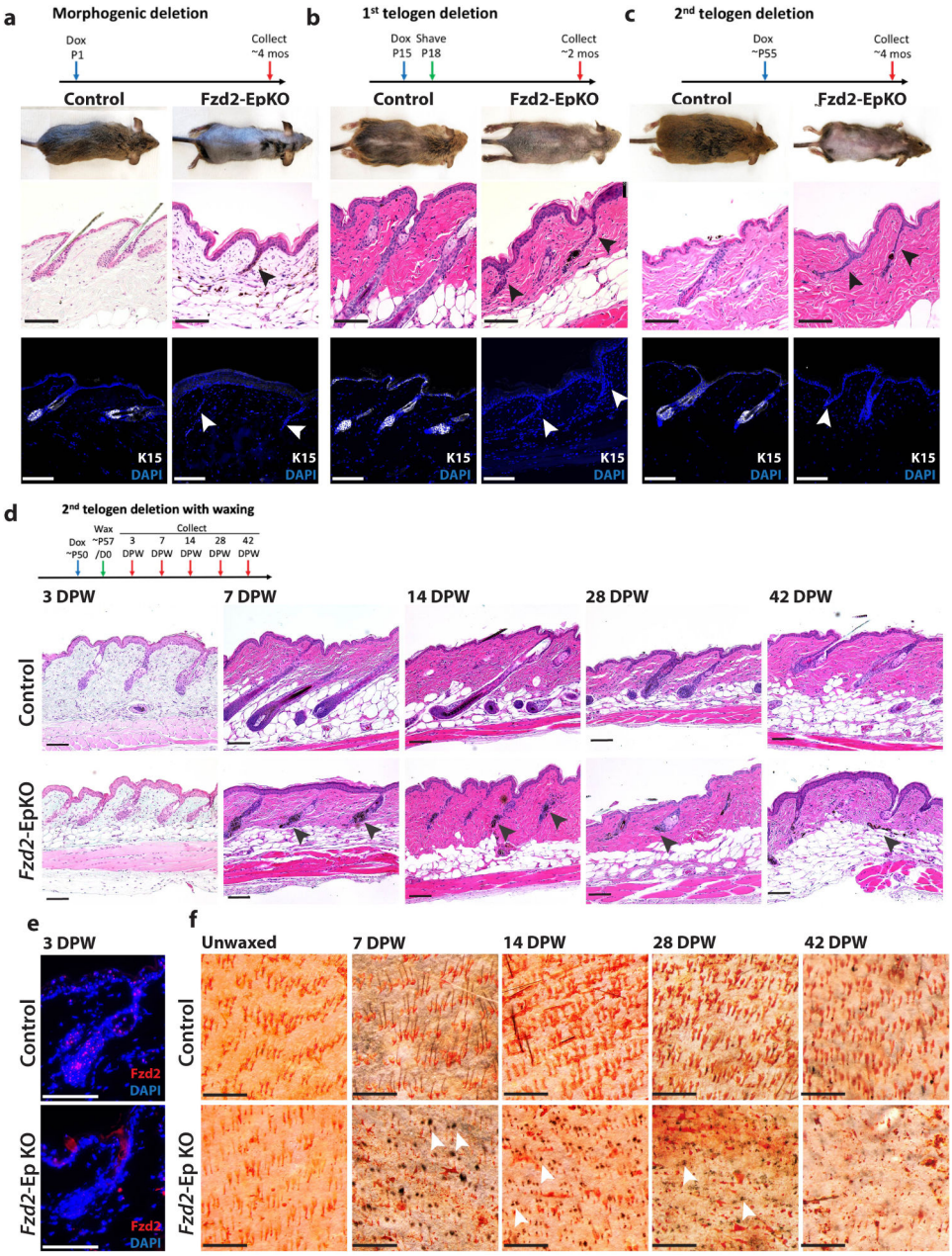

Figure 1: Fzd2 deletion results in widespread degeneration of hair follicles.

(a-c) Mice were treated with Doxycycline for deletion at (a) postnatal day 1 (P1, morphogenic), (b) P15 (first hair cycle), and (c) ~P50 (second telogen). Gross images (top panels) exhibit hair loss in *Fzd2*-EpKO mice. Histology (middle panels) reveals degenerated follicles (arrowheads) and loss of bulge stem cells (lower panels, K15 immunostaining, white) in *Fzd2*-EpKO mice. Nuclei counterstained with DAPI (blue); scale bars, 100 microns. (d) Injury-induced anagen promotes loss of hair follicles over time. Disorganized melanocytes are noted (arrowheads). Scale bars, 100 microns. (e) *In situ* hybridization of *Fzd2* (red) confirms loss in *Fzd2*-EpKO mice. DAPI (blue); scale bars, 100 microns. (f)

Whole-mount Oil Red-O staining demonstrates progressive loss of sebaceous glands and abnormal melanocytic clusters (arrowheads). Scale bars, 500 microns.

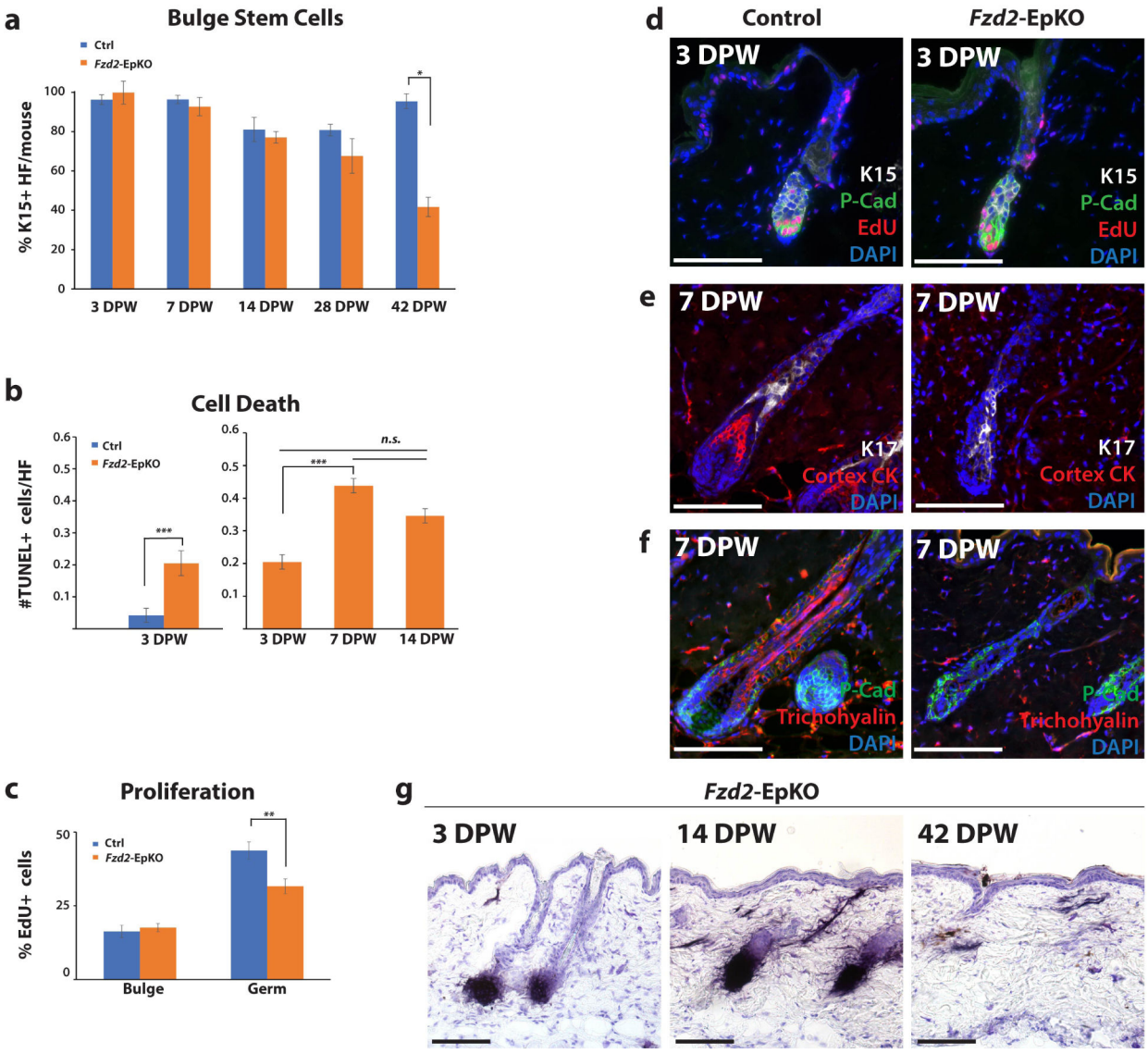

Figure 2: Hair follicle stem cells in mice lacking epidermal Fzd2 exhibit functional changes.

(a) K15 positive hair follicles per mouse: control, *Fzd2*-EpKO n=3 mice/group; * p<0.02. (b) TUNEL positive cells per hair follicle; control 3 days post-wounding (DPW) *n*=80 hair follicles (HF), *Fzd2*-EpKO 3 DPW *n*=171 HF, 7 DPW *n*=86 HF, 14 DPW *n*=53 HF; *** p<0.001, n.s., not significant. (c) Percent EdU positive cells; control *n*=53 HF, *Fzd2*-EpKO *n*=74 HF; ** p<0.005. (d-f) Immunostaining in control and *Fzd2*-EpKO skin. (d) EdU immunostaining (red), K15 (white), P-Cadherin (green), and DAPI counterstain (blue). (e) K17 (white), cortex cytokeratin (red), and DAPI counterstain (blue). (f) P-cadherin (green), trichohyalin (red), and DAPI counterstain (blue). (g) Alkaline phosphatase staining for dermal papilla, with hematoxylin counter stain. Data presented are means ± SEM. Scale bars, 100 microns.