Frizzled6 Deficiency Disrupts the Differentiation Process of Nail Development

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Nails protect the soft tissue of the tips of digits. The molecular mechanism of nail (and claw) development is largely unknown, but we have recently identified a Wnt receptor gene, *Frizzled6* (*Fzd6*), that is mutated in a human autosomal-recessive nail dysplasia. To investigate the action of *Fzd6* in claw development at the molecular level, we compared gene expression profiles of digit tips of wild-type and *Fzd6*$^{-/-}$ mice, and showed that Fzd6 regulates the transcription of a striking number of epidermal differentiation–related genes. Sixty-three genes encoding keratins (Krts), keratin-associated proteins, and transglutaminases (Tgms) and their substrates were significantly downregulated in the knockout mice. Among them, four hard Krts, Krt86, Krt81, Krt34, and Krt31; two epithelial Krts, Krt6a and Krt6b; and Tgm 1 were already known to be involved in nail abnormalities when dysregulated. Immunohistochemical studies revealed decreased expression of Krt86, Krt6b, and involucrin in the epidermal portion of the claw field in the knockout embryos. We further showed that Dkk4, a Wnt antagonist, was significantly downregulated in *Fzd6*$^{-/-}$ mice along with Wnt, Bmp, and Hh family genes; and *Dkk4* transgenic mice showed a subtly but appreciably modified claw phenotype. Thus, Fzd6-mediated Wnt signaling likely regulates the overall differentiation process of nail/claw formation.

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INTRODUCTION

Interactions between the epidermis and underlying mesenchyme drive the development of skin and its appendages, including teeth, hair, and nails/claws. The developmental mechanisms are highly conserved among species (Chuong *et al.*, 2001). Primordial nails/claws start to appear on the dorsal surface of the developing distal digits of mouse limbs at embryonic day 14–15 (E14-15), and morphogenesis is similar in primates and rodents (Hamrick, 2001). The nail/claw fold extends from the epidermis and folds inward to cover the proximal nail plate. The nail fold is succeeded by the nail matrix, which contains proliferating keratinocytes. Keratinocytes dorsal to the matrix start to express epithelial keratins (Krts) and eventually undergo apoptosis, depositing a cornified structure on the nail plate (Figure 1a). Krt1 and Krt10 are expressed during the initial stages of keratinization and are later replaced by a network of Krt5 and Krt14 that assembles into intermediate filaments and contribute to the cytoskeleton of epithelial cells.

Krts constitute a major component of the cytoskeleton of the differentiated nail plate, and consequently mutations in the corresponding genes can perturb nail formation (Chamcheu *et al.*, 2011; McLean and Moore, 2011). As development continues, cells around the nail matrix migrate into the spinous layer, stop dividing, and begin to synthesize a new set of structural proteins and enzymes characteristic of the nail plate. Additional Krts expressed in the nail matrix include epithelial Krts, Krt6A and Krt17, and hard Krts, Krt31, Krt33B, Krt34, Krt39, Krt81, Krt85, and Krt86 (Rice *et al.*, 2010; Barthélemy *et al.*, 2012). Krt filaments aggregate in the presence of filaggrin, and a series of other proteins, such as involucrin, loricrin, and proline-rich proteins, are synthesized and cross-linked by transglutaminases (Tgms). This reinforces the cornified envelope under the membrane of terminally differentiated keratinocytes (corneocytes). Nail plate formation then ensues.

Although the precise molecular signatures and branch points and their timing for nail development remain unclear, regulation of ectodermal appendage formation is partially understood, and is clearly dependent on multiple signaling pathways (Fuchs, 2008). Induction of the nail placode is signaled from the underlying mesenchyme to the overlying epidermis. At this stage, Wnt signaling has a major role, with a

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Abbreviations: Bmp2K, Bmp2-inducible kinase; Fzd6, Frizzled6; E, embryonic day; Krt, keratin; Krtap, keratin-associated protein; Lces, late cornified envelope protein; Sprr, small proline-rich protein; Tgm, transglutaminase

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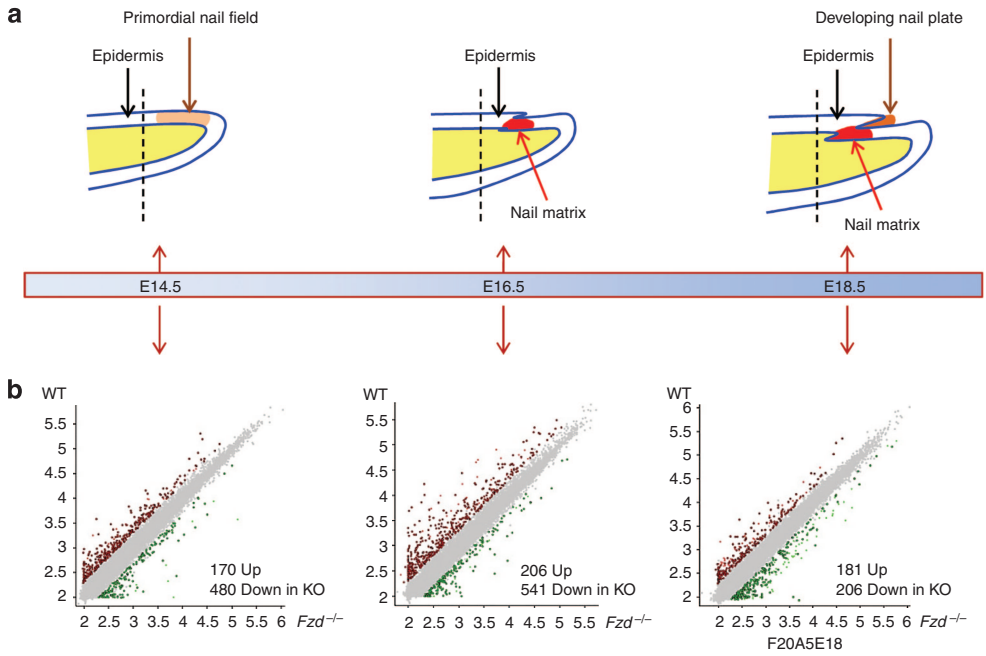

Figure 1. Expression profiling of mouse digit tips during claw development. (**a**) Schematic representation of early-stage claw development. A primordial claw/nail field is formed on the digit tips in mice at around embryonic day 14.5 (E14.5). Primitive nail matrix is discernible at E16.5, and nail matrix starts to form nail plate at around E18.5. Broken lines indicate excised region for RNA collection. (**b**) Scatter plots show number of genes differentially expressed in wild-type (WT) and *Fzd6*$^{-/-}$ digit tips at E14.5, E16.5, and E18.5. KO, knockout.

system of autocrine and paracrine pathways regulated by several feedback loops (Fuchs, 2008; Widelitz, 2008). The importance of Wnt signaling for the formation of nails has been proven from molecular studies on specific congenital human disorders (Hill *et al.*, 2006; Duverger and Morasso, 2008). Autosomal-recessive anonychia or hyponychia has been reported to be caused by mutations in the Frizzled agonist *R-spondin 4* (*RSPO4*) gene (Bergmann *et al.*, 2006; Blaydon *et al.*, 2006), and we have recently reported mutations in the *FZD6* gene in a severe form of isolated autosomal-recessive nail dysplasia (Fröjmark *et al.*, 2011). In addition, mutations in the *WNT10A* gene are associated with odontoonychodermal dysplasia (Adaimy *et al.*, 2007; Nawaz *et al.*, 2009), and mutations in the WNT-associated transcription factors *LMX1B* and *MSX1* cause Nail-Patella and Witkop syndrome, respectively (Dreyer *et al.*, 1998; Jumlongras *et al.*, 2001).

To further define the molecular architecture of genes important for nail/claw formation, we carried out gene expression profiling in a mouse model with RNAs from wild-type and *Fzd6*$^{-/-}$ digit tips at three embryonic stages, E14.5, E16.5, and E18.5, times at which early nail development is evident histologically. The *Fzd6*$^{-/-}$ mouse was originally shown to have altered hair patterning, implying that Fzd6 is involved in a planar polarity system of biological structures (Guo *et al.*, 2004; Wang *et al.*, 2010). Subsequent studies showed that the *Fzd6*-ablated mouse is a model for congenital claw dysplasia (Fröjmark *et al.*, 2011). Here we show that the *Fzd6*-ablated mouse embryos show dysregulation of many genes required for epidermal differentiation. Striking abnormalities were observed in the expression of genes encoding proteins of the cornified envelope and Krts that are critical for claw/nail development and claw/nail plate formation. The results thus clarify a range of molecular mechanisms mediated by Fzd6 in normal claw/nail development in distal digits.

RESULTS

Expression profiling reveals genes regulated by Fzd6 in developing claw

To infer candidate genes regulated by Fzd6-mediated Wnt signaling during claw development, we carried out genome-wide gene expression profiling with RNAs from digital tips of wild-type and *Fzd6*$^{-/-}$ mouse embryos at E14.5, E16.5, and E18.5 (Figure 1a). As expected, biological triplicates of hybridization were grouped with their genotypes in a hierarchical clustering dendrogram (Supplementary Figure S1a online), and we set criteria for significantly affected genes as log-intensity $\geqslant$2.0, false discovery rate $\leqslant$0.05, and fold-difference $\geqslant$2.0. analysis of variance revealed 650 genes as significantly affected in the knockout embryos at E14.5, 747 genes at E16.5, and 387 genes at E18.5 (Figure 1b). The full list of significantly affected genes is presented in Supplementary Table S1 online. Differentially expressed genes were validated using quantitative real-time reverse-transcriptase–PCR for the selected transcripts *Fzd6, Ivl, Hrnr,* and *Rptn*. Downregulation of *Ivl* ($P=0.0033$) and *Hrnr* ($P=0.0026$) was confirmed and the expression of *Rptn* was reduced, although not significantly ($P=0.099$) at E16 (Supplementary Figure S2 online). Consistent with array data, *Ivl, Hrnr,* and *Rptn* were not differentially

expressed at E14 and E18 (data not shown). Reduced expression of *Fzd6* was confirmed at all time points ($P<0.01$), as expected (Supplementary Figure S2 online).

Striking numbers of epidermal differentiation marker genes were significantly affected in *Fzd6*$^{-/-}$ digit tips from early developmental stages

As an overall index of expression profiles, we carried out principal component analysis (Supplementary Figure S1b online). Expression patterns of wild-type and *Fzd6*$^{-/-}$ mice changed progressively during development (arrows in Supplementary Figure S1b online). We found a large number of epidermal differentiation marker genes clustered downward in the knockout along principal component 1, consistent with their positive induction/regulation by Fzd6. A smaller group of additional ''Fzd6-dependent'' epidermal differentiation genes were seen with a somewhat different expression pattern along principal component 2 (Supplementary Figure S1b online). The regulated epidermal differentiation genes encoded many Krts (clustered on chromosomes 11 and 15); keratin-associated proteins (Krtaps, clustered on chromosome 11 and 16); and Tgms and their substrates, including late cornified envelope proteins (Lces, clustered on chromosome 3); S100a calcium-binding proteins (clustered on chromosome 3); small proline-rich proteins (Sprrs, clustered on chromosome 3); and trichohyalin, loricrin, involucrin, and filaggrin (clustered on chromosome 3). We next analyzed these classes further.

Krt and Krtaps genes significantly affected in *Fzd6*$^{-/-}$ mice

Krts can be classified into epithelial Krts (Krt1-24, 76-80), hair follicle-specific epithelial Krts (Krt25-28, 71-75), and hard Krts expressed in hair shafts and nails (Krt31-40, 81-86; Moll *et al.*, 2008).

By statistical analysis, we found eight hard (nail/hair) Krts significantly downregulated in *Fzd6*$^{-/-}$ mice (Figure 2a): *Krt84* and *Krt81* at E14.5; *Krt86, Krt34, Krt33b, Krt32,* and *Krt31* at E16.5; and *Krt32* at E18.5. Immunohistochemical staining revealed Krt86 expression in the suprabasal layers of epidermis in the dorsal digit tips in wild-type mice that was significantly reduced in the *Fzd6*$^{-/-}$ embryos at E16.5 (Figure 3, Krt86). Among the downregulated Krts, Krt86, Krt81, Krt34, and Krt31 (Figure 2b) have been reported to be expressed in the nail matrix, the region producing the actual nail plate (Perrin *et al.*, 2004). Concomitantly, five Krtap genes, *Krtap13-1, Krtap13, Krtap8-1, Krtap3-3,* and *Krtap3-1,* were sharply downregulated in the knockout mice at E16.5 (Figure 2a). Krtaps are known to cross-link with hard Krts to form rigid structures in hair (Rogers *et al.*, 2006).

In addition, 16 epithelial Krts were also significantly downregulated in the knockout mice (Figure 2a). Among them, *Krt6a* and *Krt6b* are mutated in the autosomal dominant pachyonychia congenita disorder, clinically characterized by hypertrophic nail dystrophy, plantar keratoderma, and epidermal cysts (McLean and Moore, 2011). *Krt6a* was significantly downregulated in the knockout mice at E14.5 and E18.5, whereas *Krt6b* was downregulated at E16.5 (Figure 2a). Immunohistochemical staining with Krt6b antibody on wild-type digits at E16.5 showed strong staining mainly in the suprabasal layers of epidermis, including squamous and granular layers and stratum corneum of the nail field, but *Fzd6*$^{-/-}$ mice showed markedly reduced staining confined mainly to the stratum corneum (Figure 3, Krt6b). Notably, *Krt79* and *Krt77* were significantly downregulated in knockout mice at E16.5, but then upregulated at E18.5 (Figure 2a).

a

Category	WT/KO	E14.5	E16.5	E18.5
Krt genes	Dn	*Krt84 (3.9)*, ***Krt81 (2.1)***, *Krt19 (3.0)*, ***Krt10 (4.1)***, ***Krt6a (3.8)***, *Krt4 (15.0)*, *Krt1 (4.0)*, *Krtdap (2.5)*	***Krt86 (5.4)***, *Krt79 (2.2)*, *Krt78 (6.7)*, *Krt77 (3.2)*, ***Krt34 (8.4)***, *Krt33b (24.4)*, *Krt32 (2.1)*, ***Krt31 (5.1)***, *Krt23 (3.0)*, *Krt13 (4.7)*, *Krt12 (9.9)*, *Krt9 (3.4)*, *Krt7 (2.5)*, ***Krt6b (4.3)***, *Krt1 (2.3)*	*Krt32 (2.0)*, *Krt13 (3.0)*, *Krt9 (2.7)*, *Krt7 (2.4)*, ***Krt6a (2.0)***
	Up	*Krt42 (25.9)*	*Krt42 (17.8)*, *Krt18 (2.5)*	*Krt79 (3.0)*, *Krt77 (2.3)*, *Krt28 (2.2)*
Krtap genes	Dn		*Krtap13–1 (10.0)*, *Krtap13 (7.5)*, *Krtap8–1 (3.5)*, *Krtao3–3 (4.5)*, *Krtap3–1 (5.2)*	

b

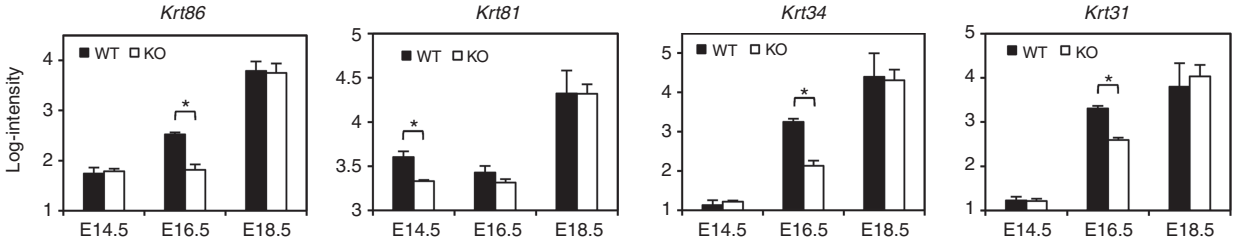

Figure 2. Keratin (Krt) and keratin-associated protein (Krtap) genes were significantly affected in *Fzd6*$^{-/-}$ digit tips. (**a**) Twenty-nine hard and epithelial krts and five Krtap genes were affected in *Fzd6*$^{-/-}$ digit tips during claw development, most of them were downregulated (Dn). Up, upregulated. Bolded genes are known genes expressed in nails or known to be responsible for nail abnormalities. Numbers in parentheses represent fold differences. (**b**) Examples of the time course of expression for four affected hard Krt genes known to be involved in nail formation are shown. *Krt86, 34,* and *31* were significantly Dn in the knockout (KO) mice at embryonic day 16.5 (E16.5), whereas Krt81 was at E14.5. WT, wild type. *$P<0.05$.

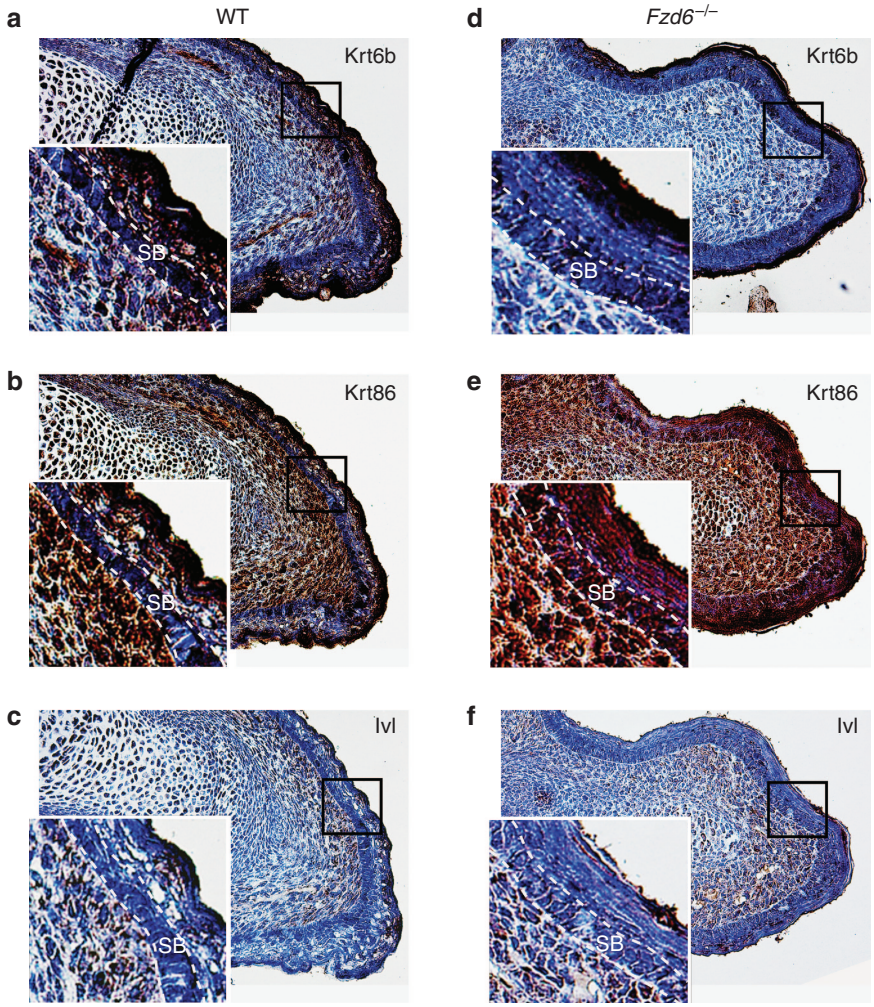

Figure 3. Immunohistochemical staining examples of the hard keratin Krt86, the epithelial keratin Krt6b, and the transglutaminase substrate Ivl in planar sections of digit tips embryonic day 16.5 (E16.5). (**a–c**) Krt86, Krt6b, and Ivl proteins are expressed in the suprabasal layers of the wild-type (WT) digit tips. (**d–f**) Only background levels were detected for the corresponding proteins in *Fzd6*$^{-/-}$ embryos. Hematoxylin (blue color) was used as background staining. Boxes indicate enlarged areas. SB, stratum basale.

By contrast, the third Krt class, hair follicle-specific epithelial Krts (with exception of mild upregulation of Krt28 at E18.5), was not affected in the knockout mice, presumably because claws/nails lack epithelial structures corresponding to the inner root sheath of hair follicles.

Hard Krts were previously shown to be direct transcriptional targets of Wnt signaling (Zhou *et al.*, 1995). The sharp downregulation of a vast number of hard Krts, epithelial Krts, and Krtaps in the *Fzd6*-targeted mice strongly suggests that Fzd6-mediated Wnt signaling regulates formation of the cytoskeleton in developing nails.

Genes significantly dysregulated in *Fzd6*$^{-/-}$ mice that are required to form the terminally differentiated cornified cell envelope

Another general finding from expression profiling was the downregulation of expression of genes encoding Tgms and their cross-linking substrates for the cornified cell envelope formation, the idiosyncratic structure in terminal differentiation (Figure 4a). The cornified envelope is an insoluble and mechanically resistant membrane structure of terminally differentiated keratinocytes (Candi *et al.*, 2005). Three Tgms, Tgm1, 3, and 5, which regulate cornified envelope formation, were sharply downregulated in the *Fzd6*$^{-/-}$ mice at E14.5 and E16.5 (Figure 4a). Among them, Tgm 1 had previously been shown to regulate cornified envelope formation in nail plates (Rice *et al.*, 2003), and mutation in the gene causes lamellar ichthyosis, affecting skin and sometimes nails (Huber *et al.*, 1995). Genes encoding Tgm substrates, including involucrin, trichohyalin, loricrin; 12 Lce proteins; 6 Sprr genes; and four S100a calcium-binding protein genes, were also significantly downregulated in the knockout mice (Figure 4a). Immunohistochemical staining of involucrin indicates that it is almost absent in suprabasal layers of the claw

a

Category	WT/KO	E14.5	E16.5	E18.5
Transglutaminases	Dn	*Tgm3 (3.6)*, ***Tgm1 (3.5)***	*Tgm5 (5.8), Tgm3 (2.1)*	
Late cornified envelope proteins	Dn		*Lce3b (3.7), Lce1m (13.9), Lce1l (81.4), Lce1i (84.3), Lce1h (24.7), Lce1f (5.0), Lce1e (10.6), Lce1d (8.0), Lce1c (111.5), Lce1b (172.4), Lce1a2 (36.6), Lce1a1 (85.4)*	
	Up			*Lce1g (2.4)*
S100 calcium-binding proteins	Dn	*S100a16 (2.7), S100a14 (4.2)*	*S100a7a (8.9), S100a6 (2.2)*	
Small proline-rich proteins	Dn	*Sprr1a (2.7)*	*Sprr3 (2.7), Sprr2h (2.3), Sprr2e (2.0), Sprr2d (3.4), Sprr2a (2.6), Sprr1a (2.1)*	*Sprr2a (2.0)*
Other Tgm substrates	Dn	*Tchh (4.5), Lor (2.7), Csta (2.3)*	*Ivl (7.1), Flg (2.9)*	

b

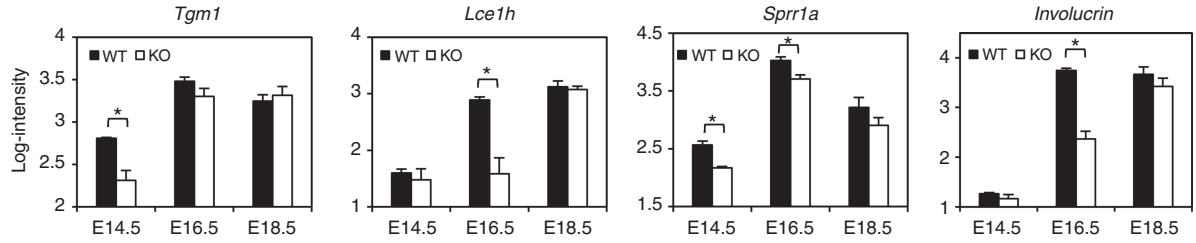

Figure 4. Transglutaminases (Tgms) and their substrates were significantly affected in *Fzd6*$^{-/-}$ mice. (**a**) *Tgms 1, 3,* and *5* were significantly downregulated in *Fzd6*$^{-/-}$ mice along with their substrates including 12 *Lces,* 4 *S100s,* 6 *Sprrs,* and *Tchh, Lor, Csta, Ivl, Flg.* Dn, downregulated; Up, upregulated. Bolded gene (*Tgm1*) is known to be responsible for nail abnormalities. Numbers in parentheses indicate fold differences. (**b**) Expression levels of *Tgm1* and its substrates *Lce1h, Sprr1a,* and *Involucrin* were shown. Note a time lag of expression pattern, *Tgm1* was significantly downregulated at embryonic day 14.5 (E14.5), *Lce1h* was at E16.5, *Sprr1a* was at E14.5 and E16.5, and *Involucrin* at E16.5. KO, knockout; WT, wild type. *$P<0.05$.

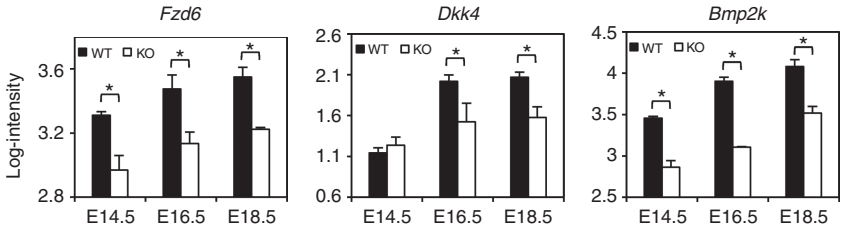

Figure 5. Signaling proteins downregulated in the *Fzd6*$^{-/-}$ mice are shown. Expression patterns of *Fzd6, Dkk4,* and *Bmp2k. Dkk4* was significantly downregulated in the knockout (KO) mice at embryonic day 16.5 (E16.5) and E18.5, but not at E14.5. Like Fzd6, Bmp2k was downregulated at all three time points. WT, wild type. *$P<0.05$.

field in *Fzd6*$^{-/-}$ mice compared with wild-type littermates (Figure 3, Ivl). Figure 4b shows expression patterns of *Tgm1* and several of its substrates (*Lce1h, Sprr1a,* and *involucrin*) in wild-type and knockout mice at early developmental stages (Figure 4b).

Downregulation of Tgms and their substrates in the knockout mice suggests that *Fzd6* affects formation of the terminally differentiated cornified envelope. Abnormal expression of Krts and cornified envelope components in *Fzd6*$^{-/-}$ mice are consistent with phenotypic observations (Fröjmark *et al.*, 2011).

Additional genes significantly altered in digit tips of *Fzd6*$^{-/-}$ mice

In addition to affecting an unusually wide range of epidermal differentiation-associated genes, ablation of *Fzd6*$^{-/-}$ also notably downregulated some signaling proteins known to control skin appendage development and differentiation. The additionally affected genes included members of Wnt, Bmp, Fgf, Tgfα, and Dhh pathways (Supplementary Table S2 online). Among them, expression changes for *Bmp2K* (Bmp2-inducible kinase) in the knockout mice were notable. Similar to *Fzd6, Bmp2K* was significantly downregulated in the knockout mice at all three time points analyzed (Figure 5). The Bmp2-induced kinase contains a nuclear localization domain and has been shown to be involved in Bmp2-induced osteoblast differentiation (Liu *et al.*, 2009). In addition to *Bmp2K, Bmp8a, Wnt2, Wnt2b, Fgf2, Fgf7,* and *Dhh* were all significantly less expressed in the knockout mice at E14.5 (Supplementary Table S2 online).

At E16.5, *Wnt10b, Dkk4,* and *Tgfα* were significantly downregulated; and at E18.5, *Dkk4* and *Fzd10* were less expressed. Dkk4 is an antagonist of Wnt pathway; and in an initial experiment to look at interactive modulation of action of factors within the Wnt system, we examined claw formation in wild-type mice bearing a *Dkk4* transgene. In previous work, although fundamental hair formation occurred unchanged in *Dkk4* transgenic mice in wild-type background, hair subtypes were altered (Cui *et al.*, 2010). For claws as well,

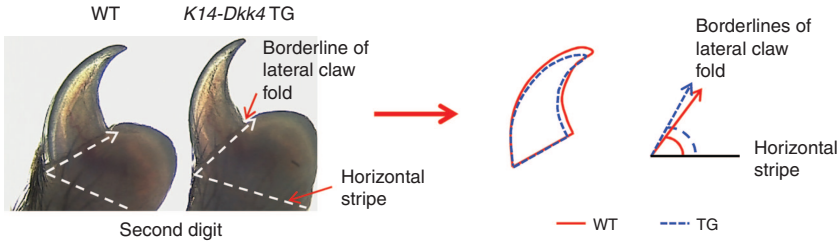

Figure 6. Claw phenotype of *K14-Dkk4* transgenic mice. Second digit tips of wild-type (WT) and *Dkk4* transgenic mice (TG) are shown. Claw is slightly thinner and the angle between lateral claw fold and horizontal skin stripes is larger in the transgenic mice compared with WT littermates.

a modulatory effect of a *Dkk4* transgene was seen at the maturation stage of development. The effect was subtle but reproducible, with an appreciable change in the thickness and angle of claws, more easily seen on the three central digits of the hind paws (in Figure 6, a claw from a transgenic mouse is sharper and is oriented 6–7° more vertically than wild-type control claws). Overall, 9 of 63 epidermal differentiation marker genes, including *Krt86, Krt34, Krt6a,* and *Krt6b,* which were downregulated in *Fzd6*$^{-/-}$ claws, were also significantly downregulated in *Dkk4* transgenic hair follicles (Supplementary Table S3 online and seeFigure S1 online in Cui *et al.*, 2010). Thus, Dkk4 may interact with a specific Wnt protein regulated by Fzd6.

Finally, we also assessed upregulated genes in *Fzd6*$^{-/-}$ mice. In contrast to a wide range of differentiation marker and signal genes downregulated in the knockout mice, upregulated genes did not cluster by function. However, expression of a small number of differentiation-related genes and genes encoding signal proteins was upregulated. The upregulated genes include *Krt18, Krt28, Krt42, Krt77, Krt79, Lce1g* (Figures 2a and 4a), and *Lrp6, Egfbp2, Wisp2* (Supplementary Table S2 online). These might represent secondary feedback effects of downregulation of the cohort of differentiation-related genes and signaling proteins.

DISCUSSION

The gene expression profile at the site and time of early claw development showed that the lack of *Fzd6* expression, associated with nail dysplasia in humans, markedly affects overall expression patterns of Krts and other proteins involved in cornified envelope formation. Our data supports a model in which the Wnt/Fzd signaling pathway, specifically involving Fzd6, has a key role in claw/nail formation through the activation of required transcription units.

We note considerable overlap in the expression of hard Krts and Krtaps in developing claw compared with shaft composition in hair follicles. They include *Krt31, Krt33b, Krt34, Krt81, Krt86, Krtap3-1,* and *Krtap3-3* (Barthélemy *et al.*, 2012). We also identified a broad spectrum of epithelial Krts in the developing claw, including *Krt1, Krt4, Krt6a, Krt9, Krt10, Krt13, Krt19,* and *Krt23*, which are not found in the mature claw plate (Rice *et al.*, 2010). In addition to these major differences in the composition of the claw plate and hair shaft, several transcripts encoding structural proteins appeared more strongly or exclusively associated with developing claws. They included *Krt6a, Krt6b, Krt7, Krt12, Krt13, Krt18, Krt23, Krt28, Krt42, Krt77, Krt78, Krtap8-1,* and *Krtap13*. These findings thus point to distinctive and characteristic Krt profiles during claw morphogenesis compared with formation of the claw plate.

Hallmarks of claw formation, including several Krts and Krtaps dysregulated in the *Fzd6*$^{-/-}$ mice, are consistent with previous findings that Wnt signaling regulates hard Krt expression (Zhou *et al.*, 1995). The pattern of dysregulated Krts (and Tgms and their substrates; see below) varied between time points, with the largest number at E16.5, the peak time of expression of *Fzd6* in digit tips during normal development (Fröjmark *et al.*, 2011; Figure 2a). This finding may suggest a requirement for spatiotemporal action of epidermal differentiation proteins and related enzymes in the early developmental stage of claw plate formation, with a few genes still affected at E18.5.

As another indication of temporal succession, *Krt6a* and *Krt6b,* mutations in which are associated with pachonychia congenita, were downregulated, but *Krt6a* was significantly downregulated at E14.5 and E18.5, whereas *Krt6b* was significantly downregulated at E16.5. Similar to *Krt6b, Krt86,* the major Krt of the claw plate, showed a ''window'' of downregulation at E16.5 in the *Fzd6*$^{-/-}$ animals. Mutations in *Krt86* are associated with monilethrix (fragile hair; van Steensel *et al.*, 2005), but this has not been reported in patients with *FZD6* mutations. Thus, the reduced *Krt86* expression does not seem to affect hair shaft formation significantly.

The cornification process has already been specified to involve a large number of structural proteins and enzymes expressed in sequential order (Candi *et al.*, 2005), and interestingly, the gene expression analysis in wild-type mice at E14.5–E18.5 showed considerable overlap with the pattern from skin as well as other epidermal appendages. But idiosyncratic to the digit tips, three key enzymes for the assembly of cornified cell envelope in stratum corneum, Tgms 1, 3, and 5, were sharply downregulated in *Fzd6* mutant mice. Similarly, a series of Tgm substrate genes, including those for structural proteins trichohyalin, involucrin, loricrin, and several Sprrs and Lces, showed significantly reduced expression, again consistent with the dysplastic nail formation in *Fzd6*$^{-/-}$ mice.

In addition, the *Fzd6*-ablated mouse clarifies critical and specific pathways for claw formation. More than 500 genes were significantly dysregulated at the three time points tested

in digit tips of *Fzd6*$^{-/-}$ compared with wild-type embryos. Consistent with observations during distal limb formation (Geetha-Loganathan *et al.*, 2008), several Wnt signaling molecules were found downregulated. Notably, Dkk4, a Wnt antagonist, has been shown to be direct target of canonical Wnt signaling (Bazzi *et al.*, 2007), suggesting a possible feedback regulation of Wnt signaling for nail/claw development mediated by Fzd6 and Dkk4. Figure 6 shows that overexpression of *Dkk4* in wild-type mice modified claw development slightly; and we found nine differentiation markers downregulated in *Fzd6* knockout mice that are also downregulated in *Dkk4* transgenic mice. Thus, Dkk4 may inhibit expression of a specific Wnt or Wnt subset that is positively controlled by Fzd6.

In further clues to the regulatory network, the expression of several other signaling proteins was affected. Notably, *Bmp2k* was downregulated at all three time points suggesting its regulation by Fzd6-mediated signaling. The expression of *Bmp2* from mesenchymal cells has earlier been shown to be important in the differentiation of epidermal appendages (Rendl *et al.*, 2008) and for patterning of distal limbs (Maatouk *et al.*, 2009).

Overall, however, the developmental program of claws thus diverges sharply from that of other well-studied skin appendages. In particular, in contrast to hair, teeth, and sweat glands, ectodysplasin is not obviously involved (Cui and Schlessinger, 2006), and the plethora of epithelial Krts made is largely distinct from those seen in hair follicles. In fact, *Fzd6*$^{-/-}$ mice and humans with homozygous *FZD6* mutations show normal structures of epidermis and complete formation of hair follicles. Further increasing the complexity of the regulatory network, Wnt signaling has been shown to be involved in epigenetic regulation of its target genes (Sierra *et al.*, 2006), a mechanism that is likely operative in the coordinated regulation of epidermal differentiation genes. It is suggestive that four clusters of implicated cornified envelop genes (Lces, Sprrs, S100s, and trichohyalin, loricrin, filaggrin) are all nearby on chromosome 3, raising the speculative possibility that they are jointly regulated by a single Wnt-dependent epigenetic alteration. The results imply that protagonist regulators in claw/nail assembly act successively in a regulatory cascade initiated by Wnt/Fzd6; the successive waves of expression (Figures 2, 4, and 5) provide an entrée to determine how the concerted action of signaling factors and epigenetic regulation establishes the pattern of gene action.

MATERIALS AND METHODS

Mouse strain, timed mating, and gene expression profiling

Heterozygous *Fzd6*$^{+/-}$ mice (kindly provided by Dr J Nathans) were crossed to generate *Fzd6*$^{+/-}$, *Fzd6*$^{-/-}$, and wild-type offspring. Timed matings were set up to harvest embryos at E14.5, E16.5, and E18.5. The morning after mating was designated as E0.5. Three middle distal phalanges from each limb were excised and immediately frozen on dry ice. Nine digital tips from three limbs of the same embryo were pooled for one replicate, and stored at $-80\,^{\circ}C$ freezer until use. Three pooled biological replicates were prepared for each genotype at each embryonic time point. Tails were collected to isolate genomic DNAs, and genotyping was done as described previously (Fröjmark *et al.*, 2011). Animal experiments were approved by the Ethical Committee for Animal Trials, Uppsala County Court, Uppsala, Sweden.

Total RNAs were isolated from each of the pooled digit tips using the Trizol Reagent (Life Technologies, Carlsbad, CA). Total RNA (2.5 μg for each replicate) was labeled with Cy3-CTP using a Quick Amp Labeling Kit (Agilent, Santa Clara, CA) to generate the experimental target, and the Cy5-CTP-labeled reference target was produced from the mixture of the Stratagene Universal Mouse Reference RNA (composed of total RNAs from 11 mouse cell lines) and 129ES cellular RNA. Labeled targets were purified using an RNeasy Mini Kit (Qiagen, Hilden, Germany), quantified on a NanoDrop scanning spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific, Waltham, MA). An experimental target was then mixed with the reference target and hybridized to the NIA Mouse 44K Microarray v3.0 (whole-genome 60-mer oligo arrays manufactured by Agilent Technology, design ID 015087) according to manufacturer's protocol (Two-Color Microarray-Based Gene Expression Analysis Protocol, Product # G4140-90050, Version 5.0.1). Hybridized microarray slides were scanned with an Agilent DNA Microarray Scanner (model G2505-64120). Triplicate data were analyzed by analysis of variance with universal reference for normalization and carried out principle component analysis (http://lgsun.grc.nia.nih.gov/ANOVA/; Sharov *et al.*, 2005). Universal reference was not used for normalization if the slope of regression between log(Cy5) versus log(Cy3) is >0.3, and geometric mean of Cy3 is >3-fold higher than geometric mean of Cy5. All data are MIAME compliant and raw data have been deposited in GEO (GSE40763).

Quantitative real-time PCR was performed with the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen, Carlsbad, CA) then run and analyzed on the MxPro Real-Time PCR System (Stratagene). Primers for quantification were designed to span intron–exon boundaries to ensure that the reactions were cDNA specific. All reactions were performed three times and in triplicates and normalized to glyceraldehyde-3-phosphate dehydrogenase. Student's two-tailed *t*-test was used for statistical analysis. All primer sequences are available upon request.

Details of the generation of *Dkk4* transgenic mice were reported previously (Cui *et al.*, 2010). Briefly, *Dkk4* transgene was subcloned into a Krt14 vector, microinjected into pronuclei of mouse embryos, and followed by implantation into pseudo-pregnant female mice. Potential founders were mated to C57BL/6J mice to identify those passing the transgene. Two 6-month-old transgenic mice and two littermates were analyzed for nail formation in this study.

Immunohistochemistry

For histological analyses, digital tips of *Fzd6*$^{-/-}$ and wild-type littermates at day E16.5 were fixed in 4% formaldehyde and embedded in paraffin. Planar sections of 3 μm were cut from the middle digit for staining. Immunofluorescent staining was performed using anti-human/mouse Krt6b (Proteintech Group, Chicago, IL, 17391-1-AP), anti-mouse Krt86 (Santa Cruz Biotechnology, Santa Cruz, CA, sc-168332), and anti-human/mouse Involucrin (Abcam, Cambridge, UK, ab53112) using a DAKO autostainer (Dako, Agilent Technologies, Santa Clara, CA). The antibodies were detected using a DAKO Chemmate EnVision kit (Dako) according to the manufacturer's protocol. Sections were counterstained with hematoxylin.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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