Involvement of Wnt, Eda and Shh at defined stages of sweat gland development
Chang-Yi Cui*, Mingzhu Yin, Jian Sima, Victoria Childress, Marc Michel, Yulan Piao and David Schlessinger

ABSTRACT
To maintain body temperature, sweat glands develop from embryonic ectoderm by a poorly defined mechanism. We demonstrate a temporal cascade of regulation during mouse sweat gland formation. Sweat gland induction failed completely when canonical Wnt signaling was blocked in skin epithelium, and was accompanied by sharp downregulation of downstream Wnt, Eda and Shh pathway genes. The Wnt antagonist Dkk4 appeared to inhibit this induction: Dkk4 was sharply downregulated in β-catenin-ablated mice, indicating that it is induced by Wnt/β-catenin; however, its overexpression repressed Wnt target genes and significantly reduced gland numbers. Eda signaling succeeded Wnt. Wnt signaling was still active and nascent sweat gland pre-germs were still seen in Eda-null mice, but the pre-germs failed to develop further and the downstream Shh pathway was not activated. When Wnt and Eda were intact but Shh was ablated, germ induction and subsequent duct formation occurred normally, but the final stage of secretory coil formation failed. Thus, sweat gland development shows a relay of regulatory steps initiated by Wnt/β-catenin – itself modulated by Dkk4 – with subsequent participation of Eda and Shh pathways.

KEY WORDS: Exocrine gland, Skin appendage, Hair follicle, Ectodermal dysplasia, Heatstroke, Hyperhidrosis, Mouse

INTRODUCTION
The functions of the skin, the largest human organ, are expanded by various appendages. Skin exocrine glands are secretory appendages that include sweat, mammary, salivary, sebaceous, meibomian, lacrimal and preputial glands – all of which are involved in crucial human and animal physiology. Sweat glands in particular comprise a dynamic thermoregulatory organ. Evaporation of sweat from skin dissipates heat generated by high ambient temperature, physical activity or fever. Absence or malformation of sweat glands create the risk of hyperthermia, as frequently encountered in individuals with skin grafts, due to lack of sweat gland restoration, and in the hereditary genetic disorder anhidrotic/hypohidrotic ectodermal dysplasia (EDA/HED) (Cui and Schlessinger, 2006).

Analyzing the process of sweat gland formation is thus essential for understanding skin biology and also for potential regeneration of fully functional skin. Sweat gland development starts from an ectodermal placode consisting of K14+ progenitor cells (Lu et al., 2012), as seen for hair follicles (Fuchs, 2007; Millar, 2002). Nascent gland germs grow down progressively into the dermis to form a sweat duct, ending in a secretory coil. However, developing sweat glands lack detectable dermal condensations/papillae that regulate the early induction of hair follicles (Headon, 2009). In addition, distinct from other exocrine glands, which form branched structures (Mikkola and Millar, 2006; Tucker, 2007), each sweat gland forms only a single tubular duct (Sato et al., 1989). The molecular mechanism of sweat gland development remains to be elucidated.

Mice with mutations in the Eda signaling pathway provide models with which to investigate the molecular basis of the features of sweat gland development (Cui et al., 2009; Kunisada et al., 2009). Eda signaling activates NF-κB-mediated transcription through its ligand ectodysplasin, the receptor Edar and the receptor/adaptor Edaradd (Botchkarev and Fessing, 2005; Mikkola, 2008). Sweat glands fail to develop in Eda mutant Tabby mice, but are restored by an Eda-A1 transgene (Cui et al., 2003), and an adaptive variant of EDAR was recently shown to foster a moderate increase in sweat gland numbers in an East Asian population and in a related mouse model (Kamberov et al., 2013). However, neither upstream regulators nor downstream effectors of Eda in sweat glands have been defined. Here, we examine a series of mutant mice in conjunction with expression profiling to demonstrate a regulatory mechanism that operates through a Wnt-Dkk4-Eda-Shh cascade that is similar, but not identical to, other skin appendages.

RESULTS
Sweat gland induction fails in Ctnnb1 mutant (β-catenin cKO) mice
In hair follicle formation, the canonical Wnt pathway acts upstream of indispensable Eda action (Durmovicz et al., 2002; Laurikkala et al., 2001; Zhang et al., 2009). We therefore tested the Wnt pathway for a role in regulation of initiation of sweat gland development. As an initial approach, we analyzed Wnt activity in developing mouse sweat glands using TOPGAL Wnt reporter mice (DasGupta and Fuchs, 1999). During normal sweat gland development, pre-germ formation began by E16.5, but only in proximal footpads, and then spread to the distal footpads at E17.5 and E18.5 (supplementary material Fig. S1A). Thus, stages for pre-induction, pre-germ, germ and advanced germ can all be simultaneously observed at E17.5. We collected footpads from TOPGAL reporter mice at E17.5 and carried out X-gal staining. In pre-induction stages, weak but uniform Wnt activity was detected in upper dermis, immediately under the basal layer of epidermis (supplementary material Fig. S1B). When pre-germs and germs started to form, scattered Wnt-active cells appeared in epidermis, but Wnt activity declined sharply in dermis (supplementary material Fig. S1B). Wnt-active epidermal cells were then focalized in sweat gland germs, with strong activity seen in advanced germs (supplementary material Fig. S1B). These data implied Wnt involvement in sweat gland development.

To analyze Wnt control further, we generated skin-specific β-catenin mutant (β-Cat cKO) mice by crossing Ctnnb1loxP/loxP mice with K14-Cre mice. The resultant β-Cat cKO progeny were born with open eyes and without whiskers. As expected, β-catenin,
Fig. 1. Ablation of β-catenin from skin epidermis results in blocking of sweat gland induction. (A) β-catenin and Lef1 are highly expressed in sweat gland germs in wild type (arrows), but undetectable in β-Cat cKO embryos. Scale bar: 20 µm. (B) Developmental histology. Pre-germs/early germs can be occasionally observed at E16.5, germs/advanced germs at E17.5 and early coiling at around P0 (arrows in wild type). No pre-germ/germ formation in β-Cat cKO embryos. Right panels show the absence of hair follicle development in the mutant mice. Scale bars: 25 µm. (C) Cell proliferation and cell death status in cKO embryos. Right panels show the absence of hair follicle development in β-Cat cKO embryos (upper panels). Caspase 3 is not present in wild-type or β-Cat cKO embryos (lower panels). Arrows indicate pre-germs. Caspase 3 is occasionally found in cells close to the epidermal ducts in wild-type adult mice (an arrow in right panel). Scale bars: 25 µm.

which is broadly expressed in skin epidermis and locally upregulated in sweat gland germs in wild-type embryos, was absent in mutant skin (Fig. 1A, upper panels). In addition, Lef1, a partner of β-catenin in transcriptional regulation, was selectively highly expressed in localized sweat gland germ cells in wild-type controls but undetectable in mutant skin (Fig. 1A, lower panels).

The β-Cat cKO mice died within a few hours, but the early stage of sweat gland development could be studied before birth. Strikingly, β-Cat cKO mice showed no indication of sweat gland germ formation throughout the period E15.5 to birth (Fig. 1B, β-Cat cKO). We confirmed that hair follicle induction was also abrogated in the mutant mice [Fig. 1B, right panel (Huelsken et al., 2001)].

The Wnt pathway is thus required for the initiation of inductive regulation of both sweat glands and hair follicles.

By Ki67 staining, we found scattered proliferating cells in the basal layer of footpad skin both in wild-type and β-Cat cKO embryos (Fig. 1C). Overall, more positive cells were seen in wild-type than in β-Cat cKO mice. Scattered Ki67-positive cells were also found in nascent sweat gland pre-germs in wild-type embryos (Fig. 1C, arrow in upper left panel). In more advanced sweat gland germs, clusters of Ki67-positive cells had formed (Fig. 4C, upper left panel), consistent with recent findings that major proliferation occurs in hair follicles at later stages rather than the early pre-germ formation stage (Ahtiainen et al., 2014). A cell death marker, caspase 3, was not found in wild-type or β-Cat cKO footpads at embryonic stages (although a few cells close to the epidermal ducts were positive in adult footpads; Fig. 1C, lower and right panels).

We also confirmed the absence of dermal condensation/dermal papilla formation during normal sweat gland development (supplementary material Fig. S2; Introduction). Instead, we observed a connective tissue sheath surrounding sweat gland germs at early developmental stages. It became thinner but was still discernable at the subsequent stages of duct and secretory region formation (supplementary material Fig. S2, arrows; see Discussion).

Wnt/β-catenin action is prerequisite for Eda, Shh and Wnt pathway activation in sweat gland formation

To determine possible downstream targets of Wnt/β-catenin, we carried out expression profiling with footpad skin from wild-type and β-Cat cKO embryos at E15.5, E16.5 and E17.5, representing stages of pre-induction, early-induction and simultaneous ongoing early-induction, late-induction, and early-duct formation, respectively (Fig. 2A). Among 279 genes significantly altered in expression at one or more time points in the mutant embryos (Fig. 2B; supplementary material Fig. S3; full list in supplementary material Table S1), gene ontology analysis revealed a set of genes – especially those involved in tissue development, skin exocrine gland/hair/tooth/limb development or cell-cell signaling – that was significantly affected in cKO footpads (Table 1). Notably, most had not previously been analyzed as being expressed in sweat glands. Among them, 40, including seven morphogenetic genes – Atoh1, Lef1, Edar, Shh, Dkk4, Wnt10b and Fzd10 – were affected at all three developmental points. (Fig. 2. Genes regulated by Wnt/β-catenin during sweat gland development. (A) Expression profiling was carried out with whole footpad skin from wild-type and β-Cat cKO embryos at E15.5, E16.5 and E17.5. The number of genes significantly affected in β-Cat cKO embryos. Forty genes were affected at all three developmental time points. (C) qRT-PCR assays confirmed significant downregulation of Edar, Shh, Dkk4, Wnt10b and Fzd10 in β-Cat cKO footpads. ***P<0.001 for others. Data are means±s.e.m. (D) Edar protein (arrow) was localized to the membrane of sweat gland germ cells in wild-type, but not in the mutant, embryos. Dashed lines demarcate the epidermal-dermal junction. Scale bar: 20 µm.)
Table 1: Functional annotation of genes affected in β-Cat cKO footpads during development

<table>
<thead>
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<th>Category</th>
<th>Gene symbol</th>
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<tr>
<td>Tissue development</td>
<td>Shh, Edar, Wnt7a, Wnt3a, Lef1, Tnf, Tbx3, Tpr73, Emx2, Elf5, Tcfcp21, Pthih, Col2a1, Otor, Atoh1, Rora, Gjb6, Tmie, Pitr, Etv4, Celsr3, Ocel1, Chl1, Mmp9, Ivi, Hmr, Spry1b, Spry2, Spry2d, Krt6a, Krt6b, Shh, Edar, Wnt3a, Lef1, Tbx3, Tcfcp21</td>
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<tr>
<td>Exocrine gland development</td>
<td>Fzd10, Wnt10b, Wnt3a, Dkk4, Lef1, Fzd10, Kremen2, Epgn, Tnf, Gjb5, Gjb4, Gjb6, Smpd3, Hrmt, Ereg, Nod2, Ivi, Hmr, Spry1b, Spry2, Spry2d, Krt6a, Krt6b, Pigs1, Shh, Wnt7a, Wnt3a, Tpr73, Tcfcp21, Pthih, Pitr, Atoh1, Etv4, Celsr3, Ocel1, Chl1, Emx2</td>
</tr>
<tr>
<td>Hair follicle, tooth and limb development</td>
<td>Shh, Edar, Wnt7a, Lef1, Tnf, Mpz2, Tbx3</td>
</tr>
<tr>
<td>Cell-cell signaling</td>
<td>Shh, Edar, Wnt10b, Wnt7a, Wnt3a, Dkk4, Lef1, Fzd10, Kremen2, Epgn, Tnf, Gjb5, Gjb4, Gjb6, Smpd3, Hrmt, Ereg, Nod2, Ivi, Hmr, Spry1b, Spry2, Spry2d, Krt6a, Krt6b, Pigs1, Shh, Wnt7a, Wnt3a, Tpr73, Tcfcp21, Pthih, Pitr, Atoh1, Etv4, Celsr3, Ocel1, Chl1, Emx2</td>
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<td>Cell/tissue differentiation</td>
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Dkk4 as an antagonist of sweat gland induction by Wnt/β-catenin

Consistent with previous evidence of Dkk4 as a direct Wnt target (Bazzi et al., 2007), it was sharply downregulated in β-Cat cKO embryos. As a Wnt antagonist, however, it might then exert negative feedback. We therefore examined sweat gland dynamics in mice bearing a skin-specific Dkk4 transgene (Cui et al., 2010). Dkk4 was highly expressed in sweat gland germlines in wild type, and more intensely in the Dkk4 transgenic mice (Fig. 3A). Wnt activity was noticeably reduced in epidermis in the transgenic footpads, as fewer epithelial cells were positive for X-gal staining compared with wild type (supplementary material Fig. S1B,C). However, the transgenic embryos showed prolonged Wnt activity in dermis detectable even when pre-germs/germs have started to form (supplementary material Fig. S1B,C); this likely reflects disruption by the Dkk4 transgene of reciprocal signaling interactions between epidermis and dermis. We found that sweat gland numbers were significantly reduced in the transgenic mice. The iodine-starch sweat test revealed a corresponding reduction of about 40% in sweating spots in adult transgenics (Fig. 3B,C), and histological analysis of serial sections further confirmed a decreased number of sweat glands in transgenic mice, consistent with reduced numbers of X-gal-positive cells during development. The gland clusters were halved in size (Fig. 3D, P75). Fewer sweat glands were also evident in developing transgenics (Fig. 3D, P8). Immunostaining with Lef1 and Edar antibodies revealed fewer sweat gland germlines in the transgenic embryos, but induction at the same time as in wild type [E16.5 (Fig. 3E, lower panels in Lef1 and Edar) and E17.5 (Fig. 3E, upper panels)], and showed normal Lef1 and Edar expression (Fig. 3E). Individual sweat glands formed in the adult mutant mice appeared normal, with fully open secretory regions and sweat ducts (Fig. 3D, P75), as well as positive sweat tests. The results thus indicate that β-catenin activity determines the number of initiation sites for sweat glands; Dkk4 antagonism would effectively lower β-catenin activity and therefore the number of sweat glands that are initiated.

As expected, expression profiling and qRT-PCR assays with footpad skin from wild-type and transgenic embryos showed that Dkk4 expression was dramatically upregulated in transgenic embryos about 160-fold at E15.5 and nearly 80-fold at E16.5. Reflecting an incomplete suppression of sweat gland induction by the transgene, changes in expression for downstream genes were milder. Supplementary material Table S2 lists significantly affected genes and supplementary material Table S3 shows functional annotation. Edar expression showed a downward trend of 30% at E15.5, when Shh was the most strikingly affected morphogen (Fig. 3F). By E16.5, both Edar and Shh were sharply downregulated, and Eda, Fzd10 and Wnt10b were also statistically significantly reduced (Fig. 3F). Wnt7a and Lef1 were also slightly lower, but Wnt7b and Ctnnb1 were unaffected, and may thus be regulated in a manner unresponsive to Dkk4 levels.

Disruption of Eda signaling results in abortive sweat gland pre-germ formation

Turning to the Eda pathway, the genes whose expression was significantly affected in β-catenin cKO footpads (Table 2) included Edar – like Dkk4, previously shown to be a direct Wnt target (Zhang et al., 2009). In addition, mice depleted of Eda pathway genes, including Tabby, Downless and Crinkled mutants, lack sweat glands (Grünewald, 1971); however, early stage developmental defects have not been characterized. We found that Tabby mice do form pre-germ like structures at early developmental stages. A few elevated clusters of basal keratinocytes in footpads (Fig. 4A, arrows in Ta) were faintly positive for Lef1 at E16.5, and more intensely and extensively stained at E17.5; by E18.5 the staining was subsiding (Fig. 4B, Ta) and thereafter disappeared. Thus, full ‘gland germs’ never formed in Tabby mutants. In wild-type controls, pre-germs, germs and early stage straight ducts are apparent at these stages. Lef1 expression was comparatively found in the clustered pre-germ and germ cells, though only in the tip of growing ducts (it became undetectable in advanced ducts starting to coil; Fig. 4B, wild type). Similar to wild-type pre-germs (Fig. 1C), some pre-germ such as cells in Tabby mutants were positive, but weakly so, for K167 (Fig. 4C, left panels). Caspase 3 was not present in either wild-type or Tabby footpads (Fig. 4C, right panels).

Thus, Eda and Dkk4 are both located downstream of Wnt/β-catenin (Fig. 2C; Table 2), but have distinctive effects on sweat gland development (Figs 3 and 4). Nevertheless, because both affect

Table 2: Morphogenetic genes affected at all three developmental time points

<table>
<thead>
<tr>
<th>Gene</th>
<th>β-Cat cKO/wild type</th>
<th>E15.5</th>
<th>E16.5</th>
<th>E17.5</th>
</tr>
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<tbody>
<tr>
<td>Lef1</td>
<td>0.4</td>
<td>0.5</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Edar</td>
<td>0.5</td>
<td>0.3</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Shh</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Dkk4</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Wnt10b</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Fzd10</td>
<td>0.5</td>
<td>0.4</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Atoh1</td>
<td>0.2</td>
<td>0.1</td>
<td>0.2</td>
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</table>
Shh expression profoundly (Fig. 3F and Fig. 4D) (Kunisada et al., 2009), we asked whether they interact in their effects. We generated Dkk4 transgenic Tabby mice (TaDk4TG) (see Materials and Methods). Sweat glands were not formed in TaDk4TG mice, but immunostaining with Lef1 revealed formation of pre-germ-like structures that were similar to Tabby pre-germs (Fig. 4E). Again, this is consistent with the action of Dkk4 feedback at the initiation of sweat glands, and of Eda at the subsequent pre-germ formation.

Shh is downstream of Eda and regulates final secretory region formation

Morphogenetic genes, such as Edar, Wnt10b and Fzd10, were significantly affected in β-Cat cKO mice but not in Tabby mice, consistent with pure Wnt-mediated regulation independent of Eda (Fig. 4D). However, as seen in β-Cat cKO, expression profiling in Tabby mice indicates that Shh is the most strikingly affected gene from induction through secretory region formation (Kunisada et al., 2009). qRT-PCR assays confirmed sharp downregulation at early developmental stages (confirmed in Fig. 4D). These results suggested that Shh is activated downstream of Eda.

To increase resolution of its action, we analyzed skin-specific Shh mutant mice (Shh cKO) (Cui et al., 2011). Consistent with a regulatory location for Shh downstream of the Wnt-Eda cascade, qRT-PCR assays showed unchanged expression levels for Eda, Edar, Wnt10b and Fzd10 in the Shh mutant mice at E15.5 and E16.5; Dkk4 was transiently highly expressed only at E15.5 (supplementary material Fig. S4A). At the histological level, as in conventional knockout mice (Chiang et al., 1999), hair follicle formation was arrested at the early germ stage (Fig. 5A; see Discussion). By contrast, sweat gland germs formed and developed further to form sweat ducts in the cKO mice (Fig. 5B, arrows in cKO P8 show sweat ducts). Consistent with normal induction and duct formation, Lef1 and Edar proteins were normally expressed in the nucleus and membrane of sweat gland germs in the mutant mice (Fig. 5C).

However, in absence of Shh, formation of the final secretory region showed a characteristic defect. The coiling process was initiated, but its progression was curtailed in the cKO mice, yielding a secretory region much smaller than wild type, clearly discernible at P5 and decisive at P8 (Fig. 5B). Immunostaining for K14 and K8, markers for the sweat duct and secretory regions, respectively, further confirmed that sweat ducts appeared normal in the Shh cKO mice but secretory regions were rudimentary (Fig. 5D, secretory regions circled with broken lines). Ki67-positive cells were clearly sparser than in the wild type at both P5 and P8 (supplementary material Fig. S4B, left panels), but caspase 3 was negative in both mutant and wild type (supplementary material Fig. S4B, right panels). Thus, the developmental defect of secretory region formation appears to reflect depressed cell proliferation rather than accelerated loss.

Involvement of Bmp and FoxA1 in auxiliary regulation

Two other genes are strongly implicated in sweat gland formation. At an early stage, ablation of one, Bmpr1a, results in failure of sweat gland formation, and when Bmp was suppressed by its antagonist noggin (Nog), hair follicles formed in mouse footpads, where they are usually not seen (Leung et al., 2013; Plikus et al., 2004). As an aid to
determining its time of action, our expression profiling showed no significant changes in expression of any Bmp pathway genes in β-Cat cKO footpads. In further targeted qRT-PCR assays, Bmp4 expression was unchanged in the mutant embryos at all time points analyzed (supplementary material Fig. S5A). Nog was slightly upregulated in the mutant embryos at E15.5, but unchanged at E16.5 or E17.5, and Bmp2 was slightly downregulated only at E17.5. In addition, the pattern of expression was very similar in Tabby footpads (supplementary material Fig. S5B), i.e. in the absence of Eda. Immunostaining for Bmpr1a was undetectable at pre-germ/germ stages in wild-type, β-Cat cKO or Tabby footpads, but showed weak positivity only in advanced germs in wild-type mice (supplementary material Fig. S5C). By contrast, its downstream effector pSmad1/5 was highly expressed in sweat gland germs in wild type, broadly expressed in the basal layer of β-Cat cKO embryos and positive for pre-germ like structures in Tabby (supplementary material Fig. S5D). Thus, Bmp likely acts upstream of the Wnt-Eda cascade, as in hair follicles (Botchkarev et al., 1999), but its role requires further characterization.

The other pathway known to be involved in sweat gland maturation, which requires Foxa1, acts at the late stage of development of sweating capacity (Cui et al., 2012; Sato et al., 1989). Sweat gland development includes successive induction, duct formation and secretory region formation (Fig. 6). Putatively implicated at an early stage in Wnt/β-catenin activation are the Bmp/Nog pathway and engrailed 1, a negative regulator of β-catenin (Bachar-Dahan et al., 2006; Botchkarev et al., 1999). We find here that Wnt/β-catenin, once activated, then controls the regulatory hierarchy for sweat gland induction and duct formation involving Eda/Edar and Dkk4 (Fig. 6). It likely sets the number of gland initiation sites.

Dkk4 and Dkk1 are members of the Wnt antagonist Dkk family, which suppresses Wnt signaling by binding to Wnt co-receptors Lrp5/6 (Niehrs, 2006). In skin, however, Dkk1 is expressed in mesenchyme surrounding hair germs, whereas Dkk4 is in the epidermal part of hair germs (Andl et al., 2002; Bazzi et al., 2007). Dkk1 completely blocked hair follicle induction when overexpressed in mice (Andl et al., 2002); however, Dkk4 transgenic mice showed comparable numbers of hairs with wild-type controls, though hair subtypes were severely distorted (Cui et al., 2010).

Dkk4, but not Dkk1, was significantly downregulated in β-Cat cKO footpads. Dkk4 is regulated by canonical Wnt (Bazzi et al., 2007) and has also been shown to be directly regulated by Edar during hair follicle development (Zhang et al., 2009). As in hair follicles, Dkk4 is selectively expressed in the epidermal compartment of sweat gland germs (Fig. 3A). However, our data suggest that it is primarily regulated by Wnt rather than Eda in sweat glands (Fig. 2C). We showed that Dkk4 levels are lowered at early developmental stages in hair follicles (e.g. E13.5, E14.5) (Cui et al., 2006), but lowering occurs at later stages in sweat glands (E17.5, E18.5) in Tabby (Fig. 4D; Kunisada et al., 2009). The apparent functional effect of Dkk4 in sweat glands is also very different from structures assembled during development (Cui et al., 2012; Sato et al., 1989). Sweat gland development includes successive induction, duct formation and secretory region formation (Fig. 6). Putatively implicated at an early stage in Wnt/β-catenin activation are the Bmp/Nog pathway and engrailed 1, a negative regulator of β-catenin (Bachar-Dahan et al., 2006; Botchkarev et al., 1999). We find here that Wnt/β-catenin, once activated, then controls the regulatory hierarchy for sweat gland induction and duct formation involving Eda/Edar and Dkk4 (Fig. 6). It likely sets the number of gland initiation sites.
that in hair follicles. Overexpressed Dkk4 blocked induction of about 40% of mouse sweat glands, but had no apparent effect on hair follicle number. Differential regulatory networks may thus delimit Dkk4 action in various skin appendages.

We also found that several Wnt target genes were moderately but significantly downregulated in the Dkk4 transgenic sweat glands, consistent with a negative-feedback interaction of Dkk4 with Wnt (Fig. 6). Because the glands formed in the presence of various levels of Dkk4 are structurally and functionally very similar, the increased level of Dkk4 apparently only limits the probability of successful sweat gland germ formation, leading to an overall reduction in number.

Wnt/β-catenin also directly activates the downstream Eda pathway. Ablation of β-catenin thus results in a complete absence of sweat gland induction, and Eda mutant Tabby mice show only primitive pre-germ-like structures. Eda is then required throughout duct formation (Cui et al., 2009). Shh intervenes later (Fig. 5 and below).

Various appendages display distinctive signaling interaction patterns

Although skin appendages are thought to form by reciprocal signaling interactions between ectoderm and mesenchyme (Millar, 2002), the use and phase of action of signaling pathways vary. Here, we discuss steps of sweat gland development compared with other skin appendages.

In hair follicles, use and phasing are similar to sweat glands, especially at the early developmental stages. Wnt is required for early induction and Eda for late induction through hair peg/sweat duct formation (Chen et al., 2012; Cui et al., 2009; Millar, 2002; Schmidt-Ullrich et al., 2006; Zhang et al., 2009) (Figs 1 and 4). The specification of appendage type may then be effected at least in part by variations in the Shh pathway (see below). In contrast to hair follicles and sweat glands, salivary gland early stage development depends instead on Fgf and Pitx2 pathways (Jaskoll et al., 2004b; Tucker, 2007). Eda and Shh pathways are then required for late-stage branching morphogenesis (Häärä et al., 2011; Patel et al., 2011). In early stage mammary gland development, yet another pattern is seen. Both Fgf and Wnt are required for induction, and Eda and PTHrP for ductal and branching morphogenesis (Lindfors et al., 2013; Voutilainen et al., 2012).

At the structural level, a striking feature seen in hair follicles, the dermal papilla, is not found in the exocrine appendages that have been studied (Headon, 2009). Wnt and Shh pathways are required for the formation of well-described dermal papillae for hair follicles (Chen et al., 2012; Millar, 2002; Zhang et al., 2009); however, salivary glands and mammary glands have no apparent dermal papillae. Instead, they form rather broad areas of dermal mesenchyme (Harunaga et al., 2011; Mikkola and Millar, 2006). As shown by heterotypic tissue recombination experiments, dynamic dermal-epidermal signaling interactions nevertheless occur during both salivary gland and mammary gland development (Kratochwil, 1969; Kusakabe et al., 1985). We find that sweat glands are also comparably surrounded by sheaths of connective tissue (supplementary material Fig. S2), though less abundantly than in salivary or mammary gland mesenchyme. We infer that sweat gland development also requires dermal-epidermal interactions, with the sweat gland mesenchyme/ connective tissue sheath likely providing dermal signaling.

Shh action: an example of variable involvement and timing in different skin appendages

Shh is key to post-induction development of several skin appendages (Chuong et al., 2000). In hair follicles, Shh is required for hair germ invagination and dermal papilla formation but not for hair germ induction (Chiang et al., 1999; St-Jacques et al., 2013; Voutilainen et al., 2012).
et al., 1998). In exocrine appendages, it was dispensable for mammary gland development (Gallego et al., 2002; Michno et al., 2003), but required for late stage salivary gland branching morphogenesis (Jaskoll et al., 2004a). In sweat glands, Shh is not required for germ formation or for subsequent duct invagination (Fig. 5), but is indispensable for final stage secretory coil formation, more similar to its role in salivary glands.

How can Shh vary in its determinative role in different skin appendage lineages? Among the possible factors to be assessed are: specific local niche matrices; pre-determinative differences in effectors in placodes; and the involvement of additional pathways such as that regulating sweating capacity, which is initiated by FoxA1 (for example) (Cui et al., 2012). However, the level and/or timing of Shh expression might be an important effector. Suggestively, inhibition of Shh signaling resulted in glandular tissue formation in back skin, which otherwise harbors only hair follicles (Grütt-Linde et al., 2007). In addition, consistent with this notion, we found that Shh expression was very low in early sweat gland induction stages and then increased sharply during postnatal secretory coil formation (Kunisada et al., 2009).

The expression profiling of appendages derived from wild type compared with the mutant mouse models also provided additional genes as candidates for involvement in sweat gland development (Table 1). For example, among the known targets downstream of Wnt/β-catenin, Epgn was upregulated in the β-catenin-ablated mice from early induction stages (Fig. 6; supplementary material Table S1); overexpression of this gene has recently been shown to suppress primary hair formation and promote sebaceous gland hyperplasia (Dahlhoff et al., 2010). By contrast, Tbx3 and Mmp9 were downregulated at late induction/early duct formation stages. Tbx3 mutations cause the Ulnar-mammary syndrome that affects limb, apocrine sweat gland and hair follicle development (OMIM#181450), and might be involved in late stage eccrine sweat gland formation. We believe that Mmp9, which is also downregulated in Tabby mutants (Kunisada et al., 2009), is also involved in tissue remodeling during sweat duct invagination. How the candidate genes might affect differential cell lineage specification and further development now becomes an area for future study.

MATERIALS AND METHODS

Mouse models

All research was conducted according to the guidelines as defined by the Office of Animal Care and Use in the NIH Intramural Program, and all animal study protocols were approved by the NIA Institutional Review Board. We used six genetically modified mouse strains for this study. The Tg(TCF/Lef1-lacZ)34Eu/J (TOPGAL reporter, stock #046232) mice, B6.129-Ctnnb1tm2Kwa/J (Ctnnb1loxP/loxP, stock: #004152) mice, the B6.129-Shh+tm2Amc1/J (ShhloxP/loxP, stock: #004293) mice, the Tg(KRT14-cre)1Amc/J (K14-Cre, stock: #004782) mice and the C57BL/6J A^−/−;Tbx3^−/− (Tabby, stock: #000338) mice were purchased from the Jackson Laboratory (Bar Harbor, MA, USA) and set up in colonies at the NIA animal facility. Skin-specific β-catenin mutant mice (β-Cat cKO) and skin-specific Shh mutant mice (Shh cKO) were generated by crossing the Ctnnb1loxP/loxP mice or the ShhloxP/loxP mice with the K14-Cre mice. Genotyping for TOPGAL, Ctnnb1, Shh and K14-Cre mutant mice was carried out by PCR with protocols provided by the Jackson Laboratory. Tabby genotyping was carried out by PCR and subsequent DdeI or AvaI digestion, as previously described (Cui et al., 2006).

The skin-specific Dkk4 transgenic mice (K14-Dkk4) were generated in our laboratory and have been reported previously (Cui et al., 2010). Briefly, the full-length open reading frame of mouse Dkk4 cDNA with insertion of a Flag tag in the 3’ end was subcloned into a K14 promoter vector and microinjected into pronuclei of one-cell C57BL/6J mouse embryos. Potential founders were mated to C57BL/6J mice to establish transgenic lines. Dkk4 transgenic TOPGAL mice were generated by crossing K14-Dkk4 transgenic mice with TOPGAL reporter mice, and Dkk4 transgenic Tabby male mice by crossing K14-Dkk4 transgenic male mice with Tabby female mice.

**Timed-mating, RNA isolation, expression profiling and qRT-PCR**

Timed-mating was set up for TOPGAL, β-Cat cKO, Tabby, Shh cKO and Dkk4 transgenic mice to obtain embryos. Except for footpads, mouse skin is covered entirely by hair; by contrast, sweat glands are located exclusively in the footpads, facilitating selective analyses. We collected non-hairy footpad skin from embryos at E15.5, E16.5 and E17.5 under a dissection microscope, and fixed in 10% formaldehyde (Ricca Chemical) for histological analysis or snap-froze them in dry ice and stored them in a −80°C freezer for RNA isolation. Frozen whole footpad skin samples from β-Cat cKO (E15.5, E16.5 and E17.5) or Dkk4 transgenic (E15.5 and E16.5) embryos were then separated into two groups for each genotype at each time point for biological duplicates, a minimum replication, but sufficient to reveal a number of crucial genes in sweat glands, including Foxa1 and Shh (Kunisada et al., 2009). Total RNAs were isolated with Trizol reagent (Invitrogen), and cyanine-3-labeled cRNAs were hybridized to the NIA Mouse 44K Microarray v3.0 (Agilent Technologies). Duplicated data were analyzed by ANOVA (Kunisada et al., 2009). Genes with FDR<0.05, fold difference>2.0 and mean log intensity>2.0 were considered to be significant for Dkk4 transgenic mice. Hybridization data have been deposited in the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE50859 for β-Cat cKO and GSE50862 for Dkk4 transgenic embryos. One-step Taqman qRT-PCR was carried out with ready-to-use probe/primer sets for Eda, Edar, Shh, Dkk4, Wnt10b, Fzd10, Wnt7a, Ctnnb1, Lef1, Bmp2, Bmp4 and Nog (Applied Biosystems). Each of the two sets of RNAs for each genotype (wild-type, β-Cat cKO, Tabby, Dkk4 transgenic or Shh cKO) was assayed in triplicate by qRT-PCR. Reactions were normalized to GAPDH and data were analyzed using Student’s t-test.

**X-gal staining, general histology, immunofluorescent staining, in situ hybridization and sweat test**

X-gal staining was carried out with TOPGAL and Dkk4 transgenic TOPGAL embryos using a lacZ tissue staining kit (rep-lacZ, InvivoGen). Briefly, 12 μm frozen sections from E17.5 mouse footpads were fixed in 0.5% glutaraldehyde for 10 min at room temperature and then incubated with X-gal staining solution overnight at room temperature. Positive signals were visualized after washing sections in PBS. For general histology, footpad skin fixed in 10% formaldehyde was dehydrated and embedded in paraffin (P3558, Sigma). Sections (5 μm) were cut, deparaffinized and ‘unmasked’ by heating at 121°C for 2 min in an Antigen Unmasking Solution (H-3300, Vector Laboratories). Hematoxylin and Eosin (H&E) staining was carried out according to the manufacturer’s standard protocol (Sigma). Indirect immunofluorescence staining was carried out with primary and secondary antibodies listed below. Sections were blocked with a serum-free protein blocking solution (X0909, Dako) for 30 min before antibody application, and antibodies were diluted in an Antibody Dilution solution (S0809, Dako).

For immunofluorescent staining, rabbit antibodies against β-catenin (95857, Cell Signaling, 1:1000 dilution), Lef1 (#2230, Cell Signaling, 1:300), Edar (18032-1-AP, Proteintech, 1:1500), Kif7 (ab15580, Abcam, 1:100), caspase 3 (#9661, Cell Signaling, 1:200), Bmpra (pa2-27280, Thermo Scientific, 1:200), pSmad1/5/8 (#9516, Cell Signaling, 1:50) and K14 (PRB155P, Covance, 1:1500), and guinea pig antibodies for K8 (GPK8, Progen, 1:200) and K14 (GP-C-K14, Progen, 1:1500) were incubated with deparaffinized, antigen-unmasked sweat gland sections. AlexaFluor-conjugated secondary antibodies were used to detect primary antibodies (Invitrogen), and images were taken using a Deltavision Microscope System (Applied Precision).
In situ hybridization, a 1 kb fragment spanning exons I-IV of mouse Dkk4 cDNA was amplified by PCR and subcloned into a pGEM-T vector (Promega). Primers used were: forward, 5’-TGGAGCTCGGAGAGAACCGAGTAGA-3’; reverse, 5’-TGGAGTGAACATGCTGCCTGTA-3’. Mouse footpads were fixed in 4% paraformaldehyde, dehydrated with 30% sucrose and embedded in OCT compound. Cryosections (20 μm) were hybridized with 1 μg/ml digoxin-labeled antisense probes at 65°C in hybridization solution (50% formamide, 5× SSC, 300 μg/ml yeast tRNA, 100 ng/ml heparin, 1 mM EDTA, 1× Denhardt’s solution, 0.1% Tween 20, 0.1% CHAPS, 5 mM EDTA), followed by washing with 2× SSC and 0.2× SSC, and incubated with anti-digoxin antibody (Roche). Positive signal was detected with NBT/BCIP solution (Roche).

For the sweat test, a hind paw of a wild-type or mutant mouse was painted with isopropanol solution (2%). Once dry, the surface was painted with starch-iodine (10 g starch/10 ml castor oil). Purple sweating spots start to form 1-2 min later and peak at around 10 min.

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Competing interests
The authors declare no competing financial interests.

Author contributions
C.-Y.C. and D.S. developed the concepts and designed the experiments; C.-Y.C. and D.S. analyzed the data; C.-Y.C. and D.S. wrote the paper.

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Supplementary material
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References


