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The basement membrane of hair follicle stem cells is a muscle cell niche

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Summary

The hair follicle bulge in the epidermis associates with the arrector pili muscle (APM) that is responsible for piloerection (commonly called "goosebumps"). We show that deposition of nephronectin into the basement membrane by bulge stem cells mediates selective adhesion of $\alpha 8 \beta 1$ integrin-expressing mesenchymal cells, including APM progenitors. Nephronectin induces $\alpha 8$ integrin-positive cells to upregulate smooth muscle markers. In nephronectin-null skin, the number of APM is reduced and those that form insert above the bulge, where there is compensatory upregulation of the nephronectin family member EGFL6. Deletion of $\alpha 8$ integrin also abolishes the selectivity of APM anchorage to the bulge. Nephronectin is a Wnt target gene; epidermal β -catenin activation upregulates epidermal nephronectin and dermal $\alpha 8$ integrin expression. We conclude that by expressing nephronectin bulge stem cells provide a smooth muscle cell niche and act as tendon cells for the APM. The results also reveal a functional role for basement membrane heterogeneity in tissue patterning.

Introduction

The epidermis is maintained through self-renewal of stem cells and differentiation of their progeny to form the lineages of the interfollicular epidermis and adnexal structures,

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including the hair follicles and sebaceous glands (Watt et al., 2006). There are several distinct populations of stem cells in adult epidermis. Their properties are regulated by intrinsic transcriptional programs in response to signals from the external microenvironment, or niche (Watt and Hogan, 2000; Watt et al., 2006).

One location of epidermal stem cells is the permanent portion of the hair follicle, known as the bulge. During the resting phase of the hair growth cycle (telogen), bulge cells are in close contact with a cluster of mesenchymal cells known as the dermal papilla, and reciprocal interactions between epidermal stem cells and dermal papilla cells are essential for hair follicle formation and maintenance (Millar, 2002; Yang and Cotsarelis, 2010). A second close association between the bulge and the adjacent mesenchyme involves a smooth muscle, called the arrector pili muscle (APM), which is responsible for raising the hair follicles (piloerection) to trap body heat and express emotions. Unlike the association between the bulge and the dermal papilla, which is lost during the growth (anagen) phase of the hair growth cycle, the bulge maintains contact with the APM throughout the hair cycle (Muller-Rover et al., 2001). Although it is well known that the bulge is the permanent attachment site of the APM, how attachment of the muscle is established and maintained in the bulge is entirely unknown.

We hypothesized that bulge-arrector pili muscle interactions might involve epidermal basement membrane components. Laminin-511 has been shown to mediate epidermal-dermal papilla signalling during hair development (Gao et al., 2008), and in a range of other tissues, such as kidney and pancreas, the basement membrane is involved in bidirectional cellular interactions (Linton et al., 2007; Nikolova et al., 2006). Furthermore, the basement membrane of the skin exhibits local variation in structure and composition (Timpl, 1996). Such basement membrane heterogeneity would not only provide a potential mechanism for anchoring different stem cell populations in different regions of the epidermis, but could also result in local differences in signalling with adjacent mesenchymal cells (Akiyama et al., 1995; Fuchs, 2008; Spradling et al., 2001; Watt and Hogan, 2000).

Gene expression profiling of the bulge compartment has revealed that bulge stem cells express a number of extracellular matrix (ECM) proteins that are distinct from those of other epidermal cells (Morris et al., 2004; Ohyama et al., 2006; Tumber et al., 2004). One of these is nephronectin, an ECM protein with five EGF-like repeats, an RGD sequence, and a COOH-terminal MAM domain (Brandenberger et al., 2001). Nephronectin is an interesting candidate mediator of epidermal interactions with mesenchymal cells, since the nephronectin receptor is $\alpha 8 \beta 1$ (Brandenberger et al., 2001; Sato et al., 2009), an integrin that is expressed in the dermal papilla but not by adult epidermal keratinocytes (Driskell et al., 2009; Watt, 2002). Furthermore, the epithelial-mesenchymal interactions required for kidney organogenesis are disrupted in mice lacking $\alpha 8 \beta 1$ or nephronectin (Brandenberger et al., 2001; Linton et al., 2007; Muller et al., 1997).

In this study, we show that bulge stem cells create a specialized basement membrane containing nephronectin, which induces arrector pili muscle differentiation and anchorage to the bulge. Loss of nephronectin or $\alpha 8$ integrin expression causes delocalization of the APM. Thus the bulge ECM not only contributes to the specialized niche of hair follicle stem cells but also provides a niche for smooth muscle progenitors.

Results

Bulge stem cells deposit nephronectin

By examining published microarray data, we identified a range of ECM genes that were upregulated in bulge stem cells, including nephronectin (*Npnt*) (Figure 1A, Table S1, S2,

S3). To confirm that nephronectin was upregulated in bulge cells, Q-PCR was performed on mRNA from disaggregated dorsal skin keratinocytes that had been FACS-sorted on the basis of the expression of bulge stem cell markers, CD34 and $\alpha 6$ integrin (Figure 1B). CD34⁺/ $\alpha 6$ integrin^{high} cells were enriched for expression of the additional bulge maker *Sox9* and expressed low levels of *Sca1*, a marker of interfollicular epidermal cells (Figure S1A; Jensen et al., 2009; Trempus et al., 2003). These cells had high levels of *Npnt* in comparison with unfractionated basal cells (All Live; cells with low forward and side scatter) and CD34⁻/ $\alpha 6$ integrin^{high} non-bulge stem cells (Figure 1C). Expression levels of other ECM genes identified from the microarrays were also confirmed by Q-PCR (Figure 1A, Figure S1A).

We next examined the distribution of nephronectin protein in embryonic and adult skin. At E14.5, nephronectin was detected throughout the epidermal-dermal basement membrane, where it was colocalized with the ubiquitous basement membrane component laminin $\gamma 1$ chain (Figure 1D; Figure S1B). However, at E16.5 and E18.5, when hair follicle morphogenesis had begun, nephronectin accumulated between the hair germ and dermal condensate, but was hardly detectable along the rest of the hair follicle (Figure 1E, F, Figure S1B). Just after birth (P1, P5), nephronectin was detected in the basement membrane of keratin 15 (K15)- and Sox9-positive early bulge stem cells as well as at the base of the follicle (Figure 1G, Figure S1B, C). At this time, nephronectin deposition in the bulge was asymmetrically distributed at the posterior side (Figure 1G, H, Figure S1B).

In adult telogen and anagen skin, nephronectin was localized to the basement membrane of the bulge, hair bulb, and APM (Figure 1I, J, Figure S1B). Other ECM proteins that localised to the bulge rather than along the entire outer root sheath were periostin and fibulin-1 (Figure S1D). Tenascin-C was confined to the bulge in telogen follicles but showed more widespread distribution in anagen (Figure S1D).

In situ hybridization for *Npnt* mRNA in embryonic and adult skin confirmed that nephronectin is expressed by epidermal cells. Epithelial cells of the bulge and hair germ were strongly positive for *Npnt* mRNA, whereas the dermis was negative (Figure 1K–M). We conclude that expression of nephronectin by bulge stem cells and hair germ cells results in heterogeneity in epidermal basement membrane composition from early hair morphogenesis to adulthood.

$\alpha 8$ integrin is specifically expressed by cells of the dermal papilla and arrector pili muscle and colocalizes with nephronectin

Since $\alpha 8\beta 1$ integrin is the major nephronectin receptor (Brandenberger et al., 2001; Sato et al., 2009), we examined whether $\alpha 8\beta 1$ integrin colocalised with nephronectin in embryonic and adult skin. Whole-mount immunostaining of E14.5 dorsal skin revealed that $\alpha 8$ integrin was strongly expressed in dermal condensates (arrowheads in Figure 2A) and also widely expressed in the superficial dermis, correlating with the widespread distribution of nephronectin in the basement membrane (arrows in Figure 2A). Between E16.5–E18.5, $\alpha 8$ integrin expression in the superficial dermis was downregulated, but it was highly expressed in the dermal condensates and dermal papillae of developing hair follicles and colocalized with nephronectin (arrowheads in Figure 2B, C).

At P1 and P5, $\alpha 8$ integrin-positive dermal cell clusters were detected in association with nephronectin in the early bulge (open arrowheads in Figure 2D, E) and elongated towards the interfollicular epidermis (Figure 2E), while $\alpha 8$ integrin was downregulated in dermal papillae of hair follicles in late anagen (Figure 2D, E). In adult telogen skin, $\alpha 8$ integrin accumulated at the interface between hair germ and dermal papilla, colocalizing with nephronectin (arrow in Figure 2F). The arrector pili muscles co-expressed $\alpha 8$ integrin and nephronectin and inserted into the nephronectin-positive bulge (Figure 2F). In addition to

nephronectin $\alpha 8\beta 1$ binds to several ECM proteins, including osteopontin, tenascin-C, fibronectin, and vitronectin (Brandenberger et al., 2001; Denda et al., 1998; Schnapp et al., 1995). However, none of these proteins showed specific colocalization with $\alpha 8$ integrin (Figure S2A–D).

To identify the $\alpha 8$ integrin-positive cells associated with the early bulge cells, we stained newborn skin sections with a panel of antibodies. At P1, the $\alpha 8$ integrin-positive population expressed dermal fibroblast markers PDGFR α and HSP47 (Figure 2G, Figure S2E; Erez et al., 2010; Kuroda and Tajima, 2004). $\alpha 8$ integrin-positive cells in the dermal papilla expressed CD133 (white arrowhead in Figure 2H; (Driskell et al., 2009), but CD133 was not expressed by $\alpha 8$ integrin-positive cells at the bulge (open arrowhead in Figure 2H). $\alpha 8$ integrin-positive cells did not express the endothelial cell marker CD31, the neural crest cell derivative marker nestin or the neuron specific marker β III tubulin (Figure 2I–L). At P1, bulge-associated $\alpha 8$ integrin-positive cells did not express smooth muscle actin α -SMA (Figure 2M). However, from P5 onwards, they were strongly positive for α -SMA (Figure 2N, O) and dystrophin (Figure S2F). These results suggest that the $\alpha 8$ integrin-positive fibroblasts associated with the bulge in P1 skin are progenitors of the APM.

$\alpha 8$ integrin-positive dermal cells adhere strongly to nephronectin and upregulate smooth muscle markers

The specific colocalization of nephronectin and $\alpha 8$ integrin led us to hypothesize that nephronectin mediates adhesion of $\alpha 8$ integrin-positive dermal cells to the bulge basement membrane. To examine this, we performed solid-phase cell adhesion assays with purified nephronectin. Disaggregated P1 dorsal dermal cells were sorted on the basis of surface $\alpha 8$ integrin expression (Figure 3A). Q-PCR analysis confirmed that $\alpha 8$ integrin mRNA (*Itga8*) was upregulated in the $\alpha 8^{\text{high}}$ population, whereas cells with high or low $\alpha 8$ integrin levels showed little variation in $\beta 1$ integrin (*Itgb1*) levels (Figure 3B). Solid-phase cell adhesion assays revealed that $\alpha 8$ integrin^{high} dermal cells adhered strongly to nephronectin-coated substrates in a concentration-dependent manner, whereas $\alpha 8$ integrin^{low} dermal cells did not (Figure 3C, D). In contrast, both populations adhered equally well to laminin-coated substrates (Figure 3C, D).

Unfractionated, $\alpha 8^{\text{high}}$ and $\alpha 8^{\text{low}}$ cells were plated on nephronectin, laminin or a mixture of both for 12 hours and then collected for Q-PCR analysis (Figure 3E). We examined expression of two smooth muscle cell marker genes, α -smooth muscle actin (α -SMA; *Acta2*) and smooth muscle protein 22- α (*Sm22a*), and two dermal papilla cell marker genes, *Cd133* and *Corin*. Prior to plating, $\alpha 8^{\text{high}}$ cells expressed high levels of *Cd133* and *Corin*, consistent with their presence in dermal papillae (Figure 3E). However, expression of these markers was downregulated, regardless of whether the cells were plated on nephronectin or laminin (Figure 3E). In contrast, the $\alpha 8$ integrin^{high} population showed significant upregulation of *Acta2* and *Sm22a* when plated on nephronectin or nephronectin and laminin, but not on laminin alone (Figure 3E).

We conclude that nephronectin mediates adhesion of $\alpha 8$ integrin-positive dermal cells and stimulates expression of APM markers.

Nephronectin is required to anchor arrector pili muscles to the bulge

To investigate the function of nephronectin in vivo, we analyzed *Npnt* knockout mice (Linton et al., 2007). Analysis of haematoxylin and eosin (H&E)-stained sections of adult dorsal telogen skin did not reveal any gross abnormalities and there was no significant difference in the size of hair bulbs and dermal papillae (Figure 4A, B and data not shown). However, in *Npnt* $-/-$ skin, $\alpha 8$ integrin was absent from the basement membrane separating

the dermal papilla and hair germ and was more prominently distributed at the periphery of the dermal papilla (Figure S3A, B, C). The effect was specific to $\alpha 8$ integrin, because the distribution of the phylogenetically related RGD-binding integrin subunits, αv and $\alpha 5$, in dermal papillae was not altered (Figure S3D, E). In addition, lack of nephronectin did not affect the distribution of the ubiquitous basement membrane component, laminin $\gamma 1$ chain (Figure S3B).

To examine whether loss of nephronectin affected the APM, we stained sections of adult telogen dorsal skin with anti- α -SMA. In control skin, muscles were detected in association with $95.9 \pm 1.2\%$ of hair follicles. In *Npnt*^{-/-} skin, there was a small but significant decrease in the percentage of hair follicles with arrector pili muscles ($82.2 \pm 3.3\%$) (Figure 4C–E).

Given the obvious limitations of conventional histology for analysing the spatial relationship between the APM and the hair follicle, we developed a whole-mount labelling technique in which we could observe the interaction in three-dimensions. Arrector pili muscles were visualized by staining for α -SMA and SM22 α and hair follicles were visualised by DAPI labelling or Keratin 14 (K14) staining. There was an inverse gradient of α -SMA and SM22 α , with α -SMA being more abundant next to the bulge and SM22 α closer to the interfollicular epidermis (Figure 4F, Figure S3F). Individual muscle bundles usually branched to insert into the bulges of several neighbouring hair follicles and each follicle typically had one associated muscle bundle (Figure 4F).

Using whole-mount visualisation, we confirmed the reduced number of hair follicles with associated arrector pili muscles in *Npnt*^{-/-} skin: 99.7% of control follicles had an associated muscle, compared with 93.3% in knockout skin (Figure 4G–I). There was also a significant reduction in the number of muscle attachment sites per hair follicle (Figure 4J). However, the major effect of *Npnt* deletion was to change the site of muscle attachment to the follicle. In control mice, the APM attached to the K15-positive bulge, whereas in *Npnt* knockout mice, the attachment was higher up the follicle (Figure 4G, H, K–M). In *Npnt*^{+/-} skin, 88.3% of muscle attachments located on the bulge. However, in the knockout, only 23.2% were on the bulge, and 76.3% located above the bulge. These results show that nephronectin is required to anchor the APM to the bulge.

The observation that loss of nephronectin led to a specific upward shift in APM insertion point, rather than randomising the insertion sites, led us to investigate whether there was compensatory change in the distribution of the nephronectin family member EGFL6/MAEG. Like nephronectin, EGFL6 is an $\alpha 8\beta 1$ ligand that is expressed in mouse skin (Osada et al., 2005). In wild type and *Npnt*^{+/-} skin EGFL6 was expressed in dermal condensates at E14.5 and in the dermal sheath at E16.5–E18.5 (Figure S3G, H). In newborn skin (P1 and P5), EGFL6, like nephronectin, localized to the K15-positive bulge (Figure S3G and Figure 1). However, in adult skin EGFL6 was only deposited in the basement membrane of the upper bulge, which is CD34-positive and K15-negative (Figure S3G). *Egfl6* mRNA was highly upregulated in CD34+ $\alpha 6^{\text{high}}$ cells relative to other basal keratinocytes (Figure S3I), indicating that EGFL6 in the upper bulge is of epidermal origin.

In control skin, the arrector pili muscles inserted below the EGFL6-positive zone of the hair follicles (Figure 4N, P). However, in *Npnt*^{-/-} skin, EGFL6 deposition in the upper bulge was increased and the muscles inserted into the EGFL6-positive zone (Figure 4O, P; see also Figure S3G, J–L). Although the site of insertion of the APM was altered in *Npnt*^{-/-} skin, piloerection could still be induced by treatment with the $\alpha 1$ -adrenergic receptor agonist phenylephrine, which induces smooth muscle contraction (Figure S3M, N).

Our data reveal that nephronectin anchors the APM to the bulge and that in the absence of nephronectin the site of insertion shifts upwards to the EGFL6-positive zone (Figure 4Q).

$\alpha 8$ integrin determines specificity of arrector pili muscle attachment site to the bulge

Since the $\alpha 8\beta 1$ integrin mediates nephronectin binding, we examined whether APM anchorage to the bulge was also disrupted in $\alpha 8$ integrin (*Itga8*) knockout mice (Muller et al., 1997). H&E-stained sections of adult dorsal telogen *Itga8*^{-/-} skin, like *Npnt*^{-/-} skin, did not show any gross abnormalities (Figure 5A, B). Nephronectin deposition in the bulge was normal, but nephronectin was lacking in the APM, demonstrating that the $\alpha 8$ integrin is essential for deposition of nephronectin in the APM, but not in the bulge (Figure 5K, L). *av* integrins, which, like $\alpha 8$, mediate nephronectin adhesion (Brandenberger et al., 2001), showed normal expression in the dermal papilla and APM of *Itga8*^{-/-} skin (Figure S4A–D).

Arrector pili muscles were still associated with hair follicles in *Itga8*^{-/-} skin with a small reduction in the number of APM (Figure 5C, D, S4E), but, as in the case of *Npnt*^{-/-} skin, their selectivity for the bulge was disrupted. The proportion of muscles that attached to the K15-positive, nephronectin-positive, bulge region was decreased (Figure 5E–G) and the proportion that inserted above the bulge, in the EGFL6-positive region, was increased (Figure 5H–J). The striking difference between *Npnt*^{-/-} and *Itga8*^{-/-} skin was that whereas in the absence of nephronectin $\alpha 8\beta 1$ -positive APM cells showed selectivity for the EGFL6-positive region, APM lacking $\alpha 8\beta 1$ lost their selectivity for nephronectin-positive basement membrane and inserted into both nephronectin- and EGFL6-positive regions (Figure 4Q, Figure 5M).

We conclude that the site of APM attachment to the hair follicle is determined by the combination of $\alpha 8\beta 1$ expression on smooth muscle cells and nephronectin deposition by bulge stem cells.

Epidermal Wnt/ β -catenin signalling determines regional nephronectin and $\alpha 8$ integrin expression

Since bulge-specific expression of nephronectin creates the niche for APM cell anchorage and differentiation, we next investigated the molecular mechanism of regional nephronectin expression. The two sites of epidermal nephronectin deposition, the bulge and hair germ, are sites of active Wnt/ β -catenin signalling (Lowry et al., 2005; Nguyen et al., 2009), leading us to investigate whether nephronectin is a Wnt target gene. We activated Wnt/ β -catenin signalling by topical application of 4-hydroxytamoxifen (4-OHT) to the back skin of adult telogen K14 Δ N β -cateninER mice, which express stabilised β -catenin fused with the C-terminus of a mutant oestrogen receptor under the control of the K14 promoter (Lo Celso et al., 2004). This resulted in a 4-OHT dose-dependent increase in epidermal mRNA encoding nephronectin (Figure 6A) and the bulge Wnt effectors Tcf3 and Tcf4 (Figure S5A). In contrast, *Egfl6* mRNA levels did not change (Figure 6A). Upregulation of nephronectin protein was observed in the bulge and the ectopic hair follicles of 4-OHT-treated K14 Δ N β -cateninER mice (Figure 6B, C; see also Lo Celso et al., 2004), whereas deposition of EGFL6 was unaffected (Figure 6D, E).

Using UCSC Genome Browser, several conserved putative binding sites for Lef/Tcfs in the *Npnt* locus were identified. We therefore performed chromatin immunoprecipitation (ChIP) assays with an antibody to Tcf4, which, together with Tcf3, is specifically expressed in the bulge (Nguyen et al., 2009), and chromatin from cultured 4-OHT-treated K14 Δ N β -cateninER keratinocytes. One of the conserved sites (site 4) showed consistent enrichment for Tcf4 relative to the control FLAG antibody (Figure 6F), demonstrating that nephronectin is a direct target of Tcf4.

We then analyzed the expression of nephronectin in K14 Δ NLef1 mice that express N-terminally deleted Lef1, which lacks the β -catenin-binding site, under the control of the K14

promoter (Niemann et al., 2002). Δ NLef1 acts as a dominant-negative inhibitor of Wnt/ β -catenin signalling by blocking formation of β -catenin/Lef/Tcf complexes. We separated adult bulge and other basal keratinocytes from K14 Δ NLef1 transgenic mice by FACS and examined the expression levels of nephronectin by Q-PCR. In bulge keratinocytes, nephronectin expression was decreased, whereas in non-bulge keratinocytes, expression was upregulated (Figure 6G). Consistent with this, in K14 Δ NLef1 skin, nephronectin deposition in the bulge and hair germ was decreased, while nephronectin deposition in the interfollicular epidermis was increased (Figure 6H, I).

We also examined the effects of BMP and Notch signalling on nephronectin expression (Figure S5B, C). Inhibition of BMP signalling by expression of the BMP antagonist Noggin under the control of the K14 promoter (Sharov et al., 2009) increased nephronectin expression in the bulge. However, activation of Notch signalling by expression of the Notch intracellular domain via the K14 promoter (Estrach et al., 2006) did not affect nephronectin expression. The upregulation of nephronectin in K14Noggin skin fits well with the upregulation of Wnt signalling that occurs on BMP inhibition in this model (Sharov et al., 2009). These results indicate that activation of Wnt/ β -catenin signalling in the bulge and hair germ induces nephronectin expression, whereas Wnt/ β -catenin signalling in the interfollicular epidermis normally suppresses nephronectin.

To determine whether epidermal β -catenin activation was sufficient to induce α 8 β 1 expression in adjacent dermal cells, we examined 4-OHT treated mice expressing the Δ N β -cateninER transgene. When Wnt/ β -catenin signalling was activated in K14 Δ N β -cateninER transgenic mice, α 8 integrin was ectopically expressed by dermal cells adjacent to ectopic hair follicles, co-localizing with nephronectin (Figure 6J–L). When β -catenin was selectively activated in bulge stem cells by 4-OHT-treatment of K15 Δ N β -cateninER mice (Baker et al., 2010), nephronectin was upregulated in the bulge and there was a corresponding increase in bulge-associated α 8-integrin expressing dermal cells (Figure 6M, N). These observations establish that epidermal Wnt/ β -catenin signalling induces expression of α 8 integrin in adjacent dermal cells.

Taken together, our data show that nephronectin is a Wnt/ β -catenin target gene and that Wnt/ β -catenin signalling in epidermis determines not only region-specific nephronectin deposition but also, as a result, the location of α 8 integrin expressing mesenchymal cells.

Discussion

It has previously been suggested that heterogeneity in basement membrane composition may help to establish distinct stem cell niches; however, direct evidence has been lacking (Akiyama et al., 1995; Fuchs, 2008; Hall and Watt, 1989; Scadden, 2006; Spradling et al., 2001; Watt and Hogan, 2000). Our study is the first to identify how local variation in epidermal basement membrane composition is established and to demonstrate that the specific composition of the bulge ECM not only provides a specialized environment for bulge stem cells, but also for adjacent mesenchymal cells (Figure 7).

Sox9-positive bulge stem cells are specified in early hair follicle morphogenesis, just after birth (Nowak et al., 2008). This coincides with restriction of nephronectin deposition to the bulge basement membrane and an associated accumulation of α 8 integrin-positive mesenchymal cells, precursors of the APM. Thus, the bulge and the niche for smooth muscle progenitors are established simultaneously. Nephronectin expression is confined to the bulge basement membrane throughout adult life, irrespective of the hair cycle, and consistent with the permanent association of the APM with the bulge.

We found that recombinant nephronectin not only supported adhesion of $\alpha 8$ integrin-positive fibroblasts from neonatal dermis, but also induced expression of smooth muscle differentiation markers, consistent with reports that $\alpha 8$ integrin signalling maintains differentiation of vascular smooth muscle cells (Zargham and Thibault, 2006; Zargham et al., 2007). It therefore seems likely that during skin development APM cells differentiate from mesenchymal progenitors through adhesion to nephronectin deposited in the early bulge. The synergistic effect of nephronectin and laminin, another component of the bulge basement membrane (Figure S1B), in inducing smooth muscle markers is consistent with the positive role of laminin in intestinal smooth muscle differentiation (Bolcato-Bellemin et al., 2003).

Deletion of nephronectin resulted in upregulation of the nephronectin related protein, EGFL6, and a corresponding change in the arrector pili muscle insertion site from the bulge to the EGFL6-positive zone above. The LFEIFEIER motif that mediates the high affinity interaction of nephronectin with $\alpha 8\beta 1$ integrin is lacking in EGFL6 (Osada et al., 2005; Sato et al., 2009) and this is the likely explanation for the specific association of $\alpha 8$ -positive cells with nephronectin in wild type skin. On deletion of the $\alpha 8$ integrin, nephronectin was still expressed in the bulge, but the specificity of APM association with nephronectin was lost, and muscles inserted into both the nephronectin-positive and EGFL6-positive zones. This is probably because, in the absence of $\alpha 8\beta 1$, adhesion to nephronectin is mediated via the αv integrins, which is a lower affinity interaction (Brandenberger et al., 2001; Figure S4).

While the APM is known to attach to the bulge via a tendon, the tendon cells have not been identified (Barcaui et al., 2002; Guerra Rodrigo et al., 1975). We showed that the tendon/ligament extracellular matrix protein periostin (Horiuchi et al., 1999; Norris et al., 2007) was strongly expressed in bulge stem cells and deposited locally around the bulge. Furthermore, the gene signature of bulge stem cells includes many tendon-related genes, such as *Scx* (scleraxis), *Mitf*, *Igfbp5*, *Fbln1* (fibulin-1), *Postn* (periostin), *Tnc* (tenascin-C), *Sparc*, *Igfbp6*, and *Fgf18* (Brent et al., 2003; Jelinsky et al., 2010; Morris et al., 2004; Tumber et al., 2004). We therefore propose that bulge stem cells function as tendon cells in providing a physical connection for the APM. The immobility of bulge stem cell compartment assures that APM attachment is stable regardless of the stage of the hair growth cycle.

We did not obtain any evidence that, as has been suggested, the APM determines the location of bulge stem cells (Akiyama et al., 1995; Christiano, 2004). The delocalisation of the muscle that occurred on loss of nephronectin had no clear effect on the bulge, as judged by bulge morphology and expression of keratin 15 and CD34. Thus nephronectin expression by bulge stem cells provides a niche for APM cells, but is not an essential component of the epidermal stem cell niche.

Wnt/ β -catenin signalling is well known to play a role in controlling epidermal stem cell renewal and lineage selection and in reciprocal interactions with the dermal papilla (Alonso and Fuchs, 2003; Watt and Collins, 2008). However, a role for Wnt/ β -catenin signalling in regulating the APM was previously unknown. We demonstrate that nephronectin, unlike EGFL6, is a direct target of Wnt/ β -catenin signalling. Nephronectin expression is upregulated by Wnt/ β -catenin activation, directly or via inhibition of BMP signalling, in the epidermis and, as a result, there is a corresponding upregulation of $\alpha 8$ integrin expression in the adjacent dermis.

The consequences of activating Wnt/ β -catenin signalling were context-dependent. Activation in the bulge upregulated nephronectin, whereas activation in the interfollicular epidermis did not. Conversely, expression of Δ NLef1, which inhibits Wnt signalling, stimulated nephronectin expression in the interfollicular epidermis and downregulated

expression in the bulge. A likely explanation for the region-specific effects of Δ NLef1 is that Δ NLef1 expression in the bulge inhibits β -catenin-dependent induction of nephronectin expression by Tcf3 or Tcf4, whereas in the interfollicular epidermis Tcf/Lef transcription factors are not expressed and so Δ NLef1 regulates nephronectin expression independently of the Wnt pathway (Nguyen et al., 2006).

The APM plays an important role in thermoregulation, since it is responsible for piloerection, which traps warm air at the skin surface. Piloerection is also believed to cause constriction of the sebaceous glands, aiding release of sebum onto the skin surface (Poblet et al., 2004). Furthermore, as Charles Darwin noted in 1872, involuntary erection of hairs, feathers and other dermal appendages is an evolutionarily conserved response to emotional disturbance. Malignant tumours of the arrector pili have been reported and are known as cutaneous leiomyosarcoma (Fons et al., 2009). Our results provide new insights into the mechanism of arrector pili muscle morphogenesis and answer the long-standing question of why the arrector pili muscle is constantly attached to the hair follicle bulge (Akiyama et al., 1995).

Experimental Procedures

Generation and experimental treatment of mice

Npnt knockout mice, *Itga8* knockout mice, K14 Δ NLef1, K14 Δ N β -cateninER (D2 line) and K15 Δ N β -cateninER transgenic mice have been described previously (Baker et al., 2010; Linton et al., 2007; Lo Celso et al., 2004; Muller et al., 1997; Niemann et al., 2002). The Δ N β -cateninER transgene was activated by topical application of 4-hydroxytamoxifen (4-OHT; Sigma) dissolved in acetone. Shaved back skin was treated topically with 1.5 mg of 4-OHT in 200 μ l of acetone three times per week for 2 weeks unless otherwise specified.

Gene expression analysis

For microarray analysis, CEL format files of the gene expression profiles of K15-positive mouse bulge stem cells (GSE1096) were obtained from the NCBI's GEO (Gene Expression Omnibus) web site (Morris et al., 2004) and analyzed with Genespring X10.0 (Agilent Technologies).

Immunohistochemistry and whole-mount preparations

Immunofluorescence staining of tissue sections was performed by conventional methods.

Whole-mount immunostaining of mouse dorsal skin was performed by applying the methods established for whole-mount immunostaining of mouse tail epidermis, with some modifications (Braun et al., 2003). Embryonic and adult dorsal skin was dissected and the subcutaneous fat tissue was removed. Skin was fixed with 4% paraformaldehyde/PBS for 1 hour at room temperature and blocked with a blocking buffer for 1 hour. Skin samples were incubated with primary antibodies diluted in blocking buffer overnight at room temperature, washed with 0.2% Tween 20/PBS for 4 hours and then incubated with DAPI and secondary antibodies diluted in blocking buffer overnight at room temperature. Finally, skin samples were washed with 0.2% Tween 20/PBS for 4 hours at room temperature and mounted.

Images were acquired using a Leica TCS SP5 Tandem Scanner confocal microscope. Z-stack maximum projection images of whole-mount preparations were produced using LAS AF software (Leica).

Quantitative RT-PCR

Total RNA was isolated using an RNeasy Mini kit (Qiagen) with on-column DNase I digestion. cDNA was synthesized using SuperScript III (Invitrogen). RT-PCR was performed using SYBR green super mix (ABI). The expression levels of target genes were normalized by *Gapdh* levels utilizing a standard curve method. Primers used in this study are listed in Supplemental Information.

FACS

Mouse adult dorsal telogen keratinocytes were isolated, stained for $\alpha 6$ integrin and CD34, and sorted as described previously (Silva-Vargas et al., 2005).

For dermal cell sorting, P1 newborn dorsal skin was treated with 5 mM EDTA/PBS at 37°C for 3 hours. The epidermal sheet, including hair follicles, was peeled off and discarded. The dermis was incubated in FAD medium containing 0.2% collagenase (Gibco) and 2U/ml of DNase I (Sigma) at 37°C for 1 hour. The dermal cell suspension was stained for $\alpha 8$ integrin. Cells were sorted with a FACSAria according to $\alpha 8$ integrin expression, after gating out dead cells and cells with high forward and side scatter.

Additional Experimental Procedures

These are described in Supplemental Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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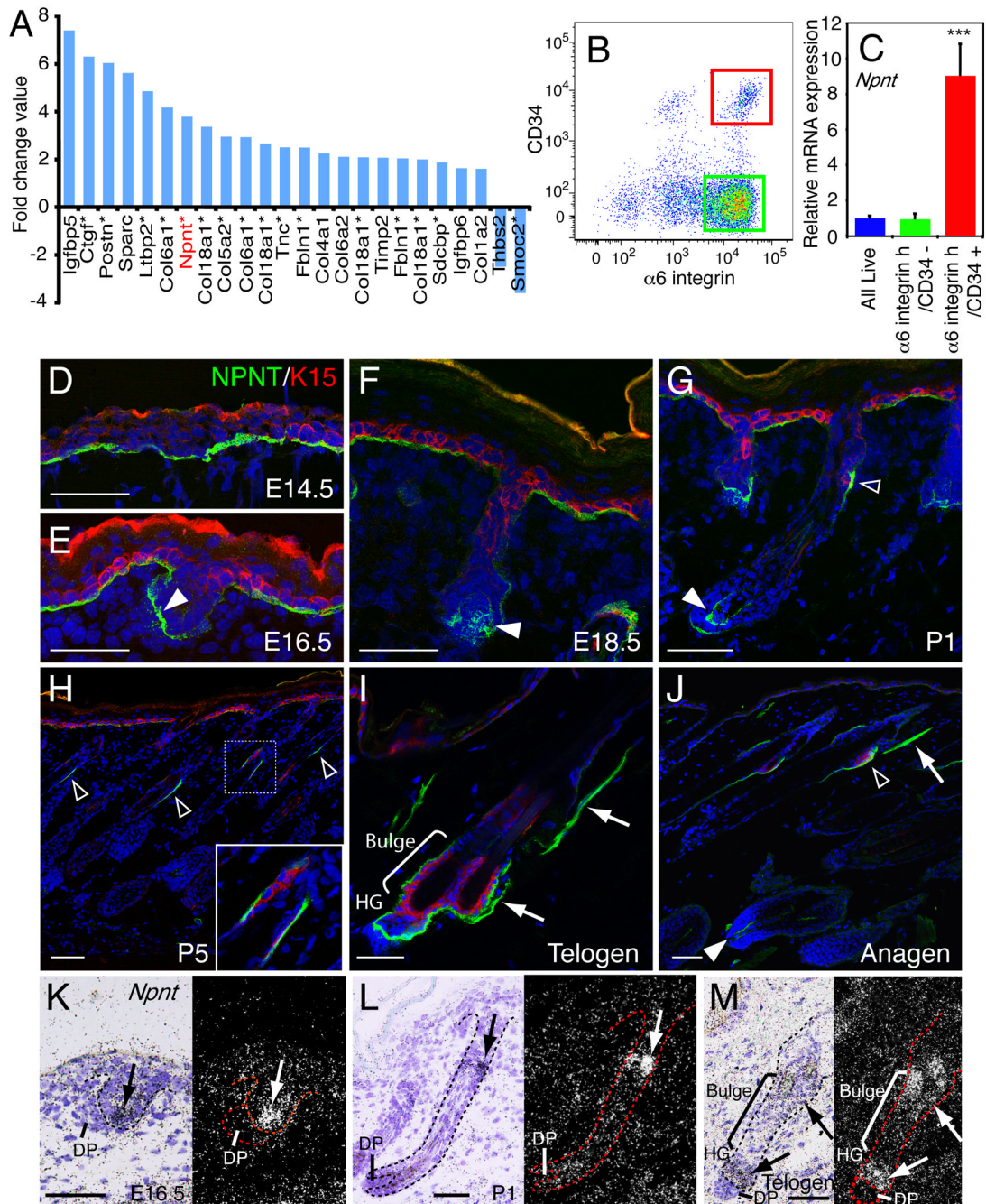


Figure 1. Nephronectin expression in skin

(A) ECM genes that are upregulated or downregulated in bulge stem cells relative to other basal keratinocytes, ranked based on \log_2 fold change value (see Table S1). Asterisks indicate the genes that are also upregulated or downregulated in mouse label retaining cells (Tables S2, S3). Some genes are listed more than once due to their multiple spots on the array. (B, C) Adult telogen dorsal keratinocytes were FACS-sorted according to $\alpha 6$ integrin and CD34 expression (B). Bulge stem cells (red gate; $\alpha 6$ integrin^{high}/CD34⁺), non-bulge basal stem cells (green gate; $\alpha 6$ integrin^{high}/CD34⁻) and all live basal cells were sorted and *Npnt* mRNA levels were determined by Q-PCR (C). Data are mean \pm SEM from three mice. (D–J) Sections of E14.5 (D), E16.5 (E), E18.5 (F), P1 (G), P5 (H), adult telogen (I) and

anagen (J) skin were immunostained for nephronectin (NPNT; green) and bulge stem cell marker K15 (red), with DAPI counterstain (blue). Note nephronectin deposition in hair germ (white arrowheads), bulge (open arrowheads) and APM (arrows). (K–M) In situ hybridization with *Npnt* probe on E16.5 (K), P1 (L), and adult telogen skin (M). Arrows indicate the strong nephronectin expression. Scale bars: 50 μ m. See also Figure S1.

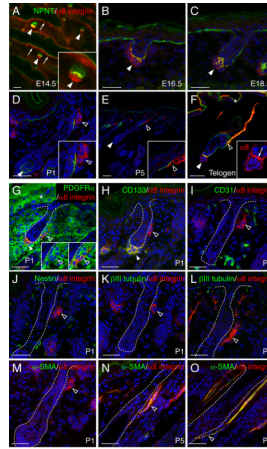


Figure 2. Nephronectin colocalizes with $\alpha 8$ integrin-positive dermal cells in dermal papilla and arrector pili muscle

(A–F) Immunostaining for nephronectin (green) and $\alpha 8$ integrin (red) in developing and adult dorsal skin. Arrows: $\alpha 8$ expression in superficial dermis (A) or at nephronectin-positive basement membrane between hair germ and dermal papilla (F); white arrowheads: dermal papilla cells; open arrowheads: $\alpha 8$ integrin-positive cells around bulge. (G–O) Expression of $\alpha 8$ integrin (red) and other dermal markers (green) in postnatal dorsal skin. Open arrowheads: $\alpha 8$ integrin-positive dermal cells adjacent to bulge. White arrowhead in G and H: dermal papilla. All sections were counterstained with DAPI (blue). Asterisk indicates non-specific staining. Scale bars: 50 μ m. See also Figure S2.

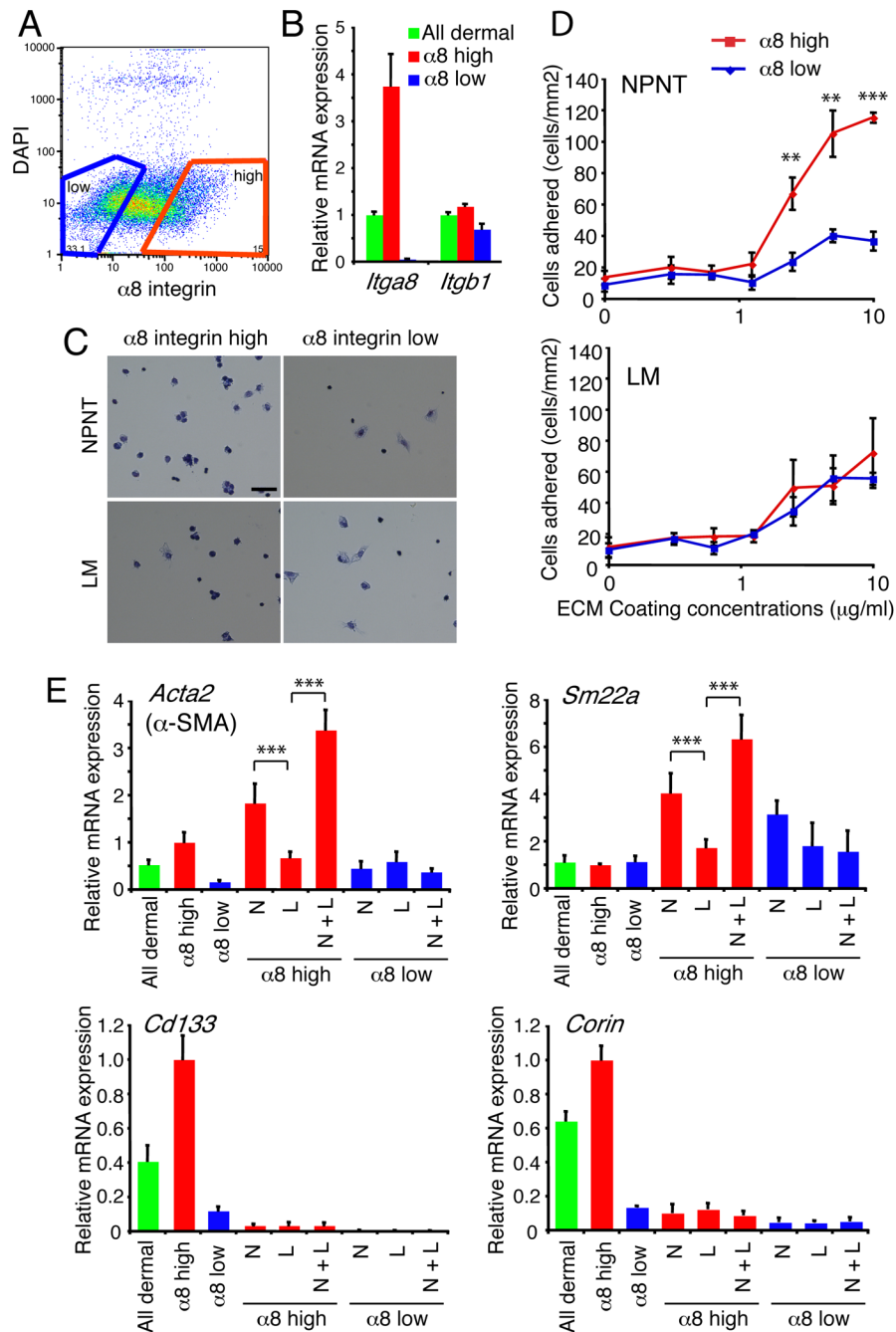


Figure 3. Adhesion and gene expression of dermal cells plated on nephronectin

(A, B) Disaggregated P1 dermal cells were FACS-sorted on the basis of $\alpha 8$ integrin levels (A), confirmed by Q-PCR for $\alpha 8$ integrin (*Itga8*) and $\beta 1$ integrin (*Itgb1*) (B). Data are means \pm SEM from three mice. (C, D) Solid-phase cell adhesion assays with FACS-sorted dermal cells plated on nephronectin (NPNT)- or laminin (LM)-coated dishes (10 $\mu\text{g/ml}$). Cells were stained with Diff Quik (C) and quantitated (D). (C) Scale bar: 50 μm . (D) Data are means \pm SEM from triplicate wells. (E) Q-PCR of smooth muscle (*Acta2* and *Sm22a*) and dermal papilla marker (*Cd133* and *Corin*) expression in unfractionated (all dermal), $\alpha 8$ integrin-high or -low populations seeded on ECM protein-coated dishes (10 $\mu\text{g/ml}$ of each protein) for 12

hours. N: nephronectin; L: laminin; N+L: nephronectin and laminin. Data are means \pm SEM from three independent wells.

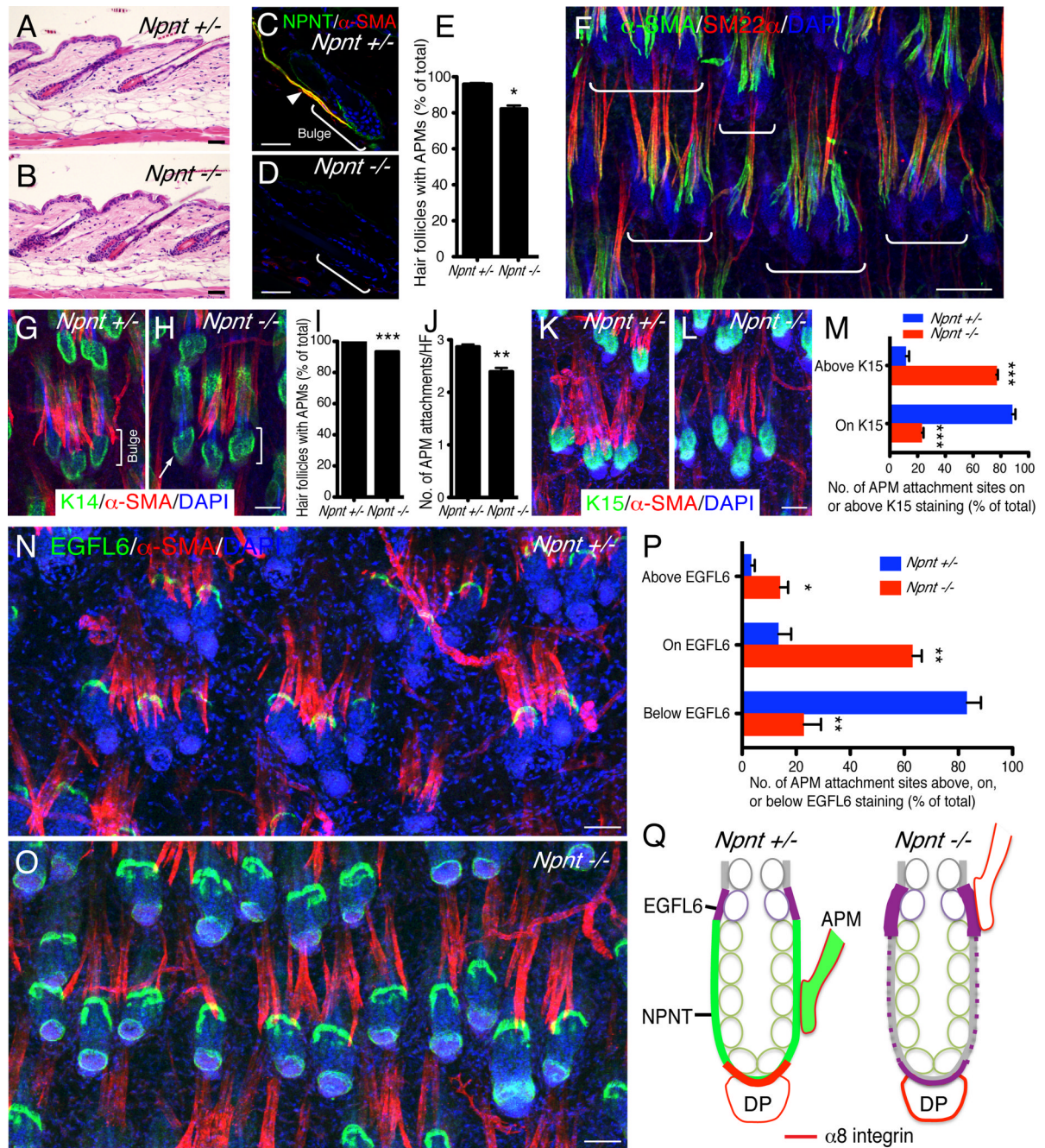


Figure 4. Nephronectin is required to anchor arrector pili muscles to the bulge

(A, B) H&E-stained adult dorsal telogen skin. (C–E) Adult dorsal skin immunostained for nephronectin (green) and α -SMA (red) and counterstained with DAPI (blue). Arrowhead indicates APM. (E) % hair follicles with arrector pili muscles. (F) Maximum projection image of dorsal wild type skin whole-mount stained for α -SMA (green), SM22 α (red), and nuclei (blue). White brackets indicate groups of hair follicles that share arrector pili muscles. (G–J) Whole-mounts (G, H) were immunostained for K14 (green) and α -SMA (red), with DAPI counterstain (blue). Arrow (H) indicates hair follicle without associated APM. Brackets indicate bulge. % follicles with arrector pili muscles (I) and number of APM attachments per hair follicle (J). (K–M) Whole-mounts were immunostained for K15 (green)

and α -SMA (red), with DAPI counterstain (blue). Position of APM attachment sites relative to K15-positive bulge was quantified (M). (N–P) Whole-mounts were immunostained for EGFL6 (green) and α -SMA (red), with DAPI counterstain (blue). Position of APM attachment sites relative to EGFL6-positive zone was quantified (P). (Q) Schematic summary of data. Green: nephronectin; purple: EGFL6; red: α 8 integrin; green circles: K15-positive bulge cells. In the presence of nephronectin, arrector pili muscles insert at the bulge. In the absence of nephronectin, EGFL6 expression in the upper bulge is increased and muscles insert in that region. α 8 integrin colocalises with nephronectin in the basement membrane of the hair germ adjacent to the dermal papilla (DP). In the absence of nephronectin EGFL6 is expressed in that region, but there is no colocalisation with α 8 integrin. All skin samples were from the back of 7 week old telogen mice. (E, I, J, M, P) Data are means \pm SEM from three mice, 100 follicles per mouse. Scale bars: 50 μ m; except for F (100 μ m). See also Figure S3.

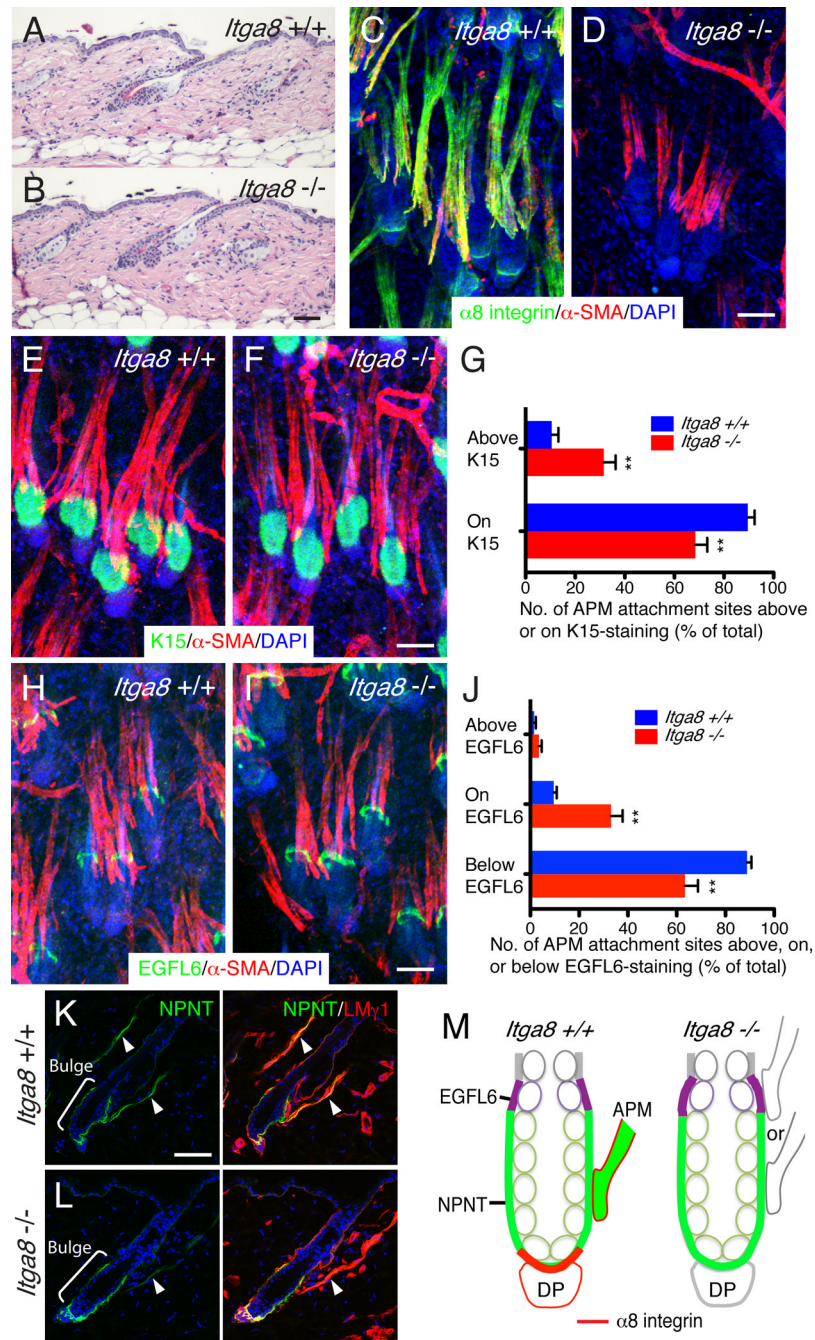


Figure 5. Deletion of $\alpha 8$ integrin disrupts specificity of APM attachment site to the bulge (A, B) H&E-stained adult dorsal telogen skin. (C, D) Skin whole-mounts were immunostained for $\alpha 8$ integrin (green) and α -SMA (red), with DAPI counterstain (blue). (E–G) Whole-mounts (E, F) were immunostained for K15 (green) and α -SMA (red), with DAPI counterstain (blue). Position of APM attachment sites relative to K15-positive bulge was quantified (G). (H–J) Whole-mounts were immunostained for EGFL6 (green) and α -SMA (red), with DAPI counterstain (blue). Position of APM attachment sites relative to EGFL6-positive zone was quantified (J). (K) Conventional cryo-sections were immunostained for nephronectin (green) and laminin $\gamma 1$ chain (red), with DAPI counterstain (blue). Arrowheads indicate APM. (M) Schematic summary of data. Green: nephronectin; purple: EGFL6; red:

$\alpha 8$ integrin; green circles: K15-positive bulge cells. In the presence of $\alpha 8$ integrin, the APM is anchored to the bulge. In the absence of $\alpha 8$ integrin, muscles lose specificity for nephronectin and can anchor to both the nephronectin-positive bulge and the EGFL6-positive upper bulge regions. In the absence of $\alpha 8$ integrin, nephronectin deposition in the APM is disrupted, but that in the bulge is unaffected. All skin samples were from the back of 7–11 week old telogen mice. (G, J) Data are means \pm SEM from four mice, 100 follicles per mouse. Scale bars: 50 μ m. See also Figure S4.

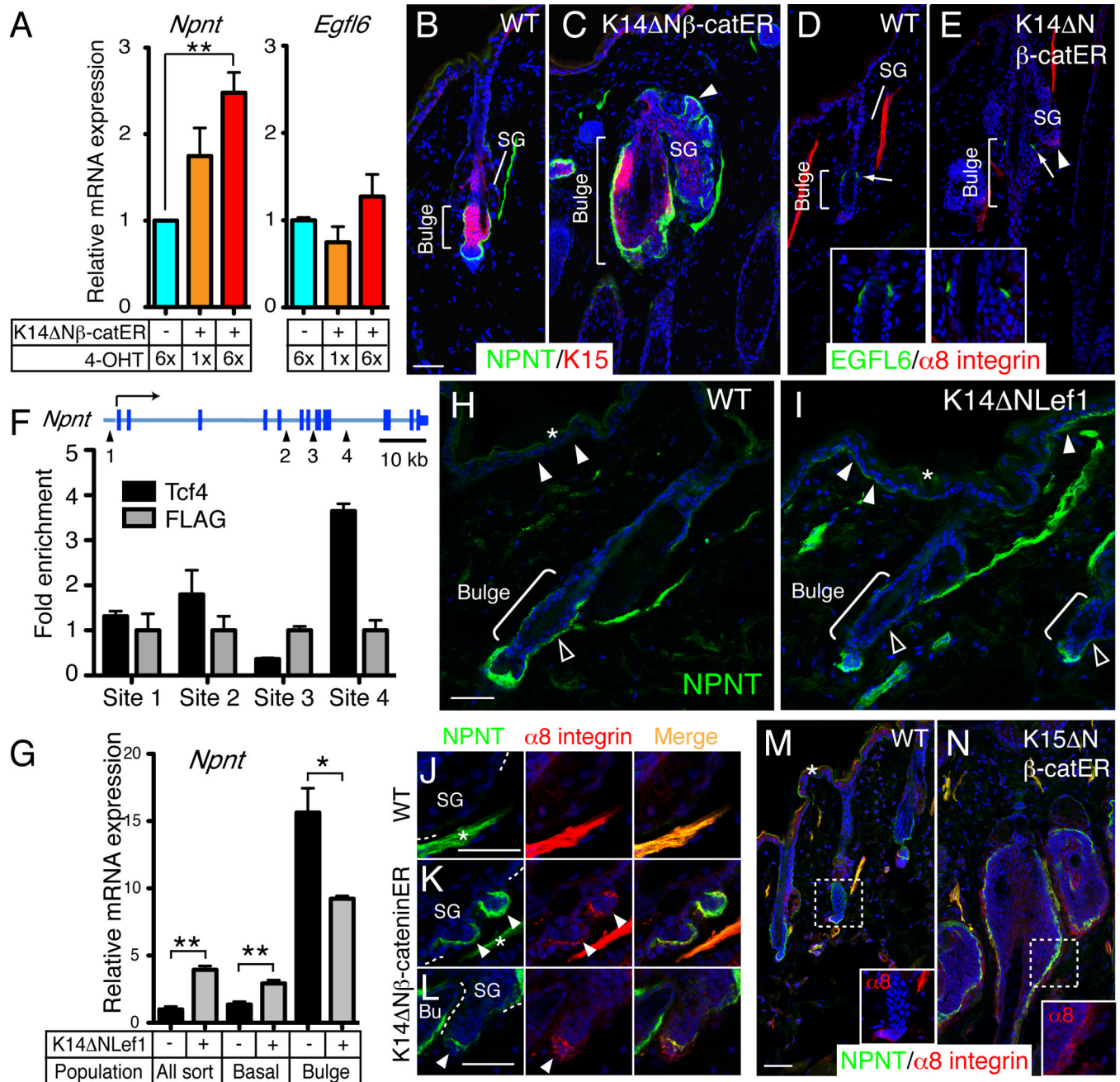


Figure 6. Regulation of regional nephronectin- $\alpha 8$ integrin interaction by Wnt/ β -catenin signalling

(A) Q-PCR of *Npnt* and *Egfl6* mRNA in keratinocytes isolated from skin of wild type ($\Delta N\beta$ -catER⁻) and K14 $\Delta N\beta$ -cateninER mice ($\Delta N\beta$ -catER⁺) that had been treated with 4-OHT for the number of times indicated. Data are means \pm SEM from three mice. (B–E) Dorsal skin of wild type and K14 $\Delta N\beta$ -cateninER mice treated 6 \times with 4-OHT and immunostained for nephronectin (green; B, C) or EGFL6 (green; D, E) and K15 (red; B, C) or $\alpha 8$ integrin (red; D, E), with DAPI counterstain (blue). Arrowheads indicate ectopic hair follicle arising from sebaceous gland (SG). Arrows indicate EGFL6-staining. Inserts show higher magnification views of EGFL6 staining around the bulge. (F) ChIP using antibodies against

Tcf4 and FLAG in K14 Δ N β -cateninER keratinocytes. Primers surrounding four conserved putative binding sites for Lef/Tcfs at the *Npnt* locus were used to detect the precipitated DNA fragments. Data are means \pm SEM of two independent experiments. (G) Q-PCR of mRNA from FACS-isolated bulge, non-bulge (basal) and total basal (All sort) keratinocytes from wild type and K14 Δ NLef1 adult telogen skin. Data are means \pm SEM from three mice. (H, I) Wild type and K14 Δ NLef1 skin immunostained for nephronectin (green) with DAPI counterstain (blue). Basement membrane of interfollicular epidermis (white arrowheads) and bulge (open arrowheads) is indicated. (J–N) Sections of wild type (J, M), K14 Δ N β -cateninER (K, L) and K15 Δ N β -cateninER (N) skin treated 6 \times (J–L) or 9 \times (M, N) with 4-OHT and immunostained for nephronectin (green) and α 8 integrin (red), with DAPI counterstain (blue). Arrowheads indicate ectopic hair follicles. Asterisks in J and K indicate arrector pili muscles. Inserts in M, N show magnified images of α 8 integrin staining around the bulge. Asterisks in H, I and M indicate non-specific staining. Scale bars: 50 μ m. See also Figure S5.

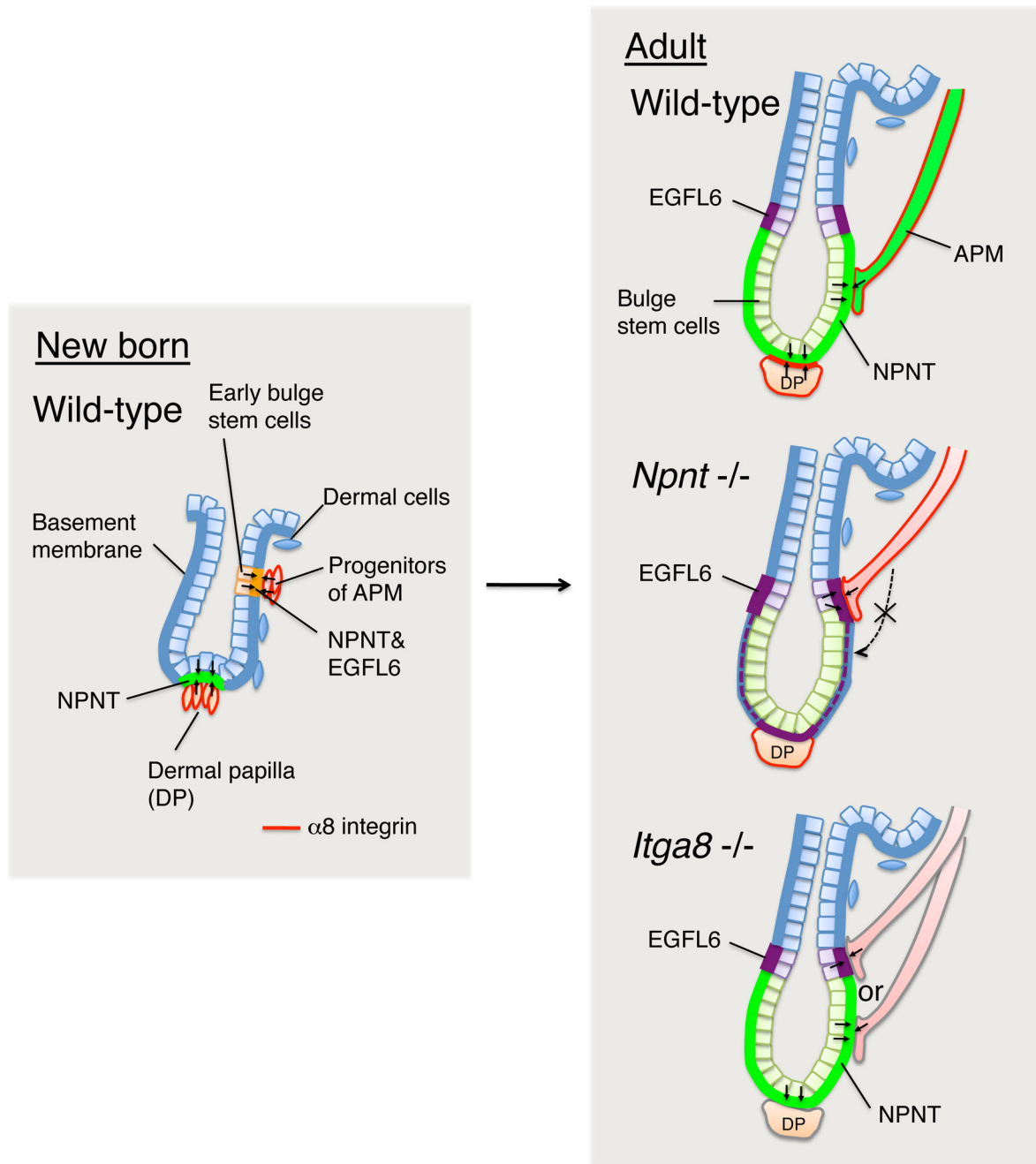


Figure 7. Model depicting role for nephronectin- $\alpha 8\beta 1$ interactions in creating the niche for the arrector pili muscle at the hair follicle bulge

During hair morphogenesis in neonatal skin, early bulge stem cells locally deposit nephronectin in the bulge basement membrane. Nephronectin induces neighbouring mesenchymal progenitors to differentiate into $\alpha 8$ integrin-positive APM cells, which adhere specifically to nephronectin, establishing a stable anchorage to the bulge that is maintained throughout adult life. In the absence of nephronectin, the APM is not anchored to the bulge, but attaches above the bulge, where there is compensatory upregulation of EGFL6. Lack of nephronectin also disturbs $\alpha 8$ integrin-mediated hair follicle-dermal papilla interactions (Figure S3). In the absence of $\alpha 8$ integrin, nephronectin is still deposited in the bulge, but

the selectivity of the APM interaction is lost and muscles are anchored both to the nephronectin-positive bulge and the EGFL6-positive upper bulge.