Current Biology *16*, 1041–1049, May 23, 2006 ©2006 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2006.04.005

Report

The miRNA-Processing Enzyme Dicer Is Essential for the Morphogenesis and Maintenance of Hair Follicles

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Summary

The discovery that microRNAs (miRNAs) play important roles in regulating gene expression via post-transcriptional repression has revealed a previously unsuspected mechanism controlling development and progenitor-cell function (reviewed in [1, 2]); however, little is known of miRNA functions in mammalian organogenesis. Processing of miRNAs and their assembly into the RNA-induced silencing (RISC) complex requires the essential multifunctional enzyme Dicer [1]. We found that *Dicer* mRNA and multiple miRNAs are expressed in mouse skin, suggesting roles in skin- and hair-follicle biology. In newborn mice carrying an epidermal-specific *Dicer* deletion, hair follicles were stunted and hypoproliferative. Hair-shaft and inner-root-sheath differentiation was initiated, but the mutant hair follicles were misoriented and expression of the key signaling molecules *Shh* and *Notch1* was lost by postnatal day 7. At this stage, hair-follicle dermal papillae were observed to evaginate, forming highly unusual structures within the basal epidermis. Normal hair shafts were not produced in the *Dicer* mutant, and the follicles lacked stem cell markers and degenerated. In contrast to decreased follicular proliferation, the epidermis became hyperproliferative. These results reveal critical roles for *Dicer* in the skin and implicate miRNAs in key aspects of epidermal and hair-follicle development and function.

Results and Discussion

Dicer and Multiple miRNAs Are Expressed in Mammalian Skin

Hair follicles develop during embryogenesis via interactions between the surface ectoderm and underlying dermal cells, and they are first apparent as a regular array of ectodermal thickenings, or placodes (reviewed in [3]). The descendants of placode cells form a lineage separate from that of adjacent surface epithelium, which develops into stratified epidermis [4]. Dermal cells are induced to cluster by placodal signals and form the hair-follicle dermal papilla, a signaling center that remains intimately associated with follicular epithelium throughout lifelong cycles of hair-follicle growth and regression. Signals from the dermal papilla to surrounding undifferentiated epithelial matrix cells cause their proliferation and differentiation into the hair shaft and inner root sheath [3]. Conversely, maintenance of dermal-papilla function requires hair-follicle epithelial signals [5]. Cyclical growth of the hair follicle is dependent on an epithelial stem cell population located in the follicle's permanent bulge region, which is established soon after birth [6]. Transient proliferation of stem cells at the onset of hair growth phases renews the rapidly proliferating matrix-progenitor-cell population [7, 8]. Hair-follicle stem cells do not contribute to the epidermis under normal homeostatic conditions, indicating that the epidermis and hair follicles remain as separate compartments in adult life [4, 9].

Bioinformatics analyses predict that conserved vertebrate miRNAs target more than 400 regulatory genes [2], suggesting that they play broad roles in biology. To date, the functions of only a handful of vertebrate miRNAs are understood (referenced in [10]). Mutations in *Dicer*, which globally disrupt miRNA processing, cause diverse developmental defects [10–12]. Loss of function of mouse *Dicer* results in early embryonic lethality [13]. Inducible deletion of *Dicer* in mouse embryonic stem (ES) cells causes defects in their proliferation, suggesting that, among other functions, *Dicer* may be essential for expansion of stem cells in the gastrulating embryo [14]; however, limited information is available regarding Dicer's functions at later stages of mammalian development [10, 15–19]. Because the epidermis and hair follicles are accessible, easy to manipulate genetically, and well characterized with respect to their morphogenesis and stem cell populations, we chose the skin as a model system to investigate the functions of *Dicer* in mammalian organogenesis.

To begin to determine whether *Dicer* plays a role in epidermal or hair-follicle development, we first asked whether *Dicer* is expressed in embryonic and postnatal skin and hair follicles. Whole-mount in situ hybridization of mouse embryos at embryonic day (E) 14.5 revealed

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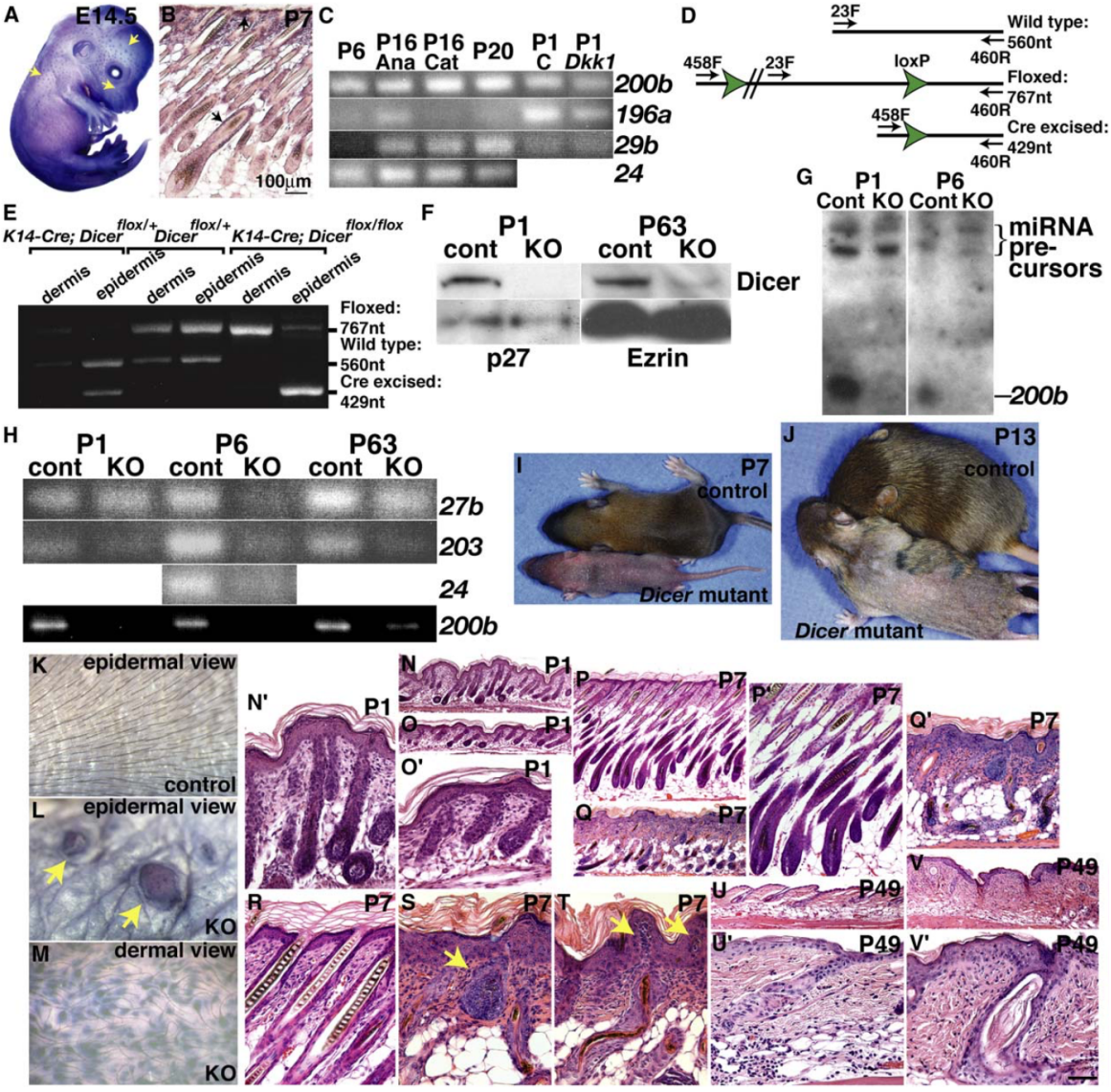

Figure 1. Expression of *Dicer* and miRNAs in the Skin and Generation of an Epidermal-Specific *Dicer* Deletion

(A) E14.5 mouse embryo subjected to whole-mount in situ hybridization with *Dicer* probe. Generalized expression is detected in the epidermis (blue staining) with elevated signal in developing hair and whisker follicles (examples indicated by arrows).

(B) Section in situ hybridization of P7 dorsal mouse skin with *Dicer* probe. Intense signal (purple-brown) is detected in the epidermis and hair-follicle outer root sheath (arrows).

(C) Semiquantitative RT-PCR analysis of expression of miRNAs *mmu-mir-200b*, *mmu-mir-196a*, *mmu-mir-29b*, and *mmu-mir-24* in mouse skin with primers specific for the mature miRNAs. The samples used were as follows: P6, isolated P6 epidermis; P16 ana, P16 posterior dorsal epidermis containing anagen-stage hair follicles; P16 Cat, P16 anterior dorsal epidermis containing catagen-stage hair follicles; P20, anterior dorsal P20 epidermis containing telogen-stage hair follicles; P1C, full-thickness P1 control dorsal skin; and P1 *Dkk1*, full-thickness dorsal skin from a P1 mouse ectopically expressing *Dkk1* in the epidermis and lacking hair follicles.

(D) Schematic depiction of PCR primers used for detection of wild-type, floxed, and Cre-excised *Dicer* alleles.

(E) Analysis of dermal and epidermal genomic DNA isolated from newborn *K14-Cre*; $Dicer^{flox/+}$, $Dicer^{flox/+}$, and *K14-Cre*; $Dicer^{flox/flox}$ mice. The $Dicer^{flox/+}$ allele is efficiently recombined in the epidermis, but not the dermis of mice carrying *K14-Cre*.

(F) Western blots of isolated dorsal epidermis at P1 (left panel) and full-thickness dorsal skin at P63 (right panel) from littermate control (cont) or *Dicer* mutant (KO) mice incubated with anti-Dicer antibody (upper panels), anti-p27 (bottom left), or anti-Ezrin (bottom right). Dicer protein is absent from KO epidermis at P1 and greatly reduced in full-thickness skin at P63.

(G) Northern blot of RNA from control and *Dicer* mutant epidermis at P1 (left panel) and P6 (right panel) hybridized with probe for *mmu-mir-200b*. miRNA precursors are detected in all lanes; the mature processed miRNA is detected in the control lanes but is absent from *Dicer* mutant

at postnatal day (P) 7 revealed prominent expression of *Dicer* in the epidermis and hair-follicle outer root sheath (Figure 1B).

To determine whether miRNAs are expressed in developing mouse skin and hair follicles, we carried out microarray analyses of miRNA expression at birth, a time point at which new hair follicles are still developing and primary hair follicles are beginning to undergo terminal differentiation. miRNA expression profiles were compared in skin from control newborn mice and skin from littermate mice that had been engineered to ectopically express the potent secreted WNT inhibitor Dickkopf 1 (DKK1) in the epidermis, resulting in complete absence of hair follicles [20, 21] (Table 1; see also Tables S1 and S2 in the Supplemental Data available online). The ten miRNAs giving the strongest hybridization signals in control newborn skin are listed in Table 1A. Several miRNAs were identified for which hybridization signals were on average more than 2.5-fold higher in control samples than in *Dkk1*-expressing samples (Table 1B), suggesting that these may be upregulated in hair follicles and/or are direct or indirect targets of WNT inhibition in the skin. Expression of *mmu-mir-200b*, *mmu-mir-196a*, *mmu-mir-29b*, *mmu-mir-27b*, and *mmu-mir-203* in control newborn skin, as well as decreased expression of *mmu-mir-200b* and *mmu-mir-196a* in *Dkk1*-expressing compared with control newborn skin, was confirmed by semiquantitative RT-PCR with primers that selectively amplify mature miRNAs (Figures 1C and 1H). Expression of all of these miRNAs and an additional miRNA, *mmu-mir-24*, was also detected in the skin at subsequent postnatal stages (Figures 1C, 1G, and 1H). Relative levels of *mmu-mir-200b* and *mmu-mir-196a* varied with the hair growth cycle, consistent with possible predominant expression of these miRNAs in hair follicles.

Table 1. miRNAs Detected with High Signal Intensity in Microarrays of Control Skin or Showing Decreased Expression in *Dkk1*-Expressing Skin

A. miRNA	Average Signal Intensity	B. miRNA	Average *n*-Fold Decrease in *Dkk1*-Expressing Skin
*mmu-mir-199a**	26.1	*mmu-mir-224*	16.6
mmu-mir-17-5p	22.3	*hsa-mir105,1,2*	5.9
mmu-mir-27a	14.7	*mmu-mir-200b*	3.7
mmu-mir-24	8.4	*mmu-mir-221*	3.7
mmu-mir-133b	7.6	*mmu-mir-200a*	3.3
mmu-mir-203	7.7	*mmu-mir-222*	2.6
mmu-mir-127	6.7	*mmu-mir-182*	2.8
mmu-mir-27b	6.5	*mmu-mir-195*	2.5
hsa-mir-106ash	6.0	*mmu-mir-15a*	2.5
mir-199as	5.4	*mmu-mir-196a*	4.1†
		mmu-mir-29b	4.0†

(A) The ten miRNAs giving the highest average signal intensities in triplicate control full-thickness skin samples at P1. Only miRNAs showing signal that was more than 2-fold above background in all three samples are listed.
(B) miRNAs showing an average decrease in signal intensity of more than 2.5-fold in *Dkk1*-expressing full-thickness P1 skin compared with control littermate skin. Background signals were subtracted before calculation of averages. With the exception of miRNAs marked †, only signals that were more than 2-fold above background in at least two of the three control samples and showed a statistically significant difference in Cy5/Cy3 ratio by a two-tailed Student's t test with $p < 0.05$ were analyzed. miRNAs marked † showed expression that was less than 2-fold above background in all three control samples.

Epidermal-Specific Deletion of *Dicer* Reduces Production of Multiple Mature miRNAs in the Epidermis and Causes Defective Hair Growth

To determine whether *Dicer* is required for development of the hair follicles or epidermis, we generated an epidermal-specific deletion of the *Dicer* gene. ES cells carrying a conditional allele of *Dicer* (*Dicer*^{flox}) in which essential exons 22 and 23 encoding the majority of both RNase III domains are flanked by loxP sites [14] were used to generate *Dicer*^{flox} mice. Cre-mediated recombination of the loxP sites in *Dicer*^{flox} yields a nonfunctional allele [14]. Mice homozygous for the floxed allele were viable and fertile with no apparent phenotype. *Dicer*^{flox} mice were crossed to a transgenic mouse line in which Cre recombinase is expressed under the control of a keratin 14 (K14) promoter, resulting in efficient deletion of target genes in the surface ectoderm, basal epidermis, and hair follicles by E14.5, the stage at which development of primary hair follicles is initiated [22].

K14-Cre; *Dicer*$^{flox/flox}$ mice were identified by genotyping of tail-biopsy DNA. Recombination of the floxed allele was confirmed by PCR analysis of DNA extracted from isolated newborn *K14-Cre*; *Dicer*$^{flox/flox}$ epidermis (Figures 1D and 1E). Western-blot analysis demonstrated that Dicer protein was absent from the epidermis in newborn *Dicer* mutant mice and was substantially reduced in P63 full-thickness skin (Figure 1F). Low levels of protein detected in mutant skin at this stage could be due to expression of Dicer in the dermis, or to less efficient *Dicer* deletion in long-surviving mutants. To determine whether lack of Dicer protein impacted mature-miRNA production, levels of selected skin miRNAs identified as described above were compared in *Dicer* mutant and control skin by northern-blot analysis and semiquantitative RT-PCR. Northern-blot analysis of

(I and J) Phenotypes of *Dicer* mutant mice with control littermates at P7 (I) and P13 (J). Note the complete lack of external hair in the *Dicer* mutant depicted at P7 (I). The mutant depicted at P13 showed a mosaic phenotype with loss of external hair over the majority of the body, but sparing the head and right flank.
(K–M) Whole mounts of dorsal skin from control littermate (K) or *Dicer* mutant (L and M) at P6 viewed from the epidermal (K and L) or dermal (M) side. Whole-mounted skin was overstained with alkaline phosphatase to reveal skin structure. Note epidermal evaginations in (L) (arrows) and failure of mutant hair shafts to penetrate the epidermis. Mutant hair follicles are misangled (M).
(N–V′) Histological analysis of *Dicer* mutant (O, O′, Q, Q′, S, T, V, and V′) and littermate control (N, N′, P, P′, R, U, and U′) skin from mice at P1 (N–O′),

mmu-mi-200b detected its precursors in control and *Dicer* mutant epidermis at P1 and P6; however, the mature miRNA was present in control but absent from *Dicer* mutant epidermis at both stages (Figure 1G). Semiquantitative RT-PCR analyses revealed reduced or absent production of multiple mature miRNAs, including *mmu-mi-200b*, in isolated skin epithelium at P6 (Figure 1H). Additionally, expression of *mmu-mi-200b* and *mmu-mir-203* was reduced or absent in full-thickness mutant skin at P1 and P63 (Figure 1H). Expression of *mmu-mir-27b* was detected at reduced levels in full-thickness mutant compared with control skin at P1 and P63 (Figure 1H). Persistence of *mmu-mir-27b* in full-thickness mutant skin at these stages might reflect inefficient *Dicer* deletion or dermal expression of this miRNA.

Newborn *Dicer* mutant mice were grossly indistinguishable from control littermates, but by P7 were stunted and lacked external hair growth (Figure 1I). Viability of the mutants was poor, with severely affected mutants dying within a few days of birth and less affected mice, including a mouse with a mosaic phenotype (Figure 1J), surviving for up to 2.5 months.

Defective Morphogenesis and Maintenance of *Dicer* Mutant Hair Follicles

Analysis of whole-mounted *Dicer* mutant skin at P6 revealed absence of external hair shafts and apparent evaginations of the epidermis (Figures 1K–1M). Hair follicles viewed from the dermal aspect of the skin were misangled and failed to display the normal anterior-posterior polarity seen in control skin (Figure 1M). Histology of *Dicer* mutant newborn skin revealed that hair-follicle growth was stunted compared to $Dicer^{flox/flox}$ or *K14-Cre*; $Dicer^{flox/+}$ littermate controls (Figures 1N–1O′). By P7, *Dicer* mutant hair follicles were misangled and wavy, weaving in and out of the plane of section (Figures 1P-1Q′). Hair-shaft structures were present but underdeveloped, and hair shafts did not extend beyond the level of the epidermis. The hair bulbs, which are usually populated by proliferating matrix cells, were much smaller than those seen in control littermate skin. Secondary, later-developing hair follicles failed to extend into the dermis (Figures 1R and 1S). A highly unusual feature of the skin at this stage was the appearance of evaginating dermal cells that became engulfed by epidermal cells (Figure 1T). The *Dicer* mutant epidermis was expanded compared to normal at P7 (Figures 1P–1T). By P49, control hair follicles were in the resting, telogen stage of the hair-growth cycle and appeared as regularly spaced uniformly oriented structures (Figures 1U and 1U′). In contrast, hair follicles had degenerated in large stretches of *Dicer* mutant skin and were replaced by cyst structures or disorganized clumps of epithelial cells within the dermis (Figures 1V and 1V′). The epidermis remained expanded at this stage (Figures 1U–1V′).

Hair-Follicle Proliferation Is Reduced, and Expression of the Progenitor Cell Marker Keratin 15 Is Absent in Newborn *Dicer* Mutant Skin

expression of markers and regulators of hair-follicle and epidermal differentiation in newborn *Dicer* mutants and littermate controls. Proliferation, assayed by either Ki67 staining or the presence of phosphohistone H3, was significantly reduced in mutant hair follicles compared with controls (1.42 ± 0.48 phosphohistone-H3-positive cells per hair follicle in control mice versus 0.71 ± 0.39 in the mutants; $p = 0.003$) (Figures 2A–2D). Epidermal proliferation at this stage was not significantly different in *Dicer* mutant and control skin (0.58 ± 0.67 phosphohistone-H3-positive cells per field at 20× magnification in the control versus 0.33 ± 0.49 in the mutant) (Figures 2C and 2D). Consistent with decreased hair-follicle proliferation, levels of phosphohistone H3 assayed by western blot of separated epidermis (containing hair-follicle epithelia) were decreased in the mutant (Figure 2M). Levels of SOX9 protein, a marker of the hair-follicle outer root sheath, were also decreased, reflecting the smaller average size of mutant follicles (Figure 2M). Epidermal stratification, judged by expression of keratins 1 and 10, was similar in *Dicer* mutant and control skin at this stage (Figures 2A and 2B and data not shown). Consistent with these data, transmission electron microscopy (TEM) of newborn *Dicer* mutant and control skin revealed that basal and suprabasal layers and stratum corneum were formed in the mutant. TEM also revealed the presence of occasional apoptotic cells in the basal layer of the mutant epidermis (Figure 2L) and in some mutant hair follicles. Quantitation of apoptotic cells revealed by TUNEL staining revealed that increased apoptosis in the developing hair-follicle bulbs did not reach the level of statistical significance (0.06 ± 0.04 TUNEL-positive cells per hair follicle in the control versus 0.12 ± 0.13 in the mutant; $p = 0.134$). However, levels of cleaved caspase 3, analyzed by immunoblotting, were markedly increased in mutant epithelial preparations (Figure 2M).

Sonic hedgehog (*Shh*) plays a key role in regulating follicular proliferation and down growth [23, 24]. In control newborn skin, *Shh* was expressed in discrete populations of matrix cells on the anterior aspects of hair follicles. *Shh* was expressed at similar levels in *Dicer* mutant newborn hair follicles (Figures 2G and 2H), indicating that decreased follicular proliferation is not a consequence of aberrant *Shh* expression. However, expression of *Shh* in mutant follicles appeared less polarized than in controls. *Notch1* regulates maintenance of inner-root-sheath precursor cells and epidermal proliferation [25, 26]. *Notch1* was expressed similarly in control and *Dicer* mutant newborn epidermis and hair follicles (Figures 2I and 2J). The signaling molecules IKKα and PI3 kinase were also expressed at similar levels in P1 control and mutant epidermis, as was RAC1, a Rho guanosine triphosphatase required for maintenance of epidermal stem cells [27] (Figure 2M).

Keratin 15 is a specific marker for hair-follicle stem cells after approximately P20, but in newborn skin it is expressed broadly in the basal layer of the epidermis [28, 29]. Expression of K15 was reduced in *Dicer* mutant newborn skin (Figures 2E and 2F). Although the significance of epidermal K15 expression is not known, ex-

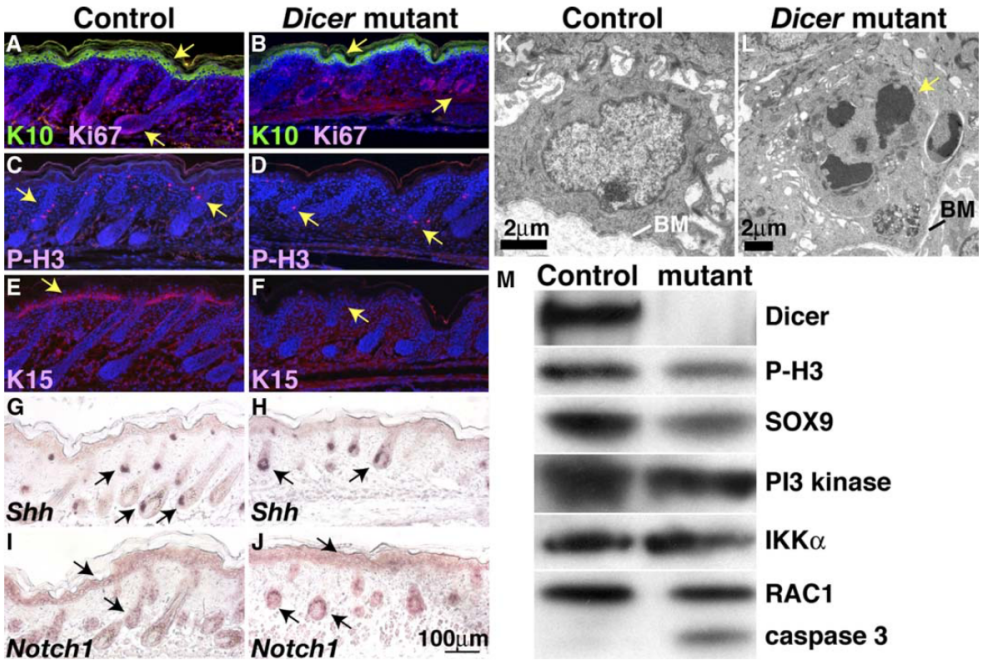

Figure 2. Expression of K15 but Not *Shh* or *Notch1* Is Affected by Loss of Epidermal *Dicer* in Newborn Skin

(A–F) Immunofluorescence of paraffin sections from newborn *Dicer* mutant (B, D, and F) and littermate control (A, C, and E) skin. (A) and (B) show immunofluorescence for the suprabasal epidermal marker K10 (green) and the proliferation-associated antigen Ki67 (red). Expression of K10 was similar in the mutant and control, but fewer Ki67-positive cells were detected in mutant hair follicles. (C and D) Immunofluorescence for the proliferation marker phosphohistone H3 (red) indicates that there are fewer proliferating cells in *Dicer* mutant than control hair follicles (arrows). (E and F) Immunofluorescence for K15 (red) reveals expression in control ([E], arrow) but not *Dicer* mutant (F) epidermis. Nuclei in panels (A)–(F) are counterstained with DAPI (blue).

(G–J) In situ hybridization of paraffin sections from newborn *Dicer* mutant (H and J) and littermate control (G and I) skin with probes for *Shh* (G and H) and *Notch1* (I and J). *Shh* is expressed similarly in control and *Dicer* newborn hair follicles (arrows in [G] and [H]). *Notch1* is expressed similarly in control and *Dicer* newborn hair follicles and epidermis (arrows in [I] and [J]). Panels [A]–[J] were photographed at the same magnification.

(K and L) TEM of newborn control (K) and *Dicer* mutant (L) skin showing apoptotic cell (arrow) next to the basement membrane (BM) in the mutant.

(M) Representative immunoblots of P1 control littermate and *Dicer* mutant epidermis incubated with antibodies to the indicated proteins. Dicer protein is absent in mutant epidermis. Expression of phosphohistone H3 (P-H3) and SOX9 is reduced and the cleaved form of caspase 3 is markedly elevated in the mutant.

K15 expression or for the survival of a subpopulation of epidermal cells that express K15. Consistent with decreased K15 expression, quantitative RT-PCR assays for CD34 mRNA, another marker for hair-follicle stem cells, demonstrated reproducible reduction in P1 *Dicer* mutant skin to 40% ± 8% of the levels detected in littermate control skin.

Two-dimensional gel electrophoresis of epidermal proteins from control and *Dicer* mutant littermates at P1 revealed an overall similar pattern of protein expression in the mutant, consistent with specific effects of *Dicer* deletion on particular cell types or pathways, rather than broad alterations in gene expression (Figure S1).

Expression of *Shh*, *Gli1*, and *Notch1* Is Lost and the Matrix-Cell Population Fails to Expand in *Dicer* Mutant Hair Follicles by P7

By P7, the *Dicer* mutant epidermis displayed a marked elevation in the numbers of both basal and suprabasal cell layers compared with control littermate epidermis. K17, and SOX9 [22, 30, 31], appeared to differentiate relatively normally in the *Dicer* mutant (Figures 3A and 3B and data not shown). Differentiation of the hair-shaft cortex and cuticle, assayed by immunofluorescence for S100A3 and AE13 [32, 33] and the inner root sheath, assayed by fluorescence for GATA3 [34] (Figures 3E, 3F, 3S, and 3T and data not shown), occurred in the *Dicer* mutant hair follicles, and these cell layers developed in a normal concentric pattern within the follicle. These data indicate that Dicer function is dispensable for the differentiation of hair-follicle matrix cells. However, the number of cells positive for each of these markers was substantially reduced in the mutant hair follicles (Figures 3E and 3F and data not shown). Proliferation in the bulb regions of P7 *Dicer* mutant hair follicles, assayed by BrdU incorporation and immunofluorescence for phosphohistone H3, was significantly reduced compared with controls (10.2 ± 1.3 BrdU-positive cells per hair follicle in control skin versus 6.8 ± 1.4 in *Dicer* mutants [p = 0.0078]; 3.3 ± 0.7 phosphohistone-H3-positive cells per hair follicle in control skin versus

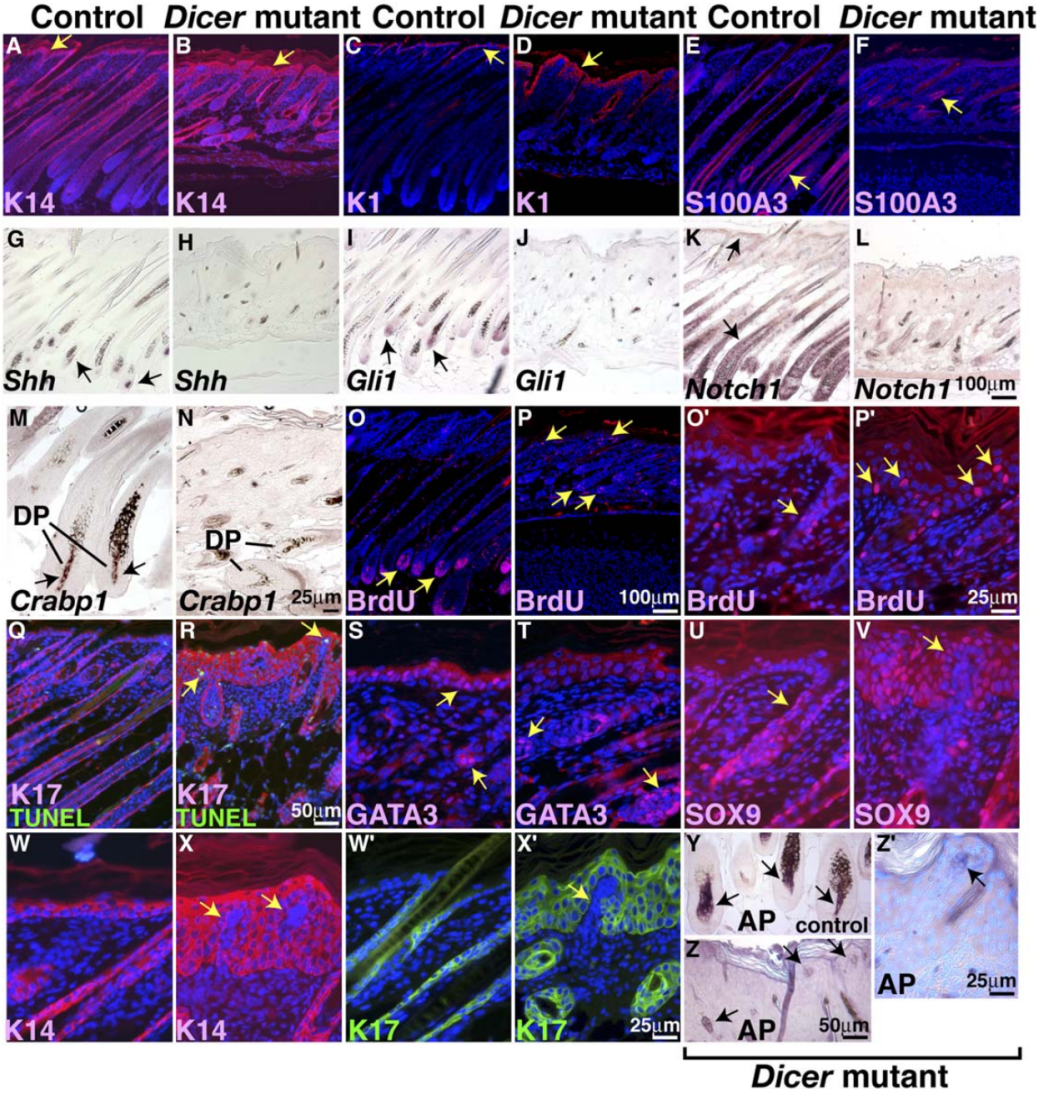

Figure 3. *Dicer* Mutant Skin at P7 Is Characterized by Lack of *Shh* and *Notch1* Expression, Proliferative Defects, and Evagination of Dermal Cells into the Epidermis

(A–F) Paraffin sections of skin from P7 *Dicer* mutant (B, D, and F) and littermate control (A, C, and E) subjected to immunofluorescence (red) for K14 (A and B), K1 (C and D), and S100A3 (E and F). Basal and suprabasal epidermal cell layers are expanded in the *Dicer* mutant. Differentiating hair-shaft cells are present in *Dicer* mutant hair follicles, but at lower numbers than in the control.

(G–N) Paraffin sections of skin from P7 *Dicer* mutant (H, J, L, and N) and littermate control (G, I, K, and M) subjected to in situ hybridization with the digoxygenin-labeled probes indicated. Positive signals appear purple brown and are indicated by arrows. Pigmented cells of the hair shaft appear black. Hybridization for *Shh*, *Gli1*, and *Crabp1* was absent in *Dicer* mutant hair follicles, and *Notch1* expression was absent from the mutant epidermis and severely reduced in hair follicles.

(O–P′) Immunofluorescence detection of BrdU in *Dicer* mutant (P and P′) and littermate control (O and O′) skin at P7. Labeled cells appear red. Their numbers are decreased in mutant hair follicles but increased in the epidermis compared with the control (arrows).

(Q and R) P7 *Dicer* mutant (R) and control (Q) skin subjected to TUNEL staining (green) and immunofluorescence for K17 (red). Note expanded K17 staining in the mutant epidermis. The frequency of TUNEL-positive cells is increased in *Dicer* mutant epidermis and hair follicles (arrows in [R]).

(S–X′) Immunoflourescence detection of GATA3 (S and T) (red), SOX9 (U and V) (red), K14 (W and X) (red), and K17 (W′ and X′) (green) in *Dicer* mutant (T, V, X, and X′) and littermate control (S, U, W, and W′) skin at P7. Note elevated SOX9 expression in *Dicer* mutant epidermis ([V], arrow) and evaginations of dermal cells into the epidermis, indicated by arrows in panels (V), (X), and (X′).

(Y–Z′) Littermate control (Y) and *Dicer* mutant (Z and Z′) P6.5 skin paraffin sections stained for alkaline phosphatase (AP) (purple). Note AP staining in control and mutant dermal papillae (arrows) and additional staining enclosed by epidermal evaginations (arrows in [Z] and [Z′]). Pigmented hair-shaft cells in (Y) appear black. Nuclei in panels (A)–(F), (O)–(X′), and (Z′) were counterstained with DAPI (blue). Panels (A)–(L), (O), and (P) were photographed at 10×; panels (Q), (R), (Y), and (Z) at 20×; and panels (M), (N), (O′), (P′), (S)–(X′), and (Z′) at 40×. DP denotes dermal papilla.

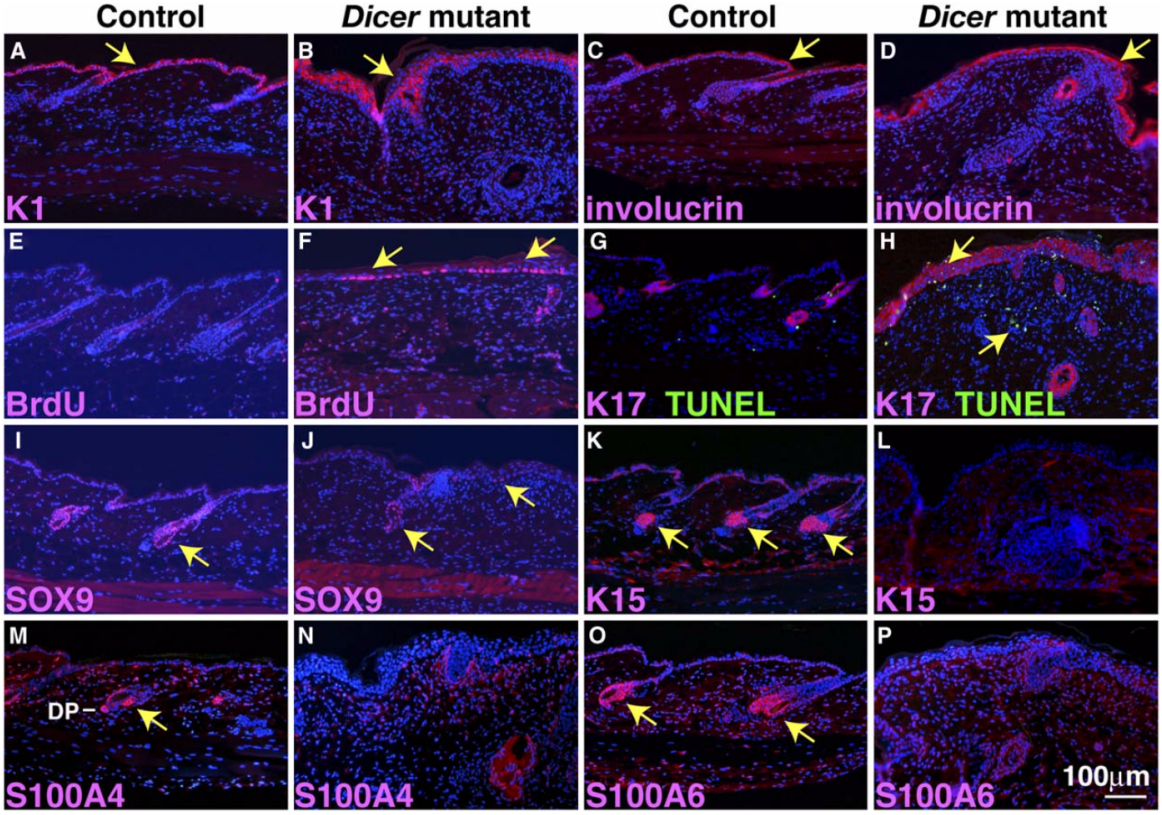

Figure 4. The Bulge Region Is Absent from *Dicer* Mutant Follicles at Later Stages

Paraffin sections of *Dicer* mutant (B, D, F, H, J, L, N, and P) and littermate control (A, C, E, G, I, K, M, and O) skin at P49 (all panels except [E] and [F]) or P63 (E and F) subjected to immunofluorescence for differentiation, proliferation, and stem cell markers and for TUNEL staining.

(A and B) K1 immunofluorescence (red) reveals expansion of suprabasal cells in *Dicer* mutant (B) compared with control (A) epidermis (arrows).

(C and D) Immunofluorescence for involucrin (red) reveals similar expression in *Dicer* mutant and control epidermis (arrows).

(E and F) Immunofluorescence for anti-BrdU (red) reveals a greater frequency of proliferating cells in *Dicer* mutant ([F], arrows) compared with control epidermis.

(G and H) Immunofluorescence for K17 (red) is specific for hair follicles in control skin (G) but in the mutant is detected at high levels in the epidermis ([H], arrow) as well as in hair-follicle remnants. TUNEL staining (green) reveals apoptotic nuclei in *Dicer* mutant (H) and control (G) skin.

(I and J) Immunofluorescence for SOX9 (red) reveals expression in control hair-follicle outer root sheath ([I], arrow) and in *Dicer* mutant hair-follicle remnants and epidermis ([J], arrows).

(K and L) Immunofluorescence for K15 (red) reveals expression in the hair-follicle stem cell containing bulge region in control follicles ([K], arrows) but not in hair-follicle remnants in *Dicer* mutant skin (L).

(M and N) S100A4 (red) is expressed in the control hair-follicle bulge and dermal papilla (DP) ([M], arrow), but specific staining is absent from *Dicer* mutant hair-follicle remnants (N).

(O and P) S100A6 is expressed in the control hair-follicle bulge ([O], arrows), but specific staining is absent from *Dicer* mutant hair-follicle remnants (P). Nuclei were counterstained with DAPI (blue). All sections were photographed at 10× magnification.

target and effector gene *Gli1* disappeared from *Dicer* mutant hair follicles by P7 (Figures 3G–3J). Similarly, *Notch1* expression was substantially reduced in *Dicer* mutant hair follicles (Figures 3K and 3L). Expression of cellular retinoic acid binding protein 1 (*Crabp1*), which normally shows specific expression in the dermal papilla [35], was absent from *Dicer* mutant follicles, indicating that loss of epithelial Dicer compromises epithelial signaling to the dermal papilla (Figures 3M and 3N). Whereas reduced *Shh* and *Notch* signaling could account for the observed defects in follicular proliferation and developmental arrest, *Dicer* function could be required either to maintain expression of these genes or to maintain the expansion or survival of the cells

immunofluorescence or BrdU incorporation, was elevated at P7 (0.5 ± 0.8 control versus 5 ± 2.4 *Dicer* mutant epidermal cells positive for phosphohistone H3 per field at 20× magnification; p = 0.008) (Figures 3O–3P′). Consistent with epidermal hyperproliferation, expression of K17 [31] was markedly increased in *Dicer* mutant epidermis (Figures 3Q, 3R, 3W′, and 3X′). Apoptosis was significantly elevated in Dicer mutant compared with control epidermis at P7 (0.2 ± 0.6 control versus 1.3 ± 0.95 *Dicer* mutant TUNEL-positive epidermal cells per field at 10× magnification; p = 0.0067) (Figures 3Q and 3R). Similar to the situation in the newborn, expression of K15 protein was reduced in *Dicer* mutant compared with control skin at P3, P6, and P7 (not

epidermis causes hyperproliferation and tumor development [26], suggesting that the observed decrease in *Notch1* expression in the *Dicer* mutant could contribute to the epidermal phenotype.

Dermal Cells Are Engulfed by *Dicer* Mutant Epidermis

A unique feature of *Dicer* mutant skin at P7 was the appearance of clusters of dermal cells apparently in the process of being surrounded by epidermal cells (Figures 3V, 3X, 3X′, Z, and Z′). Consistent with engulfment of dermal cells by the epidermis, nests of cells that failed to express K14 or K17 were detected within the expanded basal epidermis (Figures 3X and 3X′). Occasionally these cells could be detected apparently being extruded from the epidermis. Because *K14-Cre* does not cause recombination of the $Dicer^{flox}$ allele in dermal cells (Figure 1E), this phenotype is due to Dicer deficiency in the epidermis or hair follicle epithelium.

The abnormal, engulfed dermal cells were tightly clustered within the epidermis and were positive for the dermal-papilla marker alkaline phosphatase (Figures 3Y–3Z′), suggesting that they could comprise dermal papillae from later developing hair follicles that migrate outwards, rather than into the dermis in association with hair-follicle epithelium. Alternatively, these dermal-papilla-like cell clusters could be induced by abnormal signaling from the epidermis. Epithelial cells surrounding the dermal structures expressed SOX9, a marker for the hair-follicle outer root sheath (Figures 3U and 3V), suggesting either a partial fate change in the epidermal cells or migration of outer-root-sheath cells into the epidermis in association with the condensed dermal structures. Evagination of tooth and whisker-follicle epithelium, which would normally invaginate into the dermis, occurs in embryos lacking IKKα via an unknown signaling pathway that does not involve NF-κB [36]. However, IKKα protein levels were unaffected or slightly elevated in Dicer mutant skin at P1 and P6 (Figure 2M and data not shown).

Dicer Mutant Hair Follicles Are Not Maintained

Dicer mutant epidermis analyzed at P49 or P63 was thickened, with increased numbers of both basal and suprabasal layers, indicated by immunofluorescence for K14 and K1 (Figures 4A and 4B and data not shown). Involucrin was expressed in mutant epidermis, indicating that terminal differentiation of the epidermis was relatively normal (Figures 4C and 4D). The epidermis was hyperproliferative, evident histologically (Figure 1V′) and by assaying for BrdU incorporation (1 ± 1 control versus 4.5 ± 3.4 *Dicer* mutant epidermal cells positive for BrdU per field at 20× magnification at P63; $p = 0.021$) (Figures 4E and 4F). TUNEL staining revealed apoptotic cells in *Dicer* mutant skin, both in the epidermis and associated with hair-follicle remnants (Figures 4G and 4H), but the overall levels of epidermal apoptosis at P63 were not statistically significantly different than those observed in control skin (0.33 ± 0.82 control versus 0.83 ± 1.17 *Dicer* mutant TUNEL-positive epidermal cells per field at 10× magnification; $p = 0.076$). Nuclei immunofluorescence for the hair-follicle epithelial stem cell marker K15 [28, 29] and the hair-follicle bulge markers S100A4 and S100A6 [37, 38] was absent from hair-follicle remnants and cysts (Figures 4K–4P). Similarly, CD34 mRNA expression, assayed by quantitative RT-PCR, was reduced in *Dicer* mutant skin to 15% ± 7% of control levels at P63. S100A4 also marks hair-follicle dermal papillae (Figure 4M). Dermal papillae were not detected associated with the hair-follicle remnants, suggesting that the epithelial signals required to maintain these structures [5] were not produced. Reduced or absent expression of K15, CD34, and other bulge-cell markers in *Dicer* mutant skin despite persistent expression of the outer-root-sheath markers SOX9 and K17 is consistent with failure of specification or apoptosis of the hair-follicle stem cell population.

Conclusions

Loss of epithelial Dicer produces several distinct defects in the skin, affecting both the epithelium and epithelial-mesenchymal signaling. These phenotypes include absence of hair-follicle stem cell marker expression, failure of dermal papilla and hair-follicle maintenance, and epidermal evagination of clusters of dermal-papilla-like cells. In addition, hyperproliferation in the absence of significantly increased apoptosis in older mutant epidermis suggests that aging *Dicer* mutant skin might be prone to developing tumors. Our findings that multiple miRNAs are expressed in postnatal mammalian skin and that production of mature miRNAs is reduced or absent in Dicer-deficient epidermis suggest that failure of miRNA processing contributes to the *Dicer* mutant skin phenotype. Our data thus reveal the existence of a previously unsuspected mechanism for post-transcriptional regulation in the skin and pave the way for identification of specific miRNAs required for critical aspects of hair-follicle and epidermal morphogenesis and maintenance.

Supplemental Data

Supplemental Data include one figure, two tables, and detailed Experimental Procedures and can be found with this article online at: http://www.current-biology.com/cgi/content/full/16/10/1041/DC1/.

Acknowledgments

The authors thank Dr. Kenji Kizawa for S100A3 antibody, Dr. Witold Filipowicz for anti-Dicer antibody, Leroy Ash for histology, Dr. Don Baldwin for miRNA array analysis, and Dr. Qian-Chun Yu for TEM. 2D-DIGE analysis was carried out by the Proteomics Core Facility of Genomics Institute and Abramson Cancer Center, University of Pennsylvania. We are grateful to Drs. George Cotsarelis and Mark Kahn for critical reading of the manuscript and helpful discussions. S.E.M. is supported by National Institutes of Health (NIH) grants R01-AR47709 and R01-DE015342. E.P.M. is an Engelhorn Scholar of the Watson School of Biological Sciences and is supported by a predoctoral fellowship from the Department of Defense (DOD) Breast Cancer Research Program (X81XWH-05-1-0256). G.J.H. is supported by funds from the DOD Breast Cancer Research Program and the NIH.

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Accession Numbers

Primary miRNA microarray data reported in this paper may be ac-

C., Glick, A., Wysolmerski, J.J., and Millar, S.E. (2004). Canonical WNT signaling promotes mammary placode development and is essential for initiation of mammary gland morphogenesis. Development *131*, 4819–4829.

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