Epidermal progenitors give rise to Merkel cells during embryonic development and adult homeostasis

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Merkel cells (MCs) are located in the touch-sensitive area of the epidermis and mediate mechanotransduction in the skin. Whether MCs originate from embryonic epidermal or neural crest progenitors has been a matter of intense controversy since their discovery >130 yr ago. In addition, how MCs are maintained during adulthood is currently unknown. In this study, using lineage-tracing experiments, we show that MCs arise through the differentiation of epidermal progenitors during embryonic development. In adults, MCs undergo slow turnover and are replaced by cells originating from epidermal stem cells, not through the proliferation of differentiated MCs. Conditional deletion of the *Atoh1/Math1* transcription factor in epidermal progenitors results in the absence of MCs in all body locations, including the whisker region. Our study demonstrates that MCs arise from the epidermis by an Atoh1-dependent mechanism and opens new avenues for study of MC functions in sensory perception, neuroendocrine signaling, and MC carcinoma.

Introduction

The skin is an essential barrier that keeps fluids in and microbes out and is also a sensory organ, allowing animals to perceive temperature, pressure, and noxious aspects of their surrounding environment (Blanpain and Fuchs, 2006). These sensations are perceived by specific receptors expressed by different cell types located in the skin such as keratinocytes, Merkel cells (MCs), and free nerve endings and are subsequently propagated by sensory neurons of the dorsal root and trigeminal ganglia to the cells of the central nervous system, allowing animals to respond to various environmental stimuli (Lumpkin and Bautista, 2005).

MCs are neuroendocrine cells present in the basal layer of the epidermis of vertebrates (Moll et al., 2005; Boulais and Misery, 2007; Lucarz and Brand, 2007). MCs are clustered in touch-sensitive zones of the glabrous and hairy skin, called touch domes, and are densely innervated by slowly adapting type I mechanoreceptor nerve fibers. MCs express intermediate filaments of primitive and simple epithelia such as keratin 8 (K8), K18, or K20 but also express neuropeptides and many components of the presynaptic machinery such as synaptotagmin or Rab3c and transcription factors involved in neuronal cell fate determination (Haeberle et al., 2004). An electrophysiological study demonstrated that MCs are excitable cells (Yamashita et al., 1992) that express voltage-gated channels, inducing calcium influx in response to depolarization (Haeberle et al., 2004). Selective destruction of MCs by photoablation (Ikeda et al., 1994) or their loss in mice genetically deficient for MCs (Maricich et al., 2009) abolishes responses of slowly adapting type I mechanoreceptor units, which is consistent with the requirement of MCs to mediate slow adapting mechanotransduction in the skin.

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Abbreviations used in this paper: cKO, conditional knockout; CREER, estrogen receptor–inducible CRE; CREPR, Cre progesterone receptor fusion protein; HF, hair follicle; MC, Merkel cell; NCC, neural crest cell; P-cadherin, placental cadherin; SC, stem cell; TAM, tamoxifen.

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The developmental origin of MCs has remained controversial since their discovery in 1875 (Moll et al., 2005; Boulais and Misery, 2007; Lucarz and Brand, 2007). One hypothesis suggests that MCs are derived from neural crest cells (NCCs; Winkelmann, 1977) because MCs are excitable cells that synthesize neuropeptides and express presynaptic molecules and proneural transcription factors cells like many other neural crest–derived cells. In addition, lineage-tracing experiments in quails (Grim and Halata, 2000) and in mice (Szeder et al., 2003) suggest that MCs originate from neural crest stem cells (SCs). A second hypothesis posits that MCs originate from epidermal progenitors. Indeed, MCs reside in the basal layer of the epidermis and express keratins of simple epithelia like K8, K18, and K20 (Moll et al., 1996a). Further evidence against the neural crest origin of MCs is their temporal appearance. MCs are present in the epidermis before the appearance of other neural crest derivatives such as nerve endings of the skin (Narisawa and Hashimoto, 1991; Cheng Chew and Leung, 1994; Vielkind et al., 1995). In humans, MCs are identifiable and transplantable several weeks before nerves reach the fetal epidermis (Moll et al., 1986, 1990; Moll and Moll, 1992), suggesting that MCs do not originate from NCCs.

In this study, we investigated the developmental origin and adult maintenance of MCs as well as the genes involved in MC specification. Lineage-tracing experiments using epidermal-specific CRE demonstrated that all MCs, including MCs of whiskers and touch domes of the foot, are derived from epidermal cells. Conditional deletion of Atoh1/Math1 in the embryonic epidermis resulted in the absence of MCs, demonstrating that Atoh1 expression in epidermal progenitors is required for MC specification at all body locations. During adult homeostasis, maintenance of the MC pool is ensured, at least in part, by the differentiation of epidermal SCs. Our study resolves a long-standing controversy regarding the developmental origin of MCs and opens new avenues to study the role of MCs in sensory perception, neuroendocrine functions, and cancer formation.

Results and discussion

MCs express markers of embryonic epidermal progenitors

MCs reside in the vibrissae and dorsal and ventral parts of the skin epidermis as well as in the paw epidermis (Moll et al., 2005; Boulais and Misery, 2007; Lucarz and Brand, 2007). Using antibodies that specifically recognize MCs (Kim and Holbrook, 1995; Moll et al., 1995; Vielkind et al., 1995), MCs were first detected in the whisker region around embryonic day (E) 15, whereas in the back skin and in the paw epidermis, MCs were detected at E17 (Fig. 1 A and Fig. S1). At postnatal day (P) 1, all MCs coexpressed K8 and K20 (Fig. 1 B).

To determine whether MCs arise from epidermal progenitors, we assessed whether early specified MCs coexpress basal embryonic epidermal progenitor markers such as K14 or K5. At E17, soon after their specification, most of the early specified MCs of the whisker (75 ± 3%) but also of the back skin (64 ± 2%) and paw epidermis (78 ± 4%) coexpressed K14 basal epidermal marker (Fig. 1 C), suggesting that MCs may arise from epidermal progenitors. These early specified MCs also expressed placental cadherin (P-cadherin), which is a marker of embryonic epidermal progenitors (Fig. 1 D). However, shortly after their specification, MCs progressively stopped expressing markers of basal epidermis (at P1, around 20% of MCs coexpressed K14), and in adulthood, MCs no longer expressed K5 or K14 (Fig. S2). The cells coexpressing K14 and MC markers during embryonic development may be the previously described transitional cells seen by electron microscopy, which were thought to represent the transition from epidermal cells to MCs (Tachibana and Nawa, 1980). Furthermore, the demonstration that early specified MCs coexpress P-cadherin, a marker of embryonic hair follicle (HF) progenitors (Rhee et al., 2006), and the absence of MC specification in Tabby mutant mice (Vielkind et al., 1995), which present defects in HF morphogenesis, suggest that HF progenitors and MC specification could be tightly linked.

MCs originate from embryonic epidermal progenitors

To determine more directly whether MCs arise from the differentiation of embryonic epidermal progenitors, we performed genetic lineage-tracing experiments using transgenic mice expressing the CRE recombinase, specifically in epidermal progenitors (K14-CRE), which genetically marks all cells derived from embryonic epidermal progenitor cells, including the interfollicular epidermis, the sebaceous gland, and their HF (Vasioukhin et al., 2001), together with the Rosa-YFP reporter transgene (Srinivas et al., 2001). All cells expressing the MC markers K8 or Rab3c from all body locations analyzed in newborn and adult mice were YFP positive (Fig. 2, A–F), whereas neural crest–derived cells such as melanocytes (Fig. 2 G) or nerve endings (Fig. 2 H) were YFP negative, indicating that MCs present at all body locations arise from the differentiation of cells expressing K14 at one time of their development.

To determine whether some MCs can arise from NCCs, as previously reported for whisker MCs (Szeder et al., 2003), we performed genetic lineage-tracing experiments using Wnt1-CRE (Chai et al., 2000) and Pax3-CRE mice, another CRE commonly used to perform fate mapping of neural crest and presomitic mesoderm derivatives in mice (Engleka et al., 2005). Analysis of YFP expression in the paw and the back skin epidermis of adult Pax3-CRE/Rosa-YFP mice showed that MCs and epidermal cells did not arise from Pax3-expressing cells. The absence of YFP expression in K8 (Fig. 3 A)- and K14-expressing cells (Fig. 3 B) demonstrates that MCs present in these regions did not originate from NCCs. Neural crest derivatives such as nerves present in the skin arose from Pax3-expressing cells, as demonstrated by the presence of YFP in neuronal filament NF200–positive cells located in the dermis of adult mice (Fig. 3 C) as well as the presence of YFP in the terminal nerve endings making contact with MCs (Fig. 3 A). Surprisingly, in the whisker region, we observed mosaic YFP expression in HF cells that were also marked by K14 and that varied greatly from animal to animal (n = 4 mice; Fig. 3 B). In one

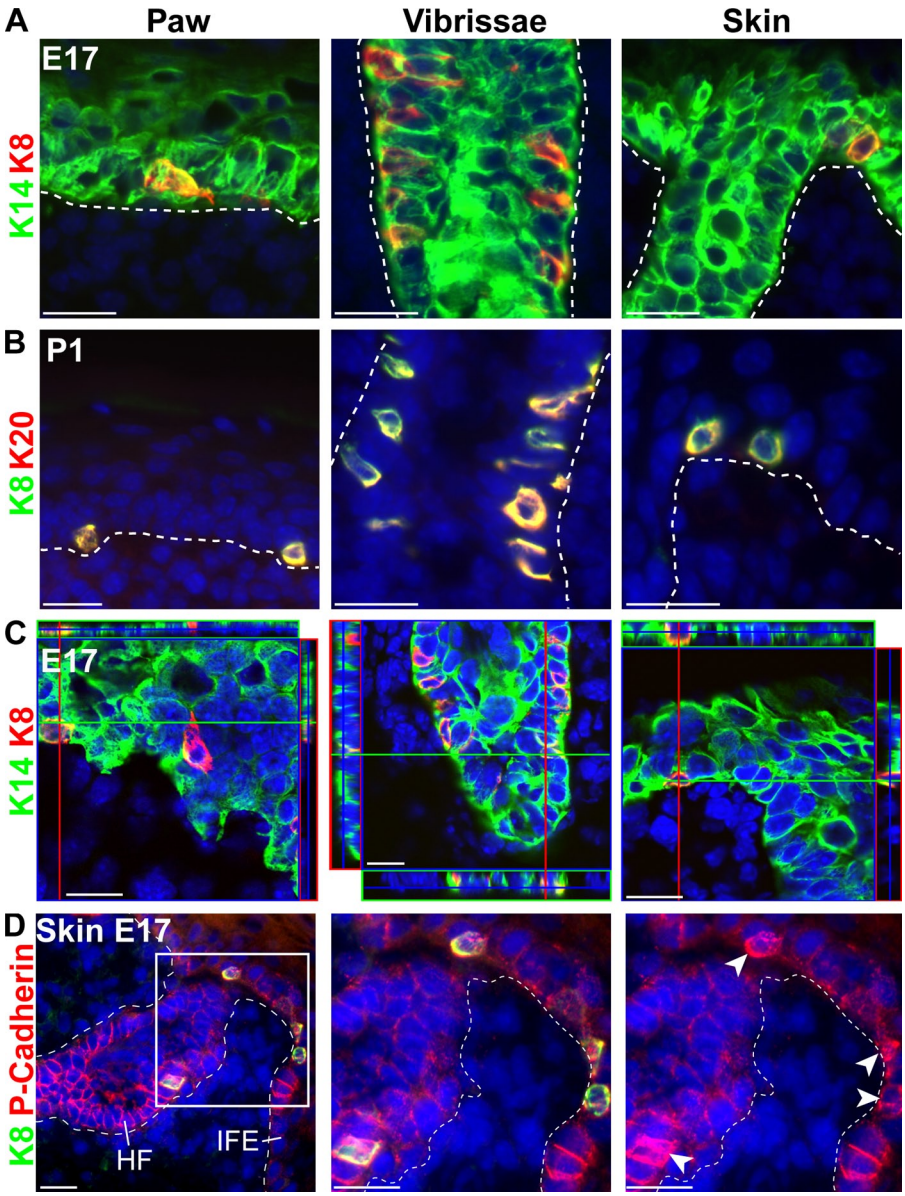

Figure 1. **MCs express epithelial markers during embryogenesis.** (A) Immunostaining of K8 and K14 performed on skin sections shows the presence of MCs in E17 mice. (B) Immunostaining of K8 and K20 performed on skin sections of 1-d-old mice. (C) Confocal microscopy analysis of K8 and K14 immunostaining on skin sections of E17 embryo shows that some MCs coexpress K8 and the epidermal marker K14. (D) Immunostaining of K8 and P-cadherin on back skin sections at E17 shows the expression of P-cadherin, an epidermal progenitor marker in MCs. The inset is magnified (middle and right). Arrowheads point to MCs. (A, B, and D) Dashed lines delineate the basal layer of the epidermis. IFE, interfollicular epidermis. Bars, 20 µm.

Pax3-CRE/Rosa-YFP mouse, no whiskers or MCs were YFP labeled, whereas in the other mice, the degree of mosaicism in the whisker varied from a few percent up to 30% of YFP-positive cells. The percentage of YFP-labeled MCs correlated perfectly with the degree of chimeric YFP expression in K14-expressing cells. Whiskers with no YFP expression in keratinocytes never presented YFP-positive MCs despite the normal expression of YFP in nerve endings (Fig. 3 A), strongly suggesting that MCs of the whisker, like MCs of the back skin and the paw epidermis, did not originate from NCCs. Wnt1-CRE is expressed at a later stage of neural crest development compared with Pax3-CRE (Chai et al., 2000; Engleka et al., 2005). In Wnt1-CRE/Rosa-YFP mice, no MCs were YFP positive in the paw and the back skin epidermis (Fig. 3 D). In the whisker region, we found that most mesenchymal cells of the face were YFP positive, as previously reported (Fernandes et al., 2004), whereas only very few whiskers (2/27) and MCs (3/261) were YFP positive (Fig. 3, D–F). These results suggest either that Pax3 and, to a lesser extent, Wnt1 are expressed in some neuroectodermal cells committed to become epidermal progenitors of the future vibrissa or that the NCCs give rise to some epidermal progenitors and MCs of the vibrissa. The variability in the chimerism between the different vibrissae within the same mouse (Fig. 3, A, D, E, and G), as well as the variability in the chimerism between different animals, is more suggestive of leaky expression of neural crest markers in some early specified epidermal progenitors rather than a fixed and predefined contribution of NCCs to the epidermal cells and MCs of the vibrissa. These results might also explain why it has been previously reported that MCs originate from NCCs (Szeder et al., 2003). With the currently available tools and these mouse models, all evidence points to MCs being derived from epidermal cells, as has been previously suggested (Moll et al., 1986, 1990; Narisawa and Hashimoto, 1991; Moll and Moll, 1992; Cheng Chew and Leung, 1994; Vielkind et al., 1995), although a minor contribution of NCCs to MCs of the whisker region could not be entirely ruled out.

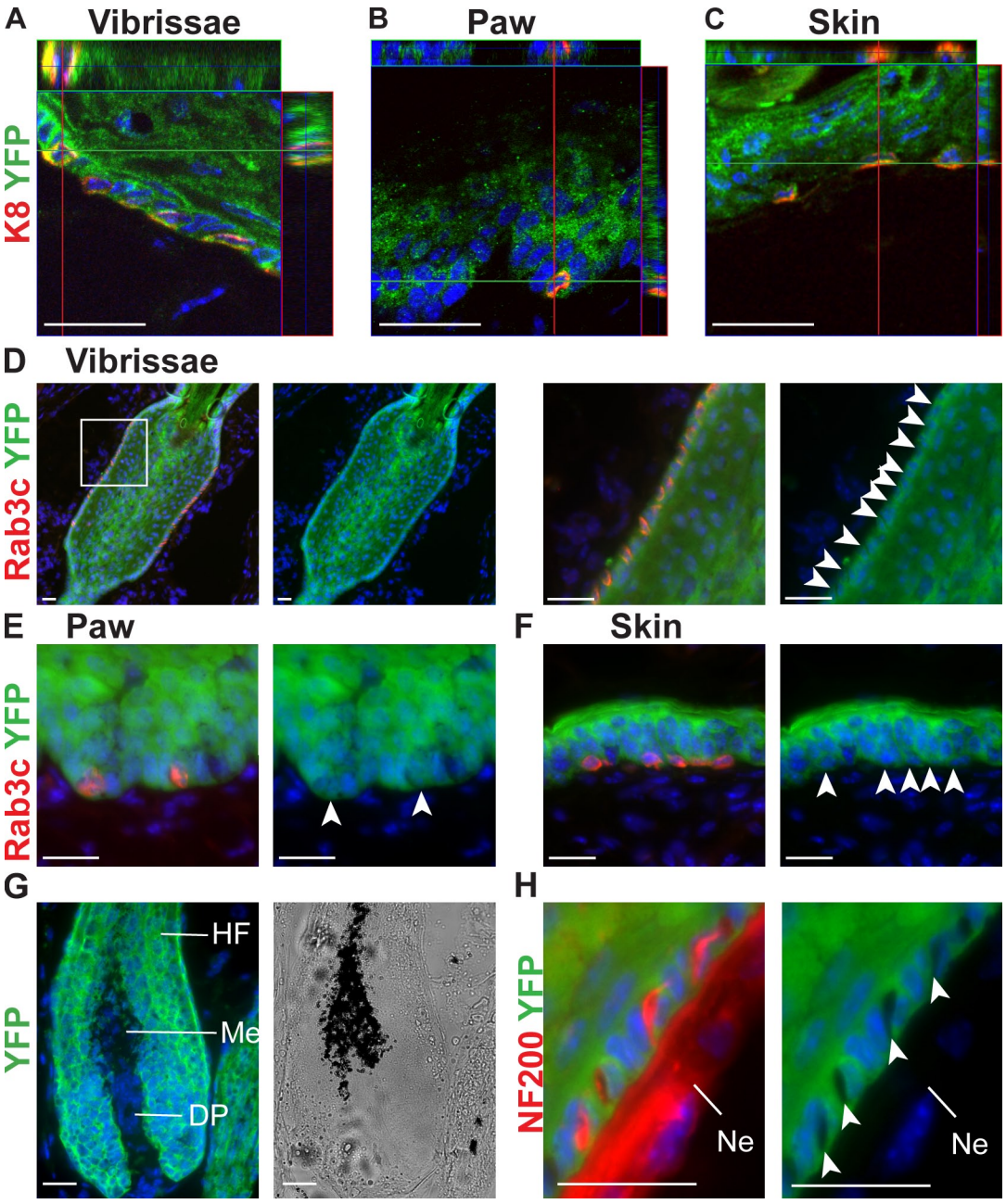

Figure 2. **MCs originate from embryonic epidermal progenitors.** (A–C) Confocal analysis of K8 and YFP immunostaining performed on vibrissae (A), paw (B), and skin (C) sections from K14-CRE/Rosa-YFP mice. (D–F) Immunofluorescence of YFP and Rab3c performed on skin sections from K14-CRE/Rosa-YFP mice. Arrowheads point to MCs. The inset in D is magnified (right). (G) Immunofluorescence of YFP performed on HF section from K14-CRE/Rosa-YFP mice (left) and bright field microscopy (right). (H) Immunofluorescence of YFP and NF200 performed on whisker section from K14-CRE/Rosa-YFP mice. Arrowheads point to nerves. DP, dermal papilla; Me, melanocytes; Ne, nerve. Bars, 20 µm.

MC turnover during adult homeostasis is ensured by epidermal progenitors

To investigate the cellular turnover of MCs during adult homeostasis, we performed clonal analysis of adult MCs using genetic fate mapping in mice. To this end, we generated transgenic mice expressing an estrogen receptor–inducible CRE (CREER) expressed in adult MCs using a promoter fragment of *K18* and crossed these mice with Rosa-YFP reporter mice. We obtained two different K18-CREER transgenic lines, in which tamoxifen (TAM) administration induced the specific labeling of adult MCs in the skin (Fig. 4 A). To assess MC turnover, we induced the expression of YFP in adult MCs in a mosaic manner (pulse) and quantified the percentage of YFP-positive MCs over time (chase). 1 wk after the administration of 15 mg TAM in K18-CREER#23/Rosa-YFP, we observed labeling of 17% (381/2162) of MCs in the vibrissa. After 3 wk of chase, the frequency of YFP-positive MCs decreased significantly, and only 9% (208/2230) of the MCs remained positive (Fig. 4 B). The decrease of YFP-labeled MCs was not the consequence of a decrease of the total number of MCs during the chase period (33 ± 2 MCs and 30 ± 1 MCs per whisker section after 1 wk and 3 wk, respectively). The progressive

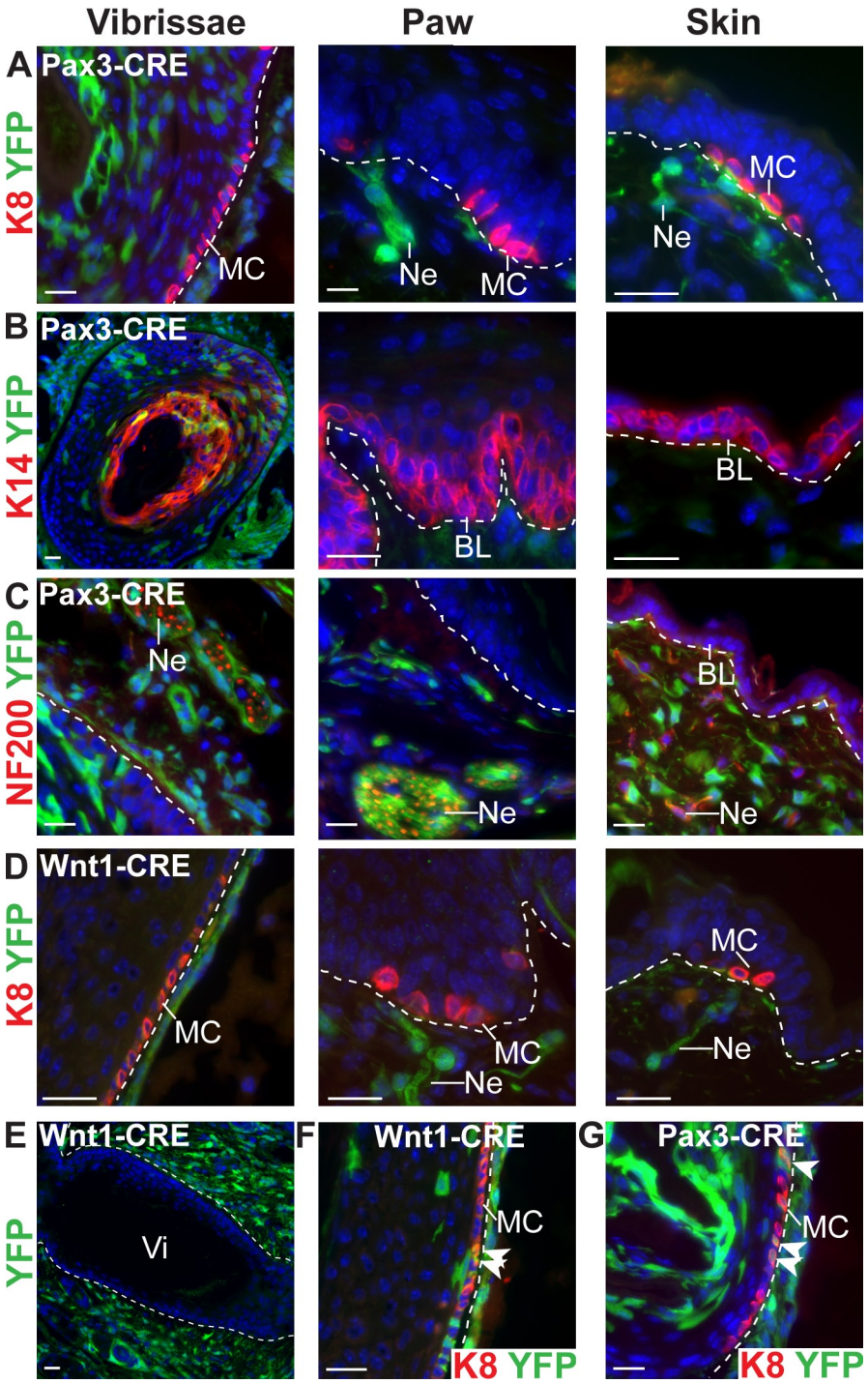

Figure 3. **MCs do not originate from the neural crest progenitors.** (A) Immunostaining of K8 and YFP performed on skin sections from Pax3-CRE/Rosa-YFP mice. (B) Immunofluorescence of K14 and YFP performed on skin sections from Pax3-CRE/Rosa-YFP mice showed that epidermal cells from the paw and back skin do not express YFP, whereas a mosaic expression of YFP could be seen in epidermal cells of the whisker. (C) Immunofluorescence of NF200 and YFP performed on skin sections from Pax3-CRE/Rosa-YFP mice. (D) Immunostaining of K8 and YFP performed on skin sections from Wnt1-CRE/Rosa-YFP mice. (E) Immunofluorescence of YFP from Wnt1-CRE/Rosa-YFP. (F and G) Immunofluorescence of K8 and YFP from Wnt1-CRE/Rosa-YFP (F) and Pax3-CRE/Rosa-YFP mice (G) showed that the percentage of MCs positive for YFP in vibrissae correlates with the degree of YFP chimerism in keratinocytes. Arrowheads point to K8– and YFP–double positive MCs. (A–E) Dashed lines delineate the basal layer (BL) of the epidermis. Ne, nerve; Vi, vibrissa. Bars, 20 μm.

loss of YFP-labeled MCs over time indicates that at least some MCs undergo a low but significant cellular turnover during adult homeostasis

To determine whether the maintenance of a steady-state number of adult MCs could be ensured by a pool of undifferentiated MCs, we determined whether some MCs proliferate during adult homeostasis. MCs are well known to be quiescent because they do not express proliferation markers (Vaigot et al., 1987; Moll et al., 1996b). To determine whether a few MCs are able to proliferate, we administrated BrdU to mice for 10 d, a protocol resulting in the labeling of most epidermal cells, including quiescent bulge SCs. Administration of BrdU for 10 d resulted in the labeling of most K14-expressing cells (Fig. 4 C), whereas all MCs of the vibrissa remained BrdU negative (Fig. 4 C), suggesting that differentiated MCs are highly quiescent during homeostasis and MC proliferation does not account for the cellular turnover observed by genetic fate mapping.

To determine whether the pool of adult MCs is maintained by epidermal progenitors or SCs, we performed lineage-tracing experiments in the whisker HF using K15–Cre progesterone receptor fusion protein (CREPR)/Rosa-YFP

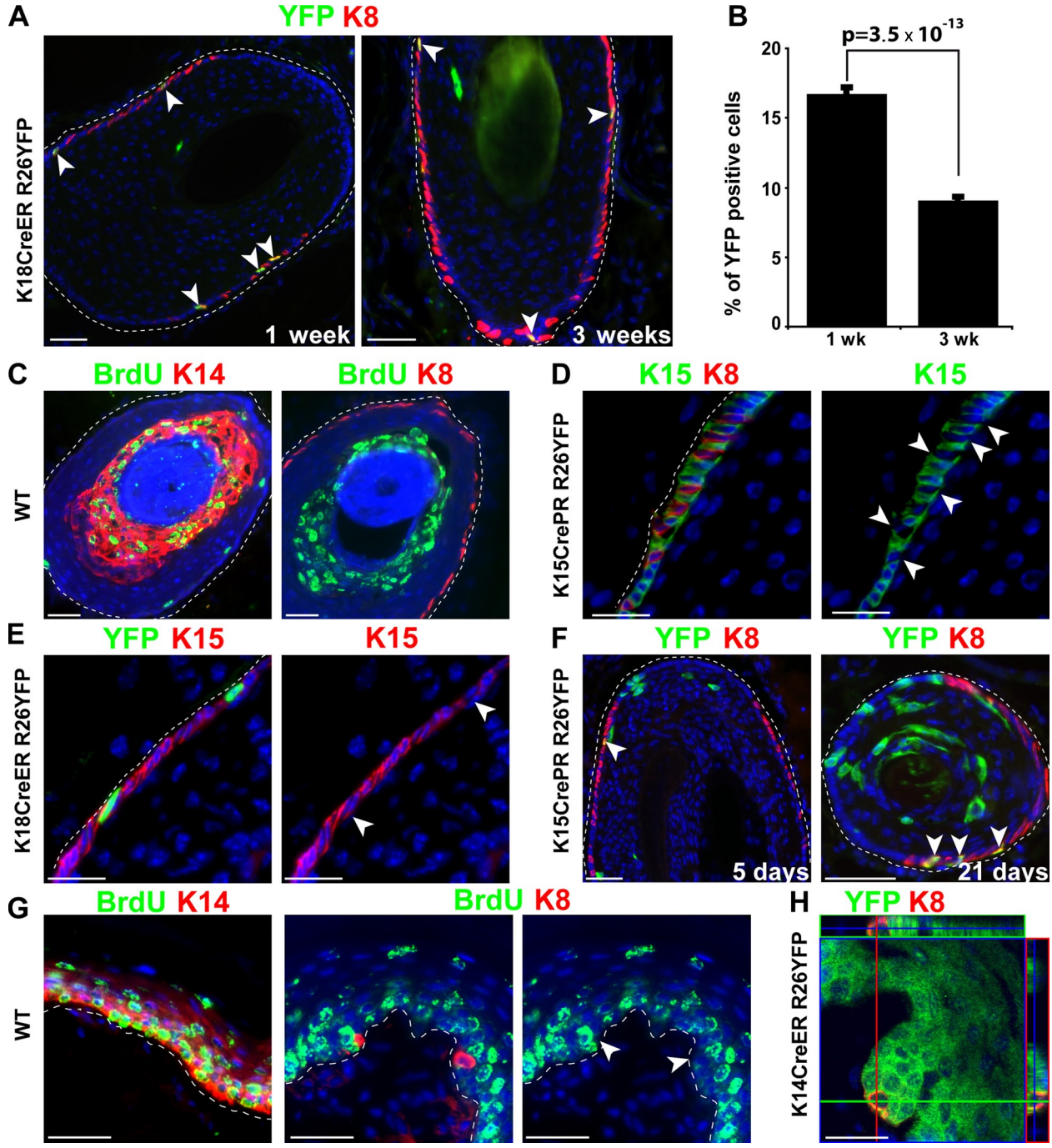

Figure 4. **MC turnover during adult homeostasis is ensured by epidermal progenitors.** (A) Immunostaining of K8 performed on vibrissa sections from K18-CREER/Rosa-YFP treated with 15 mg TAM and analyzed 1 and 3 wk after the last injection. (B) Quantification of YFP-positive cells in MCs (n = 2 mice per time point; error bars = SEM; p-value is from the paired Student's t test). (C) BrdU immunostaining in K14 (left)- and in K8 (right)-positive cells. (D) Immunostaining of K15 and K8 in the vibrissa follicle. (E) Immunostaining of K15 in vibrissa sections from K18-CREER/Rosa-YFP analyzed 1 wk after 15 mg TAM injection. (F) Immunostaining of K8 and YFP in vibrissa sections from K15-CREPR/Rosa-YFP analyzed 5 (left) and 21 d (right) after the administration of 2.5 mg RU486 per day. (G) BrdU immunostaining in K14 (left)- and in K8 (right)-positive cells. (H) Immunostaining of K8 performed on paw sections from K14-CREER/Rosa-YFP treated with TAM for 1 mo. (A and C–G) Dashed lines delineate the basal layer of the epidermis. (A and D–G) Arrowheads point to MCs (D, E, and G) and K8– and YFP–double positive MCs (A and F). WT, wild type. Bars, 20 µm.

mice, which labels HF bulge SCs and their progeny (Morris et al., 2004). In the adult vibrissa, no MCs expressed K15, as demonstrated by the absence of cells coexpressing K15 and K8 (Fig. 4 D), and K15 was not expressed in differentiated MCs targeted by the K18-CREER (Fig. 4 E). Administration of RU486 in K15-CREPR/Rosa-YFP for 5 or 21 d resulted in the induction of multiple labeled clones in basal whisker cells (Fig. 4 F). The presence of YFP-positive cells expressing K8 increased with the duration of K15-CREPR activation from 4.5% of K8/YFP-positive cells after 5 d of RU486 administration to 22.3% after 21 d (Fig. 4 F), whereas the percentage of YFP-labeled MCs relative to the total number of basal whisker

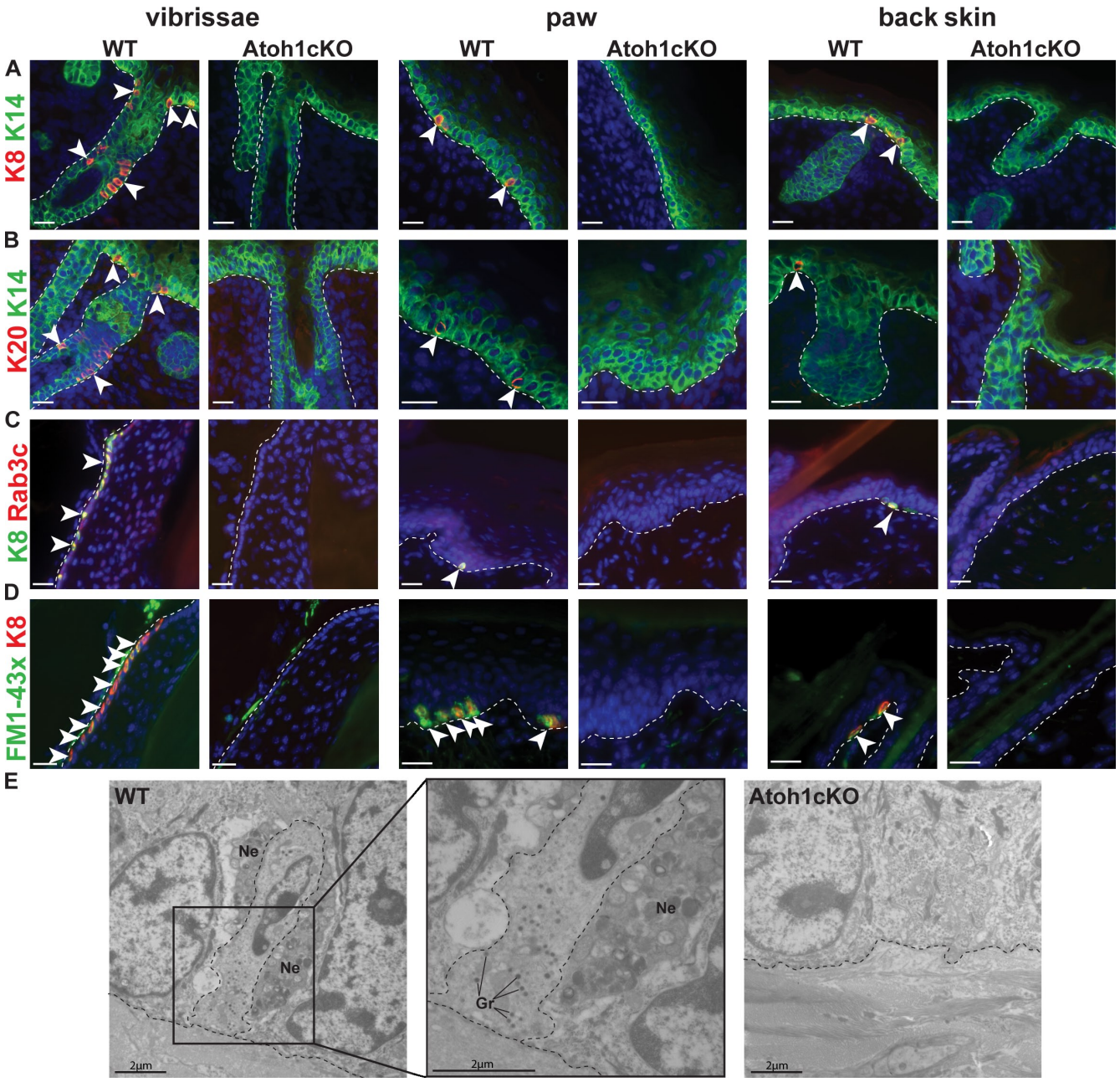

Figure 5. **Conditional deletion of *Atoh1* in embryonic epidermal progenitors results in the absence of MC specification.** (A and B) Immunofluorescence of K14 and K8 (A) and K14 and K20 (B) performed in newborn wild-type (WT) and *Atoh1* cKO mice shows the complete loss of MCs in *Atoh1* cKO mice. Arrowheads point to MCs. (C) Immunofluorescence of K8 and Rab3c in 2-mo-old wild-type and *Atoh1* cKO mice. Arrowheads point to K8– and Rab3c–double positive MCs. (D) FM1-43x administration to 1-mo-old mice resulted in the uptake of the fluorescent dye in MCs of wild-type mice but not in *Atoh1* cKO mice. FM1-43x labeling is still observed in peripheral nerve endings in *Atoh1* cKO mice. Arrowheads point to K8– and FM1-43x–double positive MCs. (E) Electron microscopy showed the absence of cells with electron-dense neurosecretory granules (Gr) contacting with nerve endings (Ne) in *Atoh1* cKO. The inset is magnified (middle). (A–E) Dashed lines delineate the basal layer of the epidermis (A–D) or the MC (E). Bars, 20 µm.

YFP-labeled cells remained constant (33% after 1 and 3 wk), demonstrating that adult epidermal progenitor cells expressing K15 and located in the basal layer of the whisker give rise to new MCs during adult homeostasis.

To determine the contribution of epidermal progenitors to the maintenance of MCs of the paw, we first performed BrdU administration for 10 d, which resulted in the labeling of the majority of K14-expressing cells (Fig. 4 G), whereas all MCs of the paw epidermis remained BrdU negative (Fig. 4 G). We next performed inducible lineage tracing in 1-mo-old K14-CREER/Rosa-YFP mice (Vasioukhin et al., 1999) by administrating 5 mg TAM every 3 d for 30 d. This prolonged TAM administration resulted in the labeling of most epidermal cells, including all of the differentiated cell types present in the skin epidermis (Fig. 4 H). This long-term K14-CREER activation resulted in YFP labeling of 25 ± 3% of MCs of the paw epidermis, demonstrating that MCs are maintained, at least in part, through the differentiation of epidermal SCs or progenitors.

Conditional deletion of *Atoh1* in embryonic epidermal progenitors results in the absence of MC specification

The molecular mechanisms that regulate MC specification remain largely unknown. Different transcription factors known to regulate cell fate specification such as Lhx3, Atoh1/Math1, Mash1, or Islet1 are preferentially expressed in neonatal mouse MCs (Haeberle et al., 2004). *Atoh1/Math1* is expressed in developing and adult MCs (Ben-Arie et al., 2000; Lumpkin et al., 2003; Haeberle et al., 2004) and in embryonic P-cadherin–positive hair progenitor cells (Rhee et al., 2006). Conditional deletion of Atoh1 in all cells of the developing trunk region using Hoxb1-CRE resulted in the absence of MC specification in the paw and the back skin epidermis, but MCs of the whisker region were still present (Maricich et al., 2009). To determine whether Atoh1 expression in epidermal progenitors and their progeny is required for MC specification in all body locations, including the whisker region, we deleted the floxed alleles of the *Atoh1/Math1* gene (Shroyer et al., 2007) in the developing skin epidermis using K14-CRE mice (Vasioukhin et al., 2001). Mice deficient for Atoh1 in the skin epidermis (*Atoh1* conditional knockout [cKO]) were born alive at a Mendelian ratio and were macroscopically indistinguishable from control mice (Fig. S3). Histological and immunofluorescence analysis of Atoh1-null epidermis showed no apparent defects in epidermal and HF differentiation (Fig. S3). However, in the absence of Atoh1, no MCs, as shown by the loss of K18, K20, or Rab3c immunoreactivity, were observed in newborn ($n = 3$) and adult ($n = 7$) mice (Fig. 5, A–C). The absence of MCs in *Atoh1* cKO is also supported by the absence of FM1-43x labeling, a fluorescent dye taken up by the recycling machinery of MCs, within the epidermis (Fig. 5 D) as well as the absence of cells presenting the typical ultrastructure features of MCs (Fig. 5 E). These data demonstrate that Atoh1 is required in epidermal progenitors and/or their progeny to specify MCs during development at all body locations, including the whisker region. In the intestine, neuroendocrine cells are derived from intestinal progenitors (Barker et al., 2007) and also require Atoh1 for their specification (Yang et al., 2001; Shroyer et al., 2007), suggesting that Atoh1 is involved in the neuroendocrine differentiation of epithelial cells in general.

Future perspectives

The absence of MCs in mice lacking Atoh1 in the epidermis offers a novel and powerful model to study the function of MCs during homeostasis and physiopathological conditions. It would be interesting in the future to define which signaling pathways control the differentiation of epidermal progenitors into MCs and whether Notch controls this process as it does during neuroendocrine cell fate specification in the intestine (Jensen et al., 2000; Yang et al., 2001). The demonstration that MCs arise from epidermal progenitors during embryonic development and adult homeostasis offers new avenues to identify the cells at the origin of MC carcinoma, a rare but devastating cancer (Moll, 2006), and to determine whether Atoh1 acts as a tumor suppressor gene in MCs, as has been recently suggested (Bossuyt et al., 2009a,b).

Materials and methods

Mice

K14-CRE (Vasioukhin et al., 2001) and K14-CREER (Vasioukhin et al., 1999) transgenic mice were provided by E. Fuchs (The Rockefeller University, New York, NY). Rosa-YFP (Srinivas et al., 2001) and K15-CREPR mice (Morris et al., 2004) were obtained from The Jackson Laboratory. Atoh1 flox/flox mice (Shroyer et al., 2007) were obtained from H.Y. Zoghbi (Baylor College of Medicine, Houston, TX). Mice colonies were maintained in a certified animal facility in accordance with European guidelines.

Generation of K18-CREER mice

The CREERT2 fragment (given by P. Chambon, Institute of Genetics and Molecular and Cellular Biology, Illkirch, France) followed by a SV40 polyadenylation signal was subcloned into a vector containing the K18 promoter and the SV40 intron (given by J. Hu, The Hospital for Sick Children, Toronto, Ontario, Canada). The resulting K18-CREERT2 fragment was microinjected into fertilized oocytes to generate transgenic mice (in the transgenic facility of the Université catholique de Louvain, Brussels, Belgium). Transgenic founders were first identified by PCR. Expression profiles of the K18-CREERT2 founders were screened with reporter Rosa-YFP mice.

CRE induction

K18-CREER/Rosa-YFP mice were treated with 15 mg TAM (Sigma-Aldrich) by i.p. injection in 23–28-d-old mice. K14-CREER/Rosa-YFP mice were treated with 5 mg TAM every 3 d for 30 d. K15-CREPR were treated with RU486 (Sigma-Aldrich) at 2.5 mg/d for the indicated time.

BrdU injection

For quantification of cell proliferation, 50 mg/kg BrdU (Sigma-Aldrich) was injected i.p. twice per day over 10 d.

Histology and immunostaining

Tissue samples were embedded in OCT (Sakura) and cut into 5–8-µm frozen sections using a cryostat (CM3050S; Leica). For Rosa-YFP mice, tissue samples were prefixed for 2 h in 4% PFA, incubated overnight in PBS + 30% sucrose at 4°C, and washed in PBS before embedding.

The following primary antibodies were used: anti-K8 (rat; 1:500; Developmental Studies Hybridoma Bank), anti-K20 (mouse; 1:200; Dako), anti-Rab3c (rabbit; 1:2,000; Abcam), anti-NF200 (mouse; 1:1,000; Sigma-Aldrich), anti-K14 (rabbit; 1:2,000; Covance), anti-GFP (rabbit; 1:1,000; Invitrogen), anti-GFP (goat; 1:2,000; Abcam), anti-β4 (rat; 1:200; BD), anti-K15 (chicken; 1:15,000; Covance), anti-K1 (rabbit; 1:1,000; Covance), anti-K5 (rabbit; 1:1,000; Covance), anti-KI67 (rabbit; 1:200; Abcam), anti–P-cadherin (rat; 1:200; Invitrogen), antiloricrin (rabbit; 1:1,000; Covance), anti-AE13 (mouse; 1:100; Abcam), and anti-AE15 (mouse; 1:100; Abcam). Immunostaining was performed as described previously (Blanpain et al., 2004). For FM1-43x experiments, 1-mo-old mice were injected i.p. with 100 µg FM1-43x in PBS (Invitrogen) and were sacrificed 24 h later. The tissue samples were prefixed for 2 h in 4% PFA, washed, and mounted in OCT.

All quantifications were performed in at least two different mice for each time point analyzed, and at least 100 MCs were counted for each condition. Errors represented the SEM.

Microscope image acquisition

Pictures of immunostaining were acquired using a microscope (Axio Observer Z1; Carl Zeiss, Inc.), camera (AxioCamMR3 or MrC5; Carl Zeiss, Inc.), and Axiovision software (Carl Zeiss, Inc.). Acquisitions were performed at room temperature using 20× 0.4 NA and 40× 0.75 NA EC Plan-Neofluar objectives (Carl Zeiss, Inc.). Confocal pictures were acquired at room temperature using a multiphoton confocal microscope (LSM510 NLO; Carl Zeiss, Inc.) fitted on an inverted microscope (Axiovert M200; Carl Zeiss, Inc.) equipped with C-Apochromat 40× 1.2 NA and 63× NA 1.2 water immersion objectives (Carl Zeiss, Inc.). Optical sections (0.35 mm thick and 512 × 512 pixels) were collected sequentially for each fluorochrome. The datasets generated were merged and displayed with the LSM510 software (Carl Zeiss, Inc.).

The imaging medium used was Glycergel (Dako) supplemented with 2.5% Dabco (Sigma-Aldrich). Fluorochromes coupled to secondary antibodies were Alexa Fluor 488 (Invitrogen), Rhodamine red-X (Jackson ImmunoResearch Laboratories, Inc.), and Hoechst or Topro3 to stain the nuclei (Invitrogen).

Electron microscopy

Skin samples were harvested from wild-type and mutant mice, fixed in 2% formaldehyde and 2% glutaraldehyde in 100 mM of cacodylate buffer,

pH 7.35, and postfixed with 2% osmium tetroxide. The samples were subsequently dehydrated in a graded series of ethanol and then embedded in Araldite (Serva). Ultrathin sections (30–60 nm) were processed on an ultramicrotome (Ultracut E; Fa. Reichert) with a diamond knife and placed on copper grids. Transmission electron microscopy was performed using an electron microscope (model 902A; Carl Zeiss, Inc.).

Online supplemental material

Fig. S1 shows the apparition of MCs during embryogenesis. Fig. S2 shows K14 expression in MCs. Fig. S3 shows that Atoh1 is not required for epidermis and HF development and differentiation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200907080/DC1.

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