# Pax3 functions at a nodal point in melanocyte stem cell differentiation

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**Most stem cells are not totipotent. Instead, they are partially committed but remain undifferentiated. Upon appropriate stimulation they are capable of regenerating mature cell types[1]. Little is known about the genetic programmes that maintain the undifferentiated phenotype of lineage-restricted stem cells. Here we describe the molecular details of a nodal point in adult melanocyte stem cell differentiation in which Pax3 simultaneously functions to initiate a melanogenic cascade while acting downstream to prevent terminal differentiation. Pax3 activates expression of Mitf, a transcription factor critical for melanogenesis[2,3], while at the same time it competes with Mitf for occupancy of an enhancer required for expression of dopachrome tautomerase, an enzyme that functions in melanin synthesis[4]. Pax3-expressing melanoblasts are thus committed but undifferentiated until Pax3-mediated repression is relieved by activated β-catenin. Thus, a stem cell transcription factor can both determine cell fate and simultaneously maintain an undifferentiated state, leaving a cell poised to differentiate in response to external stimuli.**

We identified an unappreciated expression domain for Pax3 in hair follicles (Fig. 1a–d). Pax3 immunoreactive cells in skin also express β-galactosidase in Dct-lacZ mice (Fig. 1e) as well as endogenous dopachrome tautomerase[5,6] (Dct) (Fig. 1f), and these cells are located in the bulge region of mature hair follicles where melanocyte stem cells are found[7] (Fig. 1g). We created mice in which the endogenous Pax3 locus was modified to express Cre recombinase and crossed them to β-galactosidase reporter mice to confirm the existence of Pax3 descendents in the hair follicle (Fig. 1h). Pax3 is expressed by label-retaining cells in resting, telogen stage adult follicles (Fig. 1i–k), further supporting their identity as stem cells[7]. Some Pax3-expressing cells within the hair follicle do not express Dct-lacZ or Dct (arrows, Fig. 1e) suggesting that these cells may not yet have initiated Dct expression. We examined Pax3 expression in skin from mice in which Wnt1-expressing neural crest derivatives are labelled with green fluorescent protein (GFP). Pax3 expression colocalizes with GFP suggesting that Pax3-positive cells derive from neural crest (Fig. 1l–n). The majority of Pax3-expressing cells in early anagen stage newborn follicles also express Mitf (ref. 8; Fig. 1o–q) and Sox10 (ref. 9; Fig. 1r–t).

We tested the ability of Pax3, Sox10 or Mitf, alone or in combination, to activate expression of a reporter construct containing a 3,181-base-pair (bp) *Dct* genomic fragment previously used to create Dct-lacZ mice. This fragment includes four regions of conservation (>75%) between mouse and human genomes (Fig. 2a). Sox10 and Mitf induce synergistic activation of reporter activity of over 100-fold. The addition of Pax3 to Sox10 and/or Mitf results in significant repression (Fig. 2a). The region between −80 and −350 bp upstream of the *Dct* transcription start (enhancer region 2, Fig. 2a) contains a functional Pax3, Sox10 and Mitf responsive element.

Pax3, Sox10 and Mitf are each able to associate with the enhancer as determined by chromatin immunoprecipitation (ChIP) (Supplementary Fig. S1a). Pax3 directly activates expression of *Mitf* by functioning synergistically with Sox10 (refs 2, 10). We compared the ability of Pax3 to regulate expression of *Mitf* (ref. 3) and *Dct* reporter constructs. Under conditions in which Pax3 activates expression of *Mitf*, identical concentrations of Pax3 repress the *Dct* reporter (Fig. 2b).

Potential Mitf and Sox10 binding sites are present within enhancer region 2 (Fig. 2f). The putative Mitf binding sequence, or M-box[11,12], has been reported previously and an adjacent atypical Lef/Tcf binding site contributes to Mitf-mediated *Dct* regulation[4]. Pax3 is able to bind with relatively low affinity to the enhancer sequence (Supplementary Fig. S1b). Mutation of the M-box destroys the ability of Pax3 to bind to the enhancer (Supplementary Fig. S1b)

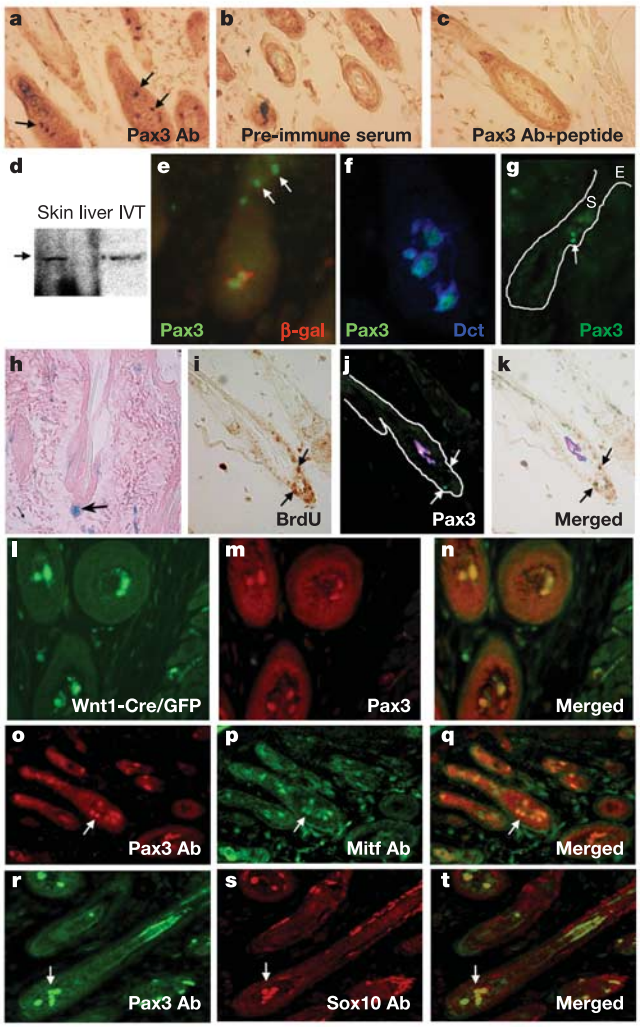


**Figure 1** Pax3 is expressed in mature hair follicles. **a**–**c**, Pax3 expression in early anagen hair follicles of 2-day-old mice is visualized using Pax3 antibody (Ab) (**a**, arrows) in comparison with controls using pre-immune serum (**b**) or antibody pre-adsorbed with Pax3 peptide (**c**). **d**, Immunoprecipitation and western analysis for Pax3 reveals a 68-kD protein in skin but not liver. *In vitro* translated (IVT) Pax3 is used as positive control (lane 3). **e**–**f**, Pax3 (green) is co-expressed with β-galactosidase driven from a Dct promoter (**e**, red) or endogenous Dct (**f**, blue) detected by immunohistochemistry in newborn skin. Pax3 is also expressed in the follicle independently of Dct (**e**, arrows). **g**, Pax3-expressing cells (green, arrow) in resting telogen stage adult hair follicles. S, shaft. E, epithelial surface. **h**, Pax3-Cre knockin mice were used to activate expression of β-galactosidase (blue, arrow). **i**–**k**, Pax3 is co-expressed with label-retaining cells, identified by 5-bromodeoxyuridine (BrdU) incorporation and long-term retention (**i**) in resting telogen stage follicles of 58-day-old mice. Pax3 is green (**j**) and an overlay of **i** and **j** is shown in **k**. **l**–**n**, Wnt1-Cre transgenic mice crossed into a Z/EG background label neural crest derivatives by expression of GFP (**l**, green), which colocalizes with Pax3 (**m**). (Overlay shown in **n**.) Pax3 expression overlaps with Mitf (**o**–**q**, arrows) and Sox10 (**r**–**t**, arrows) in early anagen P2 follicles.

and to repress basal reporter gene activity (Fig. 2c). The ability of Pax3 to repress basal activity or Mitf-dependent activation is at least partially dependent upon an intact Pax3 paired type DNA binding domain because a mutation that abolishes paired domain DNA binding (Pro to Leu at amino acid 50) diminishes repressor function (Supplementary Fig. S1d). A functional Sox10 binding site is downstream of the M-box (Supplementary Fig. S1c).

These observations suggest that Pax3 and Mitf compete for binding to the *Dct* enhancer. Saturation curves for Mitf activation and ChIP assays are consistent with Pax3 acting as a competitive inhibitor (Fig. 2d, e). When transfected at equal ratios, or with a slight excess of Mitf expression plasmid, Pax3 preferentially associates with enhancer DNA. At high ratios of Mitf:Pax3, Mitf displaces Pax3. Hence, Pax3, functioning with Sox10 (refs 2, 10), can activate expression of *Mitf* while simultaneously acting as a competitive inhibitor of Mitf-mediated activation of *Dct*.

Mutation of the Lef/Tcf binding site abolishes Pax3-mediated repression (Fig. 2g). Pax5, a factor closely related to Pax3, is able to physically interact with Lef1 (ref. 13) and both Pax5 and Pax2 can recruit Groucho co-repressors[14,15]. Lef/Tcf factors can interact with Groucho co-repressors[16]. Pax3 is able to recruit Grg4 (Fig. 2h), which is expressed in Dct- and Pax3-positive cells (Fig. 2i, j). Pax3 and Grg4 physically interact (Supplementary Fig. S1e). Endogenous Grg4 is present in 293T cells and could account for Pax3-mediated repression observed in that cell line (Supplementary Fig. S1f).

Lef/Tcfs are cofactors for nuclear β-catenin and mediate canonical Wnt signalling[17]. Co-transfection of activated β-catenin abolishes repressor activity of Pax3 (Fig. 3a) and displaces Pax3 from *Dct* enhancer DNA (Fig. 3b) thus preventing competition with Mitf (Fig. 3c). Activated β-catenin displaces Grg4 from the *Dct* enhancer (Fig. 2h), explaining the loss of Pax3-mediated repression. Lef1, Pax3 and Grg4 can form a complex in solution, and activated β-catenin displaces Pax3 (Fig. 3d).

B16 melanoma cells harbour an activating mutation in β-catenin and express Pax3, Mitf, Sox10, Grg4 and Dct. Pax3 is not located at the endogenous *Dct* enhancer in B16 cells (Fig. 3e). Transfection of dominant negative Lef1 induces endogenous Pax3 and Grg4 proteins to occupy the endogenous *Dct* enhancer (Fig. 3e) and levels of Dct RNA (data not shown) and protein (Fig. 3f) are reduced. Because β-catenin also regulates *Mitf*, we confirmed that the effects of dominant negative Lef1 were maintained in the presence of excess Mitf (Fig. 3e, f).

We examined Pax3 and Dct expression in skin from TOPGAL mice, which serve as a reporter for activated β-catenin signalling[18]. In newborn anagen stage hair follicles, cells that express both Pax3 and Dct also express β-galactosidase (Fig. 4a–c). However, when Pax3 is present in the absence of activated β-catenin signalling, Dct is not expressed (Fig. 4d–f). We expressed a soluble inhibitor of Wnt

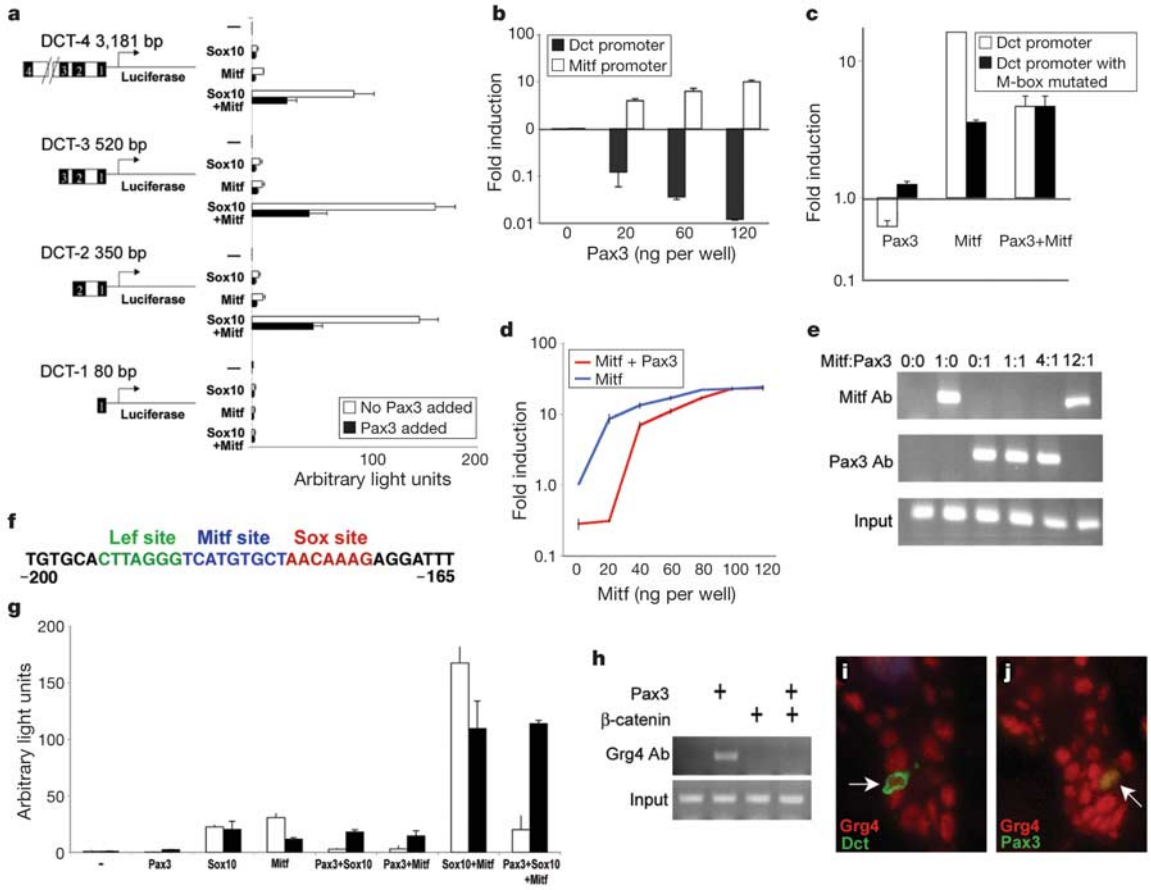


**Figure 2** Pax3 and Mitf regulate *Dct* and compete for enhancer occupancy. **a**, Deletion analysis of the *Dct* upstream sequence. Homology between human and mouse is indicated (black boxes, numbered 1–4). Transfections in 293T cells are without (white bars) or with (black bars) Pax3 protein. All transfections are expressed as the average of at least three independent experiments in triplicate, +/− s.d. **b**, Pax3 represses the *Dct* reporter while activating the *Mitf* reporter. **c**, Pax3 and/or Mitf is transfected with DCT-2 reporter (white bars) or with DCT-2 reporter with M-box mutated (black bars). **d**, Saturation curve for Mitf-mediated activation of DCT-2, with (red line) or without (blue line) 20 ng Pax3 expression construct. **e**, ChIP analysis of 293T cells transfected with Mitf and/or Pax3. Amplified DNA is from homology region 2. Primers for β-actin and luciferase failed to yield product. **f**, Sequence of *Dct* enhancer. **g**, Pax3, Mitf and/or Sox10 is transfected with DCT-2 reporter construct (white bars) or with DCT-2 reporter with the Lef1 site mutated (black bars). **h**, ChIP analysis of 293T cells transfected with Pax3 and/or activated β-catenin using Grg4 antibody to immunoprecipitate endogenous Grg4. Primers for β-actin and luciferase failed to yield product. **i**, Immunohistochemistry of 2-month-old mouse resting hair follicle using Grg4 (red) and Dct (green) antibodies. **j**, Immunohistochemistry of 2-month-old follicle using Pax3 (green) and Grg4 (red) antibodies.

signalling using an inducible system[19,20]. Consistent with previous data[21–23], we observed a marked decrease in Pax3-expressing cells in the skin of transgenic mice induced to express Dickkopf 1 (Dkk1). However, some Pax3-expressing cells were observed after late embryonic induction of Dkk1. In wild-type embryonic skin, the majority of Pax3-expressing cells also express Dct (98% of 200 cells analysed). In Dkk1-overexpressing embryos, the percentage of Pax3-expressing cells that also express Dct is markedly decreased (38% of 180 cells analysed).

We generated mouse embryos in which β-catenin was inactivated in Pax3-expressing cells[24]. We confirmed loss of β-catenin by immunohistochemistry, and homozygous floxed β-catenin embryos that carried the Pax3-Cre allele were identified at mid-gestation with neural tube defects (data not shown). At embryonic day 13.5 (E13.5), loss of β-catenin in Pax3-expressing cells resulted in the loss of Dct expression in skin, in accord with related previous work[25,26]. However, we were able to identify Pax3-expressing cells in the skin of these embryos, albeit in fewer numbers than in wild-type litter mates where we found many Pax3-expressing cells that also expressed Mitf and Dct (Fig. 4g–i). In skin lacking β-catenin in Pax3 cells, Pax3 and Mitf were co-expressed, but Dct was absent (Fig. 4j–l). Hence, β-catenin is required for Dct expression in Pax3-expressing melanocyte precursors.

Our data suggest a model to explain how expression of an upstream determination factor, such as Pax3, might initiate a lineage-specific gene programme while at the same time prevent terminal differentiation. In melanocyte precursors, we suggest that Pax3 functions with Sox10 to activate *Mitf* expression, while at the same time it prevents Mitf from activating downstream genes (Fig. 4m). This nodal checkpoint is characterized by Pax3 competition with Mitf for enhancer occupancy (Fig. 4n). Thus, the Pax3-expressing precursor is unable to fully differentiate, while Mitf is able to accumulate, resulting in a 'biological capacitor' in which the cell is primed to rapidly express downstream genes once Pax3-mediated repression is relieved. We postulate that external stimuli that result in melanocyte stem cell activation, such as injury or sun exposure, function through activation of β-catenin, resulting in displacement of Pax3 and associated Groucho co-repressors and activation of downstream gene expression.

The ability of a single factor, or complex of factors, to simultaneously activate a determination programme while preventing terminal differentiation may represent a general paradigm for developmental and stem cell biology. This paradigm predicts a class of 'pangenes' that encode related functions, reminiscent of the Greek god Pan, and Peter Pan, who were able to orchestrate complex events while never growing to maturity. For instance, Sox10 maintains neural crest lineage multipotency while inhibiting terminal neuronal differentiation[27]. During eye development, the conserved *Pax6* gene is required for specification of multiple ocular lineages. Loss of Pax6 expression in *Drosophila*, mice and man results in a complete absence of eye formation because critical developmental

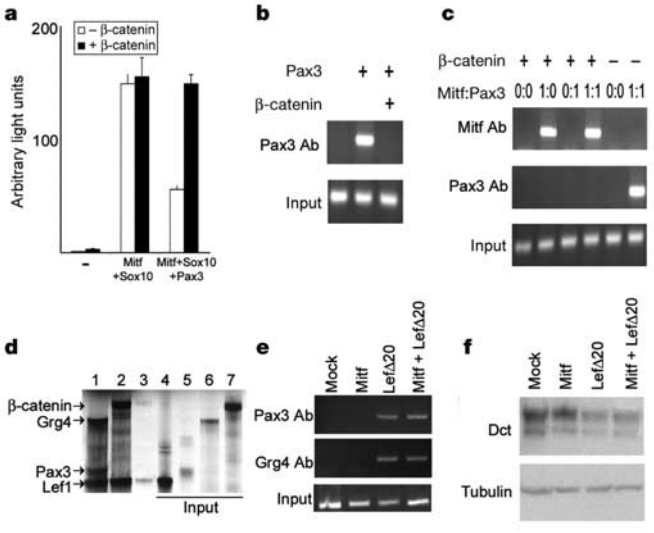


**Figure 3** Activated β-catenin modulates Pax3 activity. **a**, DCT-2 reporter is transfected alone, with Mitf and Sox10 or with Mitf, Sox10 and Pax3 without (white bars) or with (black bars) activated β-catenin. **b**, **c**, ChIP analysis from 293T cells transfected with Mitf, Pax3 and/or activated β-catenin. Primers for β-actin and luciferase failed to yield product. β-catenin displaces Pax3 from DCT-2 enhancer DNA (**b**) and β-catenin prevents Pax3 from effectively competing with Mitf for occupancy of the *Dct* enhancer site (**c**). **d**, *In vitro* translated Grg4, Pax3 and Lef1 proteins were incubated together and immunoprecipitated with anti-Lef1 antibody, which resulted in precipitation of all three proteins (lane 1). Further addition of β-catenin resulted in loss of Pax3 and Grg4 in the immunoprecipitate (lane 2). Lane 3 is negative control immunoprecipitate in the absence of Lef1 antibody. Lanes 4–7 are input Lef1, Pax3, Grg4 and β-catenin proteins, respectively. **e**, ChIP analysis in B16 melanoma cells with primers for endogenous *Dct* enhancer. Endogenous Pax3 and Grg4 proteins occupy the *Dct* enhancer when a mutant Lef1 protein that does not bind to β-catenin (LefΔ20) is present. β-actin genomic primers, negative control, provided no amplification of DNA (not shown). **f**, Western analysis of B16 cells transfected without or with dominant negative Lef1 (LefΔ20).

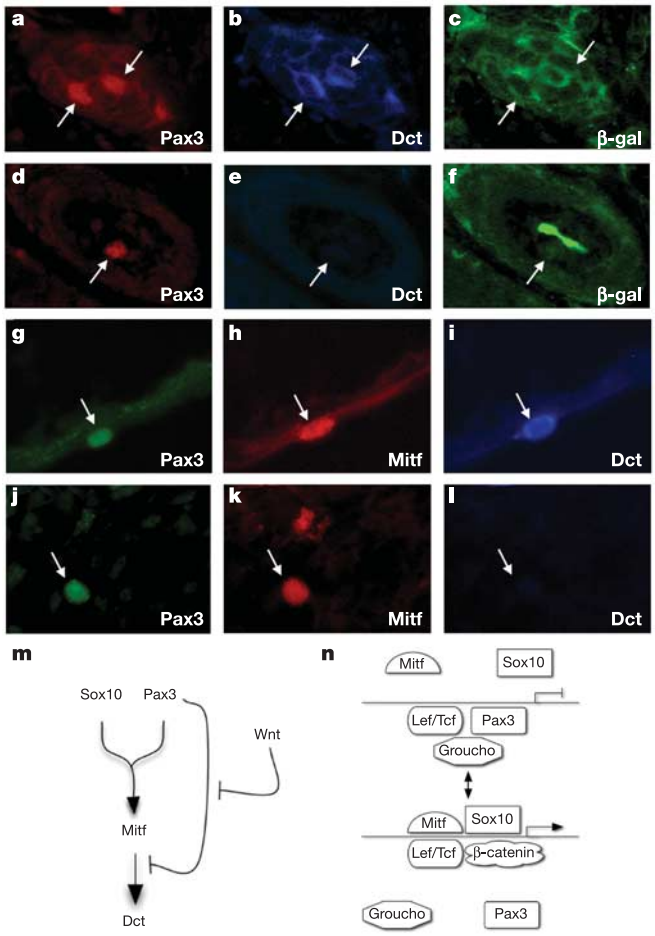


**Figure 4** Dct expression requires activated β-catenin. **a**–**f**, Pax3, Dct and β-galactosidase expression in anagen stage hair follicles of 2-day-old TOPGAL mice. Cells in the follicles express all three proteins (**a**–**c**) or only Pax3 (**d**–**f**). **g**–**l**, Pax3, Mitf and Dct expression in skin of E13.5 wild-type embryos (**g**–**i**) or embryos lacking β-catenin in Pax3-expressing cells (**j**–**l**). Cells in wild-type embryo that express all three proteins are easily identified in skin (**g**–**i**). Occasional Pax3-expressing cells, which co-express Mitf in skin, are identified in tissue-specific β-catenin mutants, but Dct expression is undetectable in these cells (**i**–**l**). **m**, **n**, Model depicting the ability of Pax3 to activate a melanogenic cascade while simultaneously competing with Mitf for activation of *Dct*, thus preventing expression of terminal differentiation markers until external stimuli abolish Pax-mediated repression.

programmes are never initiated[28]. However, inactivation of Pax6 later in development, at the retinal progenitor stage, results in loss of ability of this committed but undifferentiated cell type to maintain pluripotentiality[29]. Pax5, which can also interact with Grg4 (ref. 14), may play similar roles in lymphocyte development[30].

Adult resident stem cells have been identified in a large number of organs and provide exciting potential for tissue regeneration. A fundamental understanding of the molecular programmes regulating both differentiation and maintenance of the undifferentiated state will be required to harness this potential. Our work characterizes a critical regulatory circuit that exemplifies conservation of genetic programmes between embryonic neural crest development and adult melanocyte stem cell function. Additional nodal checkpoints, with parallel transcriptional circuits, are likely to exist in other embryonic and adult stem cells. □

## Methods

### Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded tissue fixed in 4% paraformaldehyde. Antigen was exposed using Bull's Eye reagent (Biocare Medical) and heated in a pressure cooker. Antibodies utilized were Pax3 (polyclonal sera or monoclonal supernatant, Developmental Studies Hybridoma Bank, 1:3,000 for DAB staining, 1:800 for immunofluorescence), Mitf (Vector Laboratories, 1:10), Sox10 (Chemicon International, 1:20), β-galactosidase (Promega Corporation, 1:100), Dct/Trp2 and Grg4 (Santa Cruz Biotechnology, 1:50 and 1:100, respectively). Secondary antibodies conjugated with fluorescent tags (Alexa Fluor, Molecular Probes) were used at a dilution of 1:250. For label retention studies, mice were injected subcutaneously with BrdU (10 μg per g body weight) twice daily from P20 to P27. Skin was collected at P58. BrdU antibody (Biocare Medical) was used at a dilution of 1:200.

### Cell culture, transfection and ChIP assays

293T cells and B16 cells (American Type Culture Collection) were maintained in DMEM supplemented with 10% fetal bovine serum (Invitrogen Life Technologies). A total of 0.5 μg of DNA was mixed with 10 μl Effectene (Qiagen). Luciferase activity (Luciferase assay kit, Promega Corporation) was normalized for transfection efficiency using pCMVβ (BD Biosciences/Clontech) and expressed as either fold activation compared with reporter construct alone, or as arbitrary light units. For ChIP assays, transfected cells were fixed in 1% formaldehyde and quenched in 0.125 M glycine, then processed according to the manufacturer's protocol (Upstate Biotechnology). Polymerase chain reaction (PCR) was performed with primers to the Dct enhancer region-2 GGAGAAGTACTTAGCAATGCAC AGG (F) and AGCCATCATTAAGGGGATTATAACC (R). All ChIP samples were tested for false positive PCR amplification using primers that amplify sequence from the β-actin gene (for genomic DNA contamination) and luciferase (reporter construct contamination). In all cases, these amplifications failed to yield product.

Details of methods for electrophoretic mobility shift assays, immunoprecipitation, western blotting, and constructs and mouse lines used are provided in the Supplementary Methods.

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# Toll-like receptor 3 promotes cross-priming to virus-infected cells

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**Cross-presentation of cell-associated antigens plays an important role in regulating CD8$^+$ T cell responses to proteins that are not expressed by antigen-presenting cells (APCs)[1]. Dendritic cells are the principal cross-presenting APCs *in vivo* and much progress has been made in elucidating the pathways that allow dendritic**