The EMBO Journal (2009) 28, 1890–1903 | © 2009 European Molecular Biology Organization | All Rights Reserved 0261-4189/09
www.embojournal.org

THE EMBO JOURNAL

The epidermal differentiation-associated Grainyhead gene *Get1/Grhl3* also regulates urothelial differentiation

Zhengquan Yu[1,2], Jaana Mannik[1,2,3], Amelia Soto[1,2,3], Kevin K Lin[1,2,3] and Bogi Andersen[1,2]*

[1]Department of Medicine, University of California, Irvine, CA, USA and [2]Department of Biological Chemistry, University of California, Irvine, CA, USA

Skin and bladder epithelia form effective permeability barriers through the activation of distinct differentiation gene programs. Using a genome-wide gene-expression study, we identified transcriptional regulators whose expression correlates highly with that of differentiation markers in both the bladder and skin, including the Grainyhead factor *Get1/Grhl3*, which is already known to be important for epidermal barrier formation. In the bladder, *Get1* is most highly expressed in the differentiated umbrella cells and its mutation in mice leads to a defective bladder epithelial barrier formation due to the failure of apical membrane specialization. Genes encoding components of the specialized urothelial membrane, the uroplakins, were downregulated in *Get1*$^{-/-}$ mice. At least one of these genes, *uroplakin II*, is a direct target of Get1. The urothelial-specific activation of the *uroplakin II* gene is due to selective binding of Get1 to the uroplakin II promoter in urothelial cells, which is most likely regulated by histone modifications. These results show a crucial role for Get1 in urothelial differentiation and barrier formation.

The EMBO Journal (2009) **28,** 1890–1903. doi:10.1038/emboj.2009.142; Published online 4 June 2009
Subject Categories: development
Keywords: barrier formation; epidermal differentiation; Grainyhead; uroplakin; urothelial differentiation

Introduction

One of the main functions of the urothelium is to form an effective permeability barrier, preventing the access of microorganisms and selectively controlling the movement of water and solutes between urine and the tissue. To achieve this function, urothelial cells undergo progressive differentiation steps from embryonic day (E) 12 to 18. The urothelium is considered to represent one of the tightest and most impermeable barriers in the body (Lewis and de Moura, 1982; Negrete *et al*, 1996). The mouse urinary bladder, which initially develops from the cloaca at E12, is fully formed at E14, when it can be isolated as a discrete unit (Staack *et al*, 2003). At E15, the endoderm-derived urothelium is a three-layered structure, composed of basal, intermediate, and superficial layers; basal cells are thought to progressively move towards the surface, differentiating first into intermediate cells and then into superficial cells. When fully differentiated, the superficial cells are referred to as umbrella cells. At E15, the apical membrane of the superficial cells is characterized by microvilli. As these cells differentiate, they markedly enlarge and the apical-surface microvilli are replaced by rigid ridges. At E18, a distinctive pattern of ridges is formed on the apical surface of umbrella cells, interspersed with the asymmetric unit membrane (AUM) plaques, which occupy most of the surface (Jezernik *et al*, 2000; Erman *et al*, 2006). The major protein components of the AUM plaques are the uroplakins (Upk), an evolutionarily conserved family (Garcia-Espana *et al*, 2006) of at least five integral membrane proteins: UpkIa, UpkIb, UpkII, UpkIIIa, and UpkIIIb (Wu *et al*, 1994; Sun *et al*, 1996; Deng *et al*, 2002; Min *et al*, 2006). Disruption of the *UpkII* and *UpkIIIa* genes in mice leads to impaired umbrella-cell development and compromised urothelial permeability barrier function (Hu *et al*, 2000, 2002; Kong *et al*, 2004).

Although the morphological and biochemical features of urothelial differentiation and barrier formation are well characterized, the transcriptional mechanisms that coordinate this process have just begun to be identified. In bladder tissue derived from mouse embryonic stem cells, Foxa1 and Foxa2 have been suggested as potential transcription factors involved in differentiation of urothelium (Oottamasathien *et al*, 2007). In an *in vitro* differentiation model of human urothelial cells, FOXA1 and IRF-1 have been identified as PPARγ-induced intermediary transcription factors necessary for urothelial differentiation (Varley *et al*, 2009). In recent years, aspects of the transcriptional mechanisms underlying this process in skin have been elucidated. Among the transcription factors important for terminal differentiation and barrier formation in epidermis are Klf4 (Segre *et al*, 1999) and Grainyhead transcription factor Get1/Grhl3 (Ting *et al*, 2005; Yu *et al*, 2006), which indirectly or directly activate many genes responsible for epidermal barrier formation (Dai and Segre, 2004; Koster and Roop, 2007). Grainyhead transcription factors have an evolutionarily conserved role in the regulation of epidermial barrier formation. *Drosophila* Grainyhead is required for normal cuticle strength and wound healing (Bray and Kafatos, 1991; Mace *et al*, 2005). In addition to regulating the mammalian epidermis differentiation program, Get1 has a role in keratinocyte migration, which is a crucial aspect of the wound-healing mechanism (Hislop *et al*, 2008; Yu *et al*, 2008).

We reasoned that a genome-wide characterization of the bladder differentiation program, and a comparison with the well-characterized skin-differentiation program, might

*Corresponding author. Department of Biological Chemistry, University of California, Sprague Hall, Room 206, Irvine, CA 92697-4030, USA. Tel.: +1 949 824 9093; Fax: +1 949 824 2200;
E-mail: bogi@uci.edu
[3]These authors contributed equally to this work

Received: 9 January 2009; accepted: 28 April 2009; published online: 4 June 2009

provide further insights into this problem. Similar to the urothelium, one of the primary roles of the stratified epidermis is to form an effective permeability barrier. But in contrast to the urothelium, a different gene program is activated to mediate differentiation of the epidermis; the epidermal barrier depends primarily on protein components of the cornified envelope and the composition of lipids between cells of the cornified layer (Segre, 2003). Common to both epithelia, cell–cell adhesion, in part mediated by tight-junction proteins, is important for effective barriers (Acharya *et al*, 2004; Niessen, 2007). In this paper, we define and compare the gene-expression programs in the developing skin and bladder, and identify Get1 as an important regulator of urothelial terminal differentiation and barrier formation.

Results

Identification of a shared transcriptional regulatory program in bladder and skin development

To gain insights into the common and unique transcriptional regulatory programs during bladder and skin differentiation, we profiled global gene expression in whole mouse bladder and dorsal skin at E14.5, E16.5, and E18.5. At E18.5, a total of 1540 transcriptional regulators are expressed in both the bladder and skin, whereas there are 264 and 532 transcriptional regulators uniquely expressed in the bladder and skin, respectively (Figure 1A). To enrich for transcriptional regulators important for developmental regulation, we narrowed these lists down to those that show significant change in expression during these developmental time points. Over these developmental time points, there are 114 transcriptional regulators that are differentially expressed in both the bladder and skin, whereas there are 29 and 425 transcriptional regulators differentially expressed uniquely in the bladder and skin, respectively. To further enrich for transcriptional regulators likely to be important for terminal differentiation, we correlated the differentially expressed transcriptional regulators with the expression of differentiation markers in the epithelia of the bladder and skin. Uroplakin genes, which encode terminal differentiation products of the bladder epithelium, show a gradual increase over the time course of bladder development (Figure 1B and Supplementary Figure S1A and B). In contrast, many genes that encode components of the epidermal barrier, including *loricrin, filaggrin, involucrin, periplakin*, and *envoplakin*, show a marked increase from E14.5 to E16.5, with a slight decrease at E18.5 during skin development (Figure 1B and Supplementary Figure S1A). There are 15 transcriptional regulators whose expression correlates highly with the expression of differentiation markers in both tissues (Figure 1C and Supplementary Figure S1C), while there are 17 (Figure 1A and D, and Supplementary Figure S1C) and 322 (Figure 1A and Supplementary Table S1) transcriptional regulators whose expression correlates uniquely with differentiation markers in the bladder and skin, respectively. The transcriptional regulators that show specific correlation with differentiation markers in each tissue are candidates for playing important roles in the differentiation process in the bladder (Figure 1D) and skin (Supplementary Table S1). Consistent with this idea, Foxna1, Skn1a, and Dlx3, which have been implicated in epidermal differentiation (Brissette *et al*, 1996; Andersen *et al*, 1997; Morasso *et al*, 1999; Baxter and Brissette, 2002), are all found among the factors in Supplementary Table S1. Interestingly, Get1, a known regulator of epidermal terminal differentiation (Ting *et al*, 2005; Yu *et al*, 2006), is among the transcriptional regulators showing expression highly correlated to differentiation markers in both bladder and skin epithelium (Figure 1C and Supplementary Figure S1C). We were also intrigued to find that E14.5 dorsal skin has a more similar global gene-expression profile to the bladder samples from all time points than to E16.5 and E18.5 dorsal skin (Supplementary Figure S2A and B). The similarity of E14.5 dorsal skin to the bladder is partially due to the high expression of simple epithelial keratins (e.g. *Krt8, Krt18*, and *Krt19*) and low expression of late epidermal differentiation genes (e.g. small proline rich-like 3, desmoplakin, and corneodesmosin) in E14.5 skin (Supplementary Figure S2C). E16.5 and E18.5 dorsal skin are also molecularly distinct because they undergo marked gene-expression changes during hair-follicle morphogenesis.

To gain insights into the biological processes during bladder development, we clustered the time-course profiles of the set of genes (4101 probe sets) that show significant change in expression during the time course. Functional annotation analysis identified significantly enriched Gene Ontology biological process categories within these clusters (Supplementary Figure S3A and B). We found that the expression of differentiation genes increased over the time course, whereas genes encoding cell-cycle regulators and cellular metabolic processes are highly enriched in clusters, showing decreasing expression during bladder development. These findings are consistent with decreasing epithelial proliferation as differentiation progresses. Interestingly, these same clusters contain a large number of genes encoding chromatin-modifying factors and other transcriptional regulators, suggesting more active chromatin modifications in early bladder development, perhaps related to much higher proliferation rate during this time period.

Get1 is highly expressed in umbrella cells of the mouse urothelium

Grainyhead transcription factors are regulators of epidermal barrier function and wound healing in species as divergent as *Drosophila* and mice (Mace *et al*, 2005; Ting *et al*, 2005). In mice, one of three Grainyhead genes, *Get1*, has an important role in regulation of epidermal terminal differentiation. The striking correlation of *Get1* expression with bladder epithelial differentiation markers (Figure 1C and Supplementary Figure S1C) suggests a role in bladder epithelial differentiation as well. This finding is of interest because the bladder epithelium is endodermally derived and is characterized by a differentiation program that is strikingly divergent from that of the ectodermally derived epidermis. Get1 transcripts are highly expressed in bladder epithelium at E15.5 when the differentiation program is initiated (Kudryavtseva *et al*, 2003; Auden *et al*, 2006). Get1 protein is specifically and strongly expressed in the developing (Figure 2A) and mature (Figure 2B) umbrella cells of the bladder epithelium at E16.5 and E18.5. This staining is specific because $Get1^{-/-}$ bladder epithelium shows no staining (Figure 2C). In addition, Get1 is strongly expressed in intermediate and superficial layers of the ureter epithelium (data not shown). The high expression of Get1 in the most-differentiated cells of the

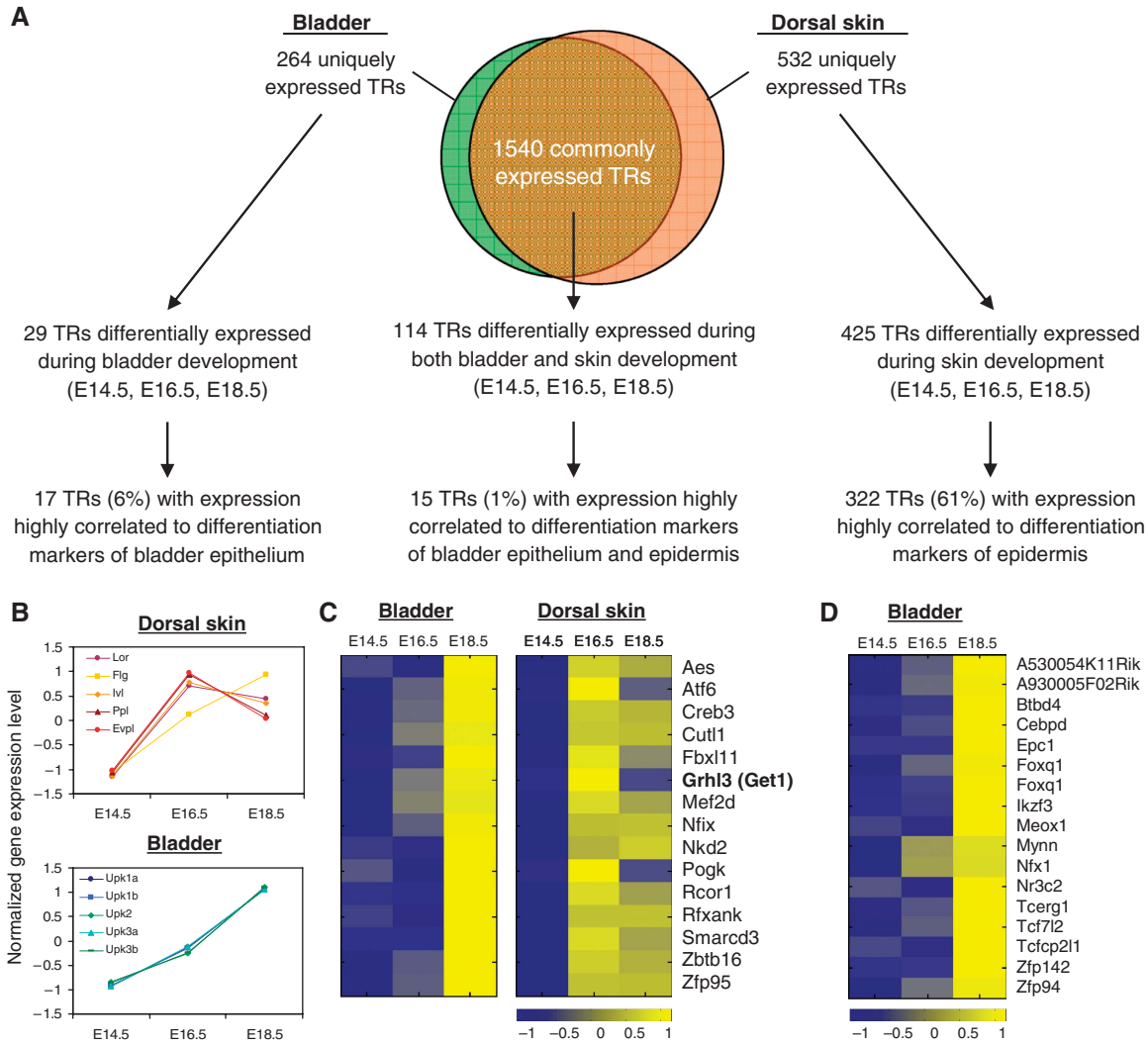


Figure 1 Shared transcriptional regulatory programs of bladder and skin differentiation. (**A**) Global analysis of common and unique transcriptional regulators regulated during bladder and skin differentiation at E14.5, E16.5, and E18.5. TR, transcription regulator. (**B**) The expression patterns of terminal differentiation markers of bladder epithelium and dorsal skin at the indicated embryonic age. (**C**) Heat map of transcriptional regulators with expression highly correlated to terminal differentiation markers of the bladder and dorsal skin. Note the *Grhl3* (*Get1*) in bold. (**D**) Heat map of transcriptional regulators that are expressed in the bladder but not in dorsal skin, and that are highly correlated to bladder terminal differentiation markers. Note that Foxq1 has multiple probe sets. For panels C and D, expression levels are normalized across time points and indicated by the colorimetric ratio scale.

bladder epithelium suggests a possible role in the terminal differentiation program and barrier formation in the bladder.

Get1 is required for formation of normal umbrella cells in the bladder urothelium

To investigate the potential role of *Get1* in terminal differentiation of the urothelium, we used *Get1*$^{-/-}$ mice previously made in our laboratory (Yu *et al*, 2006). *Get1*$^{-/-}$ mice, which die at birth, have neural tube defects, an open eye phenotype, and an epidermal barrier defect (Ting *et al*, 2003, 2005; Yu *et al*, 2006, 2008). In addition, *Get1*$^{-/-}$ mice have enlarged urinary bladders (Figure 2D and E); this phenotype is fully penetrant at E18.5. To determine the role of *Get1* in urothelial terminal differentiation, we examined the urinary bladder histology of WT and *Get1*$^{-/-}$ mice at E16.5 and E18.5, the time period during which the apical cell membranes of umbrella cells form the characteristic rigid ridges (Erman *et al*, 2006). At E16.5, the most superficial layer of the urothelium is composed of differentiating umbrella cells with a rugged apical surface (Figure 2F). In contrast, in the *Get1*$^{-/-}$ bladder, the superficial epithelial cells are more round and have a smooth surface (Figure 2G). At E18.5, the superficial umbrella cells are fully formed with the characteristic rugged apical plasma membrane in WT mice (Figure 2H), whereas the *Get1*$^{-/-}$ urothelium contains smaller cells with a smooth apical cell surface (Figure 2I), suggesting that *Get1* is required for the formation of normal umbrella cells.

To further determine the effect of *Get1* on umbrella cell maturation in the bladder, we examined the ultrastructure of umbrella cells from E18.5 WT and *Get1*$^{-/-}$ mice. Using scanning electron microscopy, we found that superficial cells of the WT urothelium are about 50 μm in diameter and are demarcated by a tight-junctional ring (Figure 3A). Their surface appears pleated with the characteristic ridges (Figure 3B) as described previously (Erman *et al*, 2006).

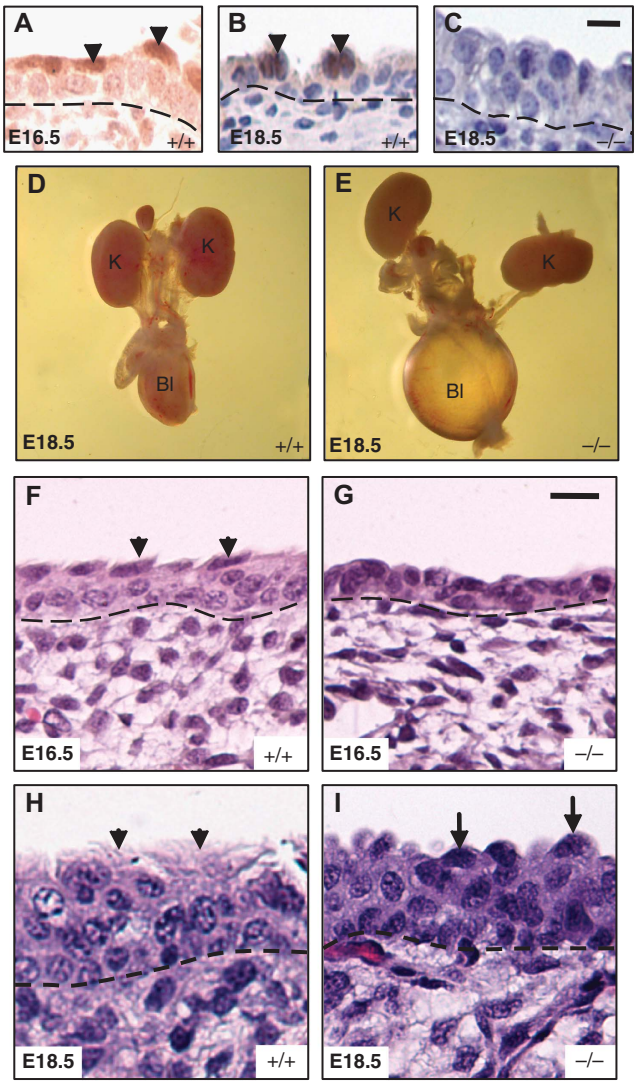


Figure 2 *Get1* is required for bladder epithelium umbrella-cell development. (**A–C**) WT mouse bladder epithelial sections from E16.5 (A) and E18.5 (B) were immunostained with Get1 antibody, whereas Get1 immunostaining in the E18.5 *Get1*$^{-/-}$ bladder was used as negative control (C). Arrowheads point to umbrella cells. Get1 is specifically expressed in the developing and mature umbrella cells of the urothelium. (**D**, **E**) Urinary bladder (Bl) and kidneys (K) at E18.5 from WT (D) and *Get1*$^{-/-}$ mice (E). The *Get1*$^{-/-}$ urinary bladder is enlarged, whereas the kidneys appear normal. (**F–I**) Hematoxylin–eosin staining of the bladder epithelium from WT (F, H) and *Get1*$^{-/-}$ (G, I) mice at E16.5 (F, G) and E18.5 (H, I). Arrowheads point to the umbrella cells in WT mice at E16.5 and E18.5. Arrows point to the surface-layer cells in *Get1*$^{-/-}$ urothelium at E18.5. The dashed lines indicate the border between the epithelium and mesenchyme. Umbrella cells are absent from the *Get1*$^{-/-}$ bladder epithelium. Scale bar: (A–C) and (F–I), 12.5 μM.

In contrast, superficial cells of the *Get1*$^{-/-}$ urothelium are much smaller, less than 10 μm in diameter (Figure 3C), and the apical surface is covered by small microvilli rather than ridges (Figure 3D). This appearance is characteristic for WT superficial epithelium, earlier in development at E15.5 (Erman *et al*, 2006). As described previously using transmission electron microscopy, the apical membrane of WT apical umbrella cells is scalloped in appearance and the cells contain abundant fusiform cytoplasmic vesicles (Figure 3E), which have an elongated form and are thought to be responsible for delivering uroplakins to the surface. In contrast, the superficial cells of the *Get1*$^{-/-}$ urothelium are round, with a much smoother apical membrane containing microvilli. In addition, the cytoplasmic vesicles, which have a discoid rather than a fusiform appearance (Figure 3F), are less abundant (Figure 3G) in *Get1*$^{-/-}$ superficial cells. Together, these results show that the *Get1* gene is critical for normal umbrella-cell formation in the bladder epithelium (Figure 3H).

Get1 is required for permeability barrier formation and terminal differentiation in bladder epithelium

Terminal differentiation of umbrella cells is critical for the establishment of the urinary bladder barrier. Therefore, to investigate the effect of *Get1* on bladder permeability barrier function, we tested the ability of the bladder epithelium to exclude methylene blue as described previously (Hu *et al*, 2000). Compared with WT bladders, methylene blue easily penetrated into the E18.5 *Get1*$^{-/-}$ bladder wall (Figure 4A–D), suggesting that in *Get1*$^{-/-}$ bladders, permeability barrier function is impaired. This finding is consistent with the impaired formation of umbrella cells. Impaired umbrella-cell differentiation was further supported by analysis of keratin (K) expression (Erman *et al*, 2006). At E16.5, K18 is normally expressed in the subapical region of the developing umbrella cells of the bladder epithelium (Figure 4E). In contrast, K18 is not detected in *Get1*$^{-/-}$ bladder epithelium (Figure 4F). K20 is another urothelial differentiation marker that is expressed in umbrella cells at a later stage (Figure 4G). In contrast, K20 is strikingly downregulated in *Get1*$^{-/-}$ urothelium at E18.5 (Figure 4H). At both E16.5 and E18.5, K6 is expressed at a low level in the basal and intermediate layers, but not in the umbrella-cell layer of the WT urothelium (Figure 4I and K). In contrast, K6, a marker for wounded epithelia, is markedly upregulated throughout the *Get1*$^{-/-}$ uroepithelium at both stages (Figure 4J and L). As K6 expression is sometimes associated with hyperproliferation, we evaluated mitotic activity in the bladder epithelium. The mitotic cell marker phospho-histone H3 is unchanged in *Get1*$^{-/-}$ urothelium, suggesting that *Get1* does not affect urothelial cell proliferation (Figure 4M–O). Together, these data indicate that *Get1* selectively regulates the urothelial terminal differentiation program.

Deletion of the Get1 gene affects multiple genes in the urothelial terminal differentiation program

To gain insights into the molecular mechanisms of bladder defects in *Get1*$^{-/-}$ mice, we carried out a microarray analysis of RNA from the bladders of three *Get1*$^{-/-}$ and three *Get1*$^{+/-}$ E18.5 mice. Statistical differential-expression analysis identified 622 differentially expressed probe sets ($P<0.00138$, false discovery rate within 10%) in the *Get1*$^{-/-}$ mice (Supplementary Figure S4). We then filtered this list based on at least 1.5-fold up- or downregulation, which resulted in 226 probe sets. Out of these 226 probe sets, 66 probe sets were regulated during the normal bladder differentiation program as described previously (Figure 1 and Supplementary Figure S3): 20 downregulated, 46 upregulated. Intriguingly, the downregulated genes in *Get1*$^{-/-}$ bladders (Figure 5A) are all in clusters 1–3 (increasing expression during wild-type bladder differentiation; Supplementary Figure S3), suggesting that *Get1* is likely to have a function

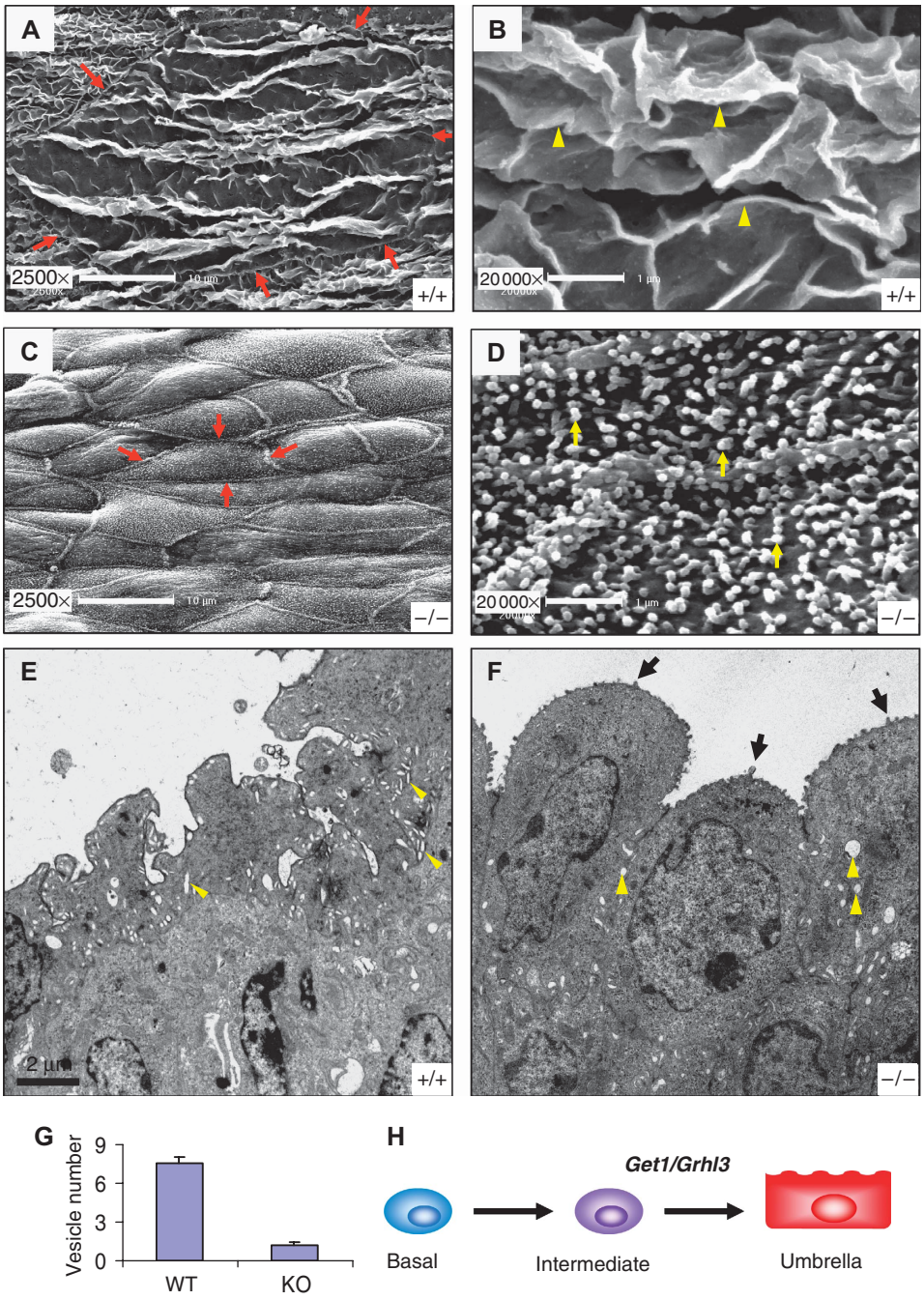


Figure 3 *Get1* is required for apical cell-membrane specialization in the bladder epithelium. (**A**–**D**) Scanning electron microscopy of the bladder epithelial surface of WT (A, B) and *Get1*$^{-/-}$ (C, D) mice at E18.5. Red arrows mark the outline of superficial cells. Yellow arrowheads point to hinge regions on the plicated surface of WT mice. Yellow arrows point to microvilli in the *Get1*$^{-/-}$ urothelium. The superficial cells of the *Get1*$^{-/-}$ urothelium are much smaller than in the WT, and their surface contains microvilli rather than ridges. (**E**, **F**) Transmission electron microscopy of WT (E) and *Get1*$^{-/-}$ (F) bladder epithelium. Arrowheads in (E) point to fusiform vesicles. Arrowheads in (F) point to discoidal vesicles. Arrows in (F) point to microvilli. The *Get1*$^{-/-}$ urothelium lacks urothelial apical plaques, and uroplakin-containing fusiform vesicles are much less prominent than in the WT. (**G**) Quantification of vesicle number in bladder superficial cells. The *Y*-axis shows the vesicle number per μm^2. The results represent the mean and s.e.m. for three WT and three *Get1*$^{-/-}$ embryos. (**H**) Model for the role of Get1 in urothelial cell differentiation.

in activating the expression of a battery of urothelial terminal differentiation genes. The uroplakins are assembled into heterodimers that form planar crystals, and these crystals eventually form the AUM plaques, which are crucial components in bladder barrier function (Sun *et al*, 1996; Sun, 2006). Transcripts of all members of the uroplakin family are significantly downregulated in *Get1*$^{-/-}$ bladders (Figure 5C and Supplementary Table S2). Among these genes, *UpkII* is strikingly downregulated 8.5-fold. Another markedly downregulated gene is *Snx31*, which is involved in endosomal recycling (Cullen, 2008) and could possibly have a function in plaque formation. In contrast, the upregulated genes are distributed among all the clusters (Figure 5B and C), and include genes associated with injury response, such as *keratin 6a*.

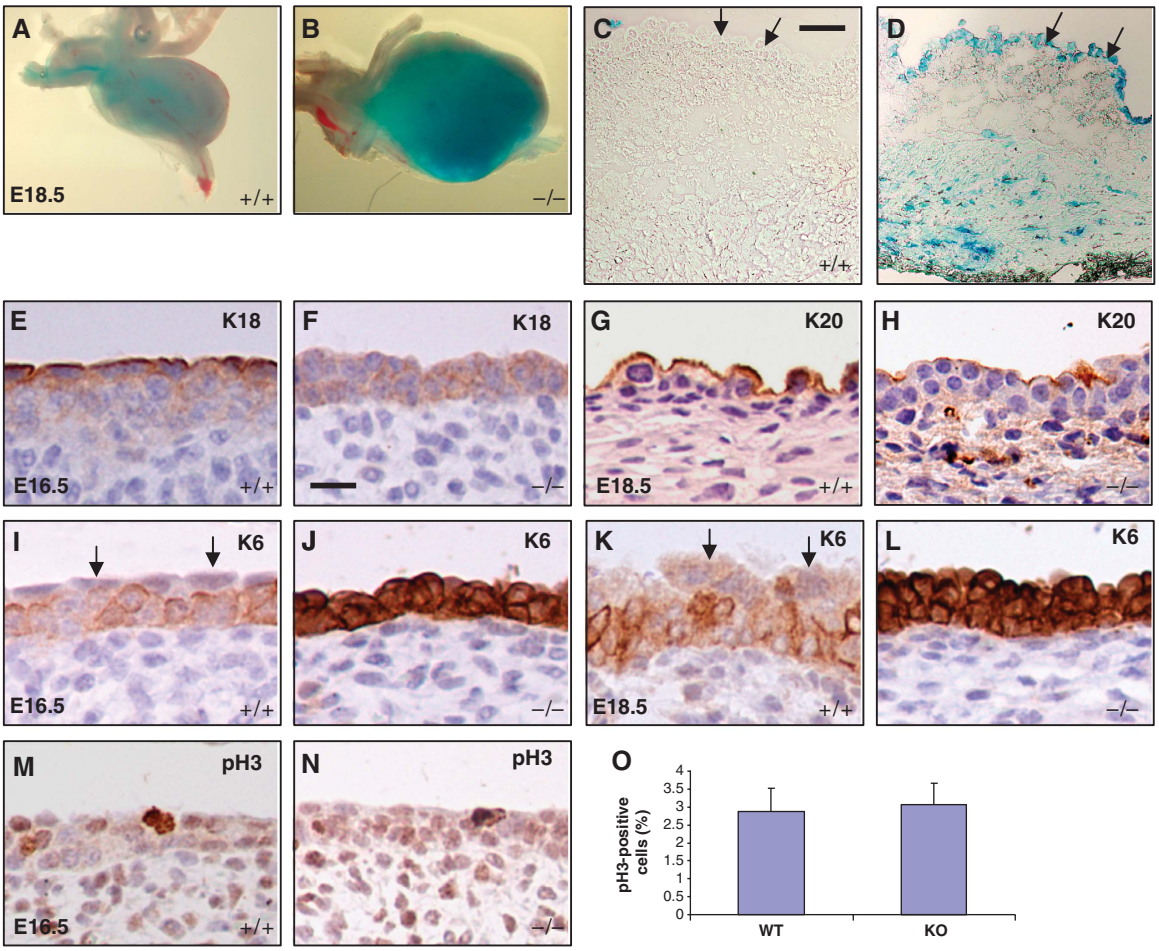


Figure 4 *Get1* is required for effective permeability-barrier formation and terminal differentiation in mouse bladder epithelium. (**A**, **B**) Gross pictures of methylene-blue penetration assay of WT (A) and *Get1*$^{-/-}$ (B) urinary bladders. (**C**, **D**) Post-sections of WT (C) and *Get1*$^{-/-}$ (D) urinary bladders after methylene-blue assay. Arrows in (C) and (D) point to the bladder epithelium. The methylene-blue dye infiltrates the bladder wall of *Get1*$^{-/-}$ mice, indicating impaired permeability barrier. (**E–N**) Immunostaining showing expression of K18 (E, F), K20 (G, H), K6 (I–L) and phospho-histone 3 (M, N) in WT (E, G, I, K, M) and *Get1*$^{-/-}$ (F, H, J, L, N) bladder epithelium at the indicated stages. Arrows in (I) and (K) point to normal umbrella cells. Expression of the umbrella-cell markers K18 and K20 is decreased, indicating impaired differentiation. The expression of K6 is markedly upregulated and extends to the surface in *Get1*$^{-/-}$ bladder epithelium, where it is normally excluded. (**O**) Quantification of phospho-histone-3-positive cells in WT and *Get1*$^{-/-}$ bladder epithelium. The number of phospho-histone-3-positive cells is normal, indicating that the cell proliferation is unchanged in the *Get1*$^{-/-}$ bladder epithelium. Scale bar: (C, D) 50 μM; (E–N) 12.5 μM.

Cell adhesion is a common mechanism contributing to barrier formation both in the epidermis and in the bladder epithelium. The deletion of *Get1* leads to defective cell–cell adhesion in the epidermis because of the downregulation of several cell-adhesion molecules (Yu *et al*, 2006). Interestingly, tight-junction structures are less prominent and there is abnormal separation of superficial cells in the *Get1*$^{-/-}$ bladder (Figure 6A and B). Some tight-junction transcripts, such as Cldn5, Cldn6, Cldn8, Cldn9 and Cldn11, are downregulated in the *Get1*$^{-/-}$ bladder epithelium, whereas others, such as Cldn2, Cldn3, and Cldn7, are upregulated (Figure 6C and Supplementary Figure S5). Transcripts for Cldn1, Cldn4, Cldn23, and Ocln, which are downregulated in *Get1*$^{-/-}$ epidermis, are not altered in the *Get1*$^{-/-}$ bladder. Therefore, *Get1* seems to regulate different cell–cell adhesion molecules in the bladder epithelium and epidermis.

The uroplakin II gene is a direct target of Get1 in bladder epithelium

All major uroplakin (UpkIa, UpkIb, UpkII, UpkIIIa, and UpkIIIb) transcripts were downregulated in *Get1*$^{-/-}$ urothelium at both E16.5 and E18.5 stages (Figures 5, 7A and B). Similarly, at least three major uroplakin proteins (UpkIb, UpkII, and UpkIIIa) that are preferentially expressed in umbrella cells were downregulated in the *Get1*$^{-/-}$ bladder epithelium at both E16.5 and E18.5 (Figure 7C–N). These results are consistent with a model whereby Get1 promotes the differentiation of the Upk-expressing umbrella cells, and suggests the possibility that Get1 might directly activate uroplakin genes.

To start investigating whether Get1 is a direct regulator of uroplakin genes, we used MatInspector (Cartharius *et al*, 2005) and BEARR (Batch Extraction and Analysis of *cis*-Regulatory Regions) (Vega *et al*, 2004) to identify high-affinity Get1 DNA-binding sites in a genomic region from 50 kb upstream to 5 kb downstream of the coding region of each of the uroplakin genes. Several high-affinity sites were identified in all five uroplakin genes, and some of these sites were conserved between human and mouse (Supplementary Figure S6A). One of these conserved Get1-binding sites resided in an evolutionarily conserved region in the *UpkII* gene, located in the region from −105 to −112 bp upstream of

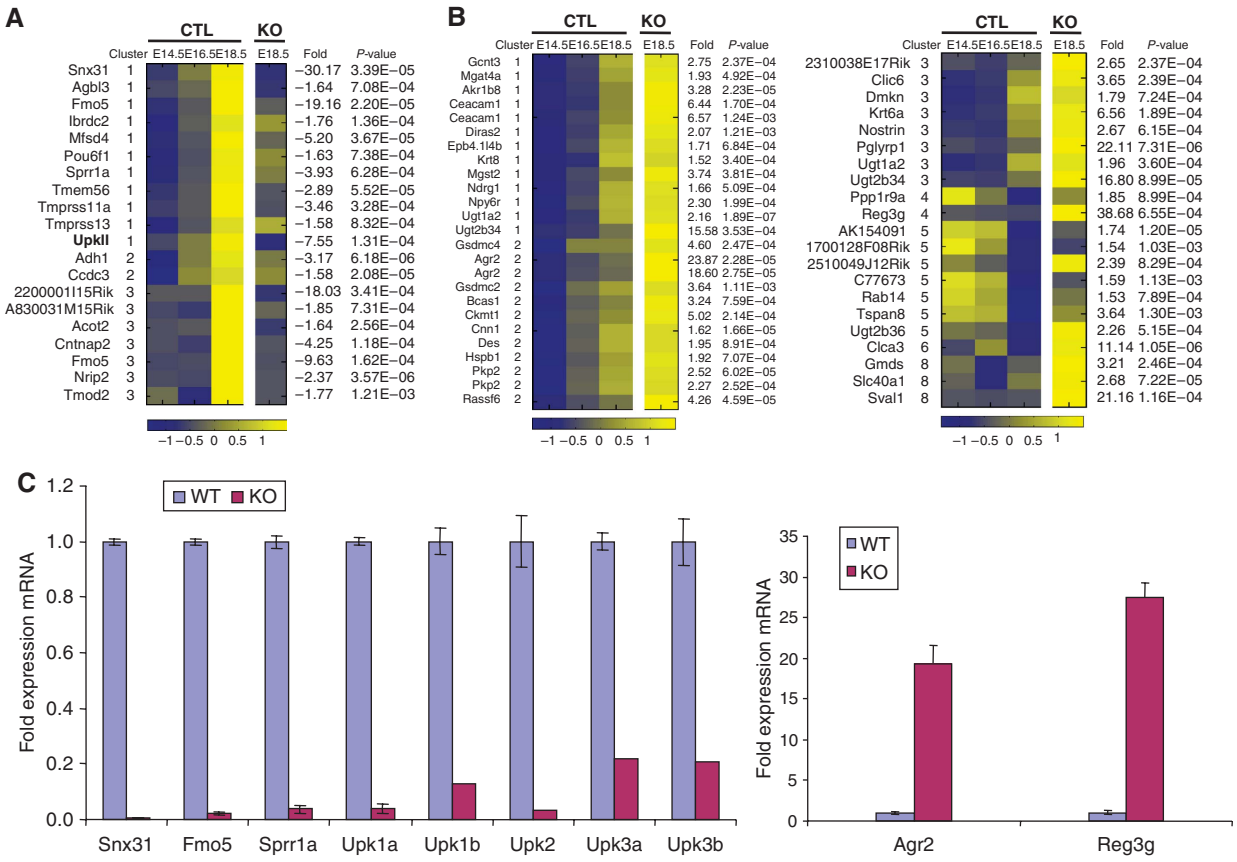


Figure 5 Identification of significantly differentially expressed genes in E18.5 *Get1*$^{-/-}$ bladder. (**A**) Downregulated and (**B**) upregulated bladder differentiation-related genes. Expression levels from microarrays are normalized across time points and indicated by the colorimetric ratio scale. The fold and *P*-value of significant differential expression, as well as the cluster membership of the WT time course, for each gene are indicated. Note that UpkII (in bold) is significantly downregulated, and that several genes have multiple probe sets. (**C**) Quantitative RT–PCR validation of selective genes that are differentially regulated in *Get1*$^{-/-}$ E18.5 bladders. Expression for each gene is normalized to 1 for WT. Results represent the mean and s.e.m. for three replicates.

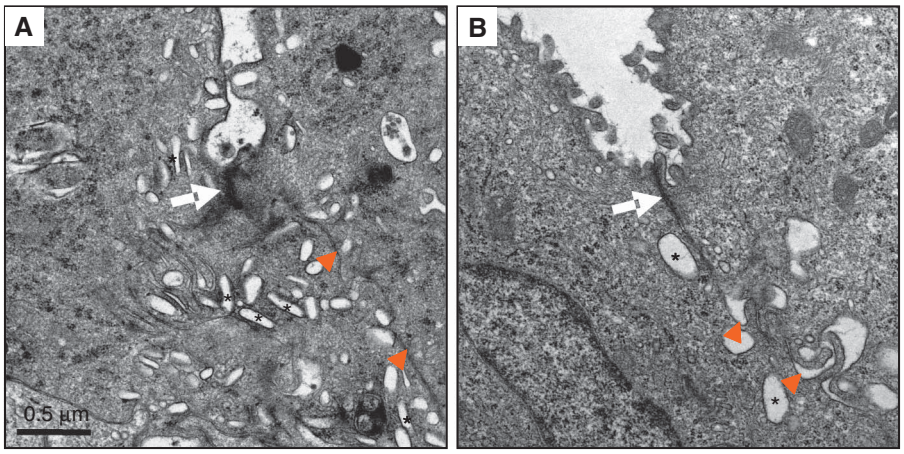
the transcription start site of the human *UpkII* gene (Figure 8A). This binding site is functional because the expression of an *UpkII* reporter plasmid in RT4 human bladder cells, which express endogenous Get1, is highly dependent on this site (Figure 8B). Furthermore, in chromatin immunoprecipitation (ChIP) experiments, Get1 binds to this region of the *UpkII* promoter in RT4 cells. This binding is specific as Get1 does not bind to another region downstream of the UpkII promoter that lacks high-affinity Get1 DNA-binding sites (Figure 8C). Together, these experiments suggest that Get1 directly regulates the expression of the *UpkII* gene in bladder epithelial cells.

Epigenetic mechanisms for the cell-specific activation of the uroplakin II gene

Although the *Get1* gene is expressed in both the epidermis and bladder epithelia, the *UpkII* gene is selectively expressed in the bladder epithelium. This suggests that other mechanisms may have a function in the selective activation of *UpkII* in the bladder epithelium. To address this question, we used normal human epidermal keratinocytes (NHEK) and the bladder epithelial cell line RT4. Like the epidermis, differentiated NHEK cells express Get1 but not UpkII, whereas RT4 cells express Get1 and UpkII (Figure 8D–F), as does the bladder. Using ChIP assays, we found that RNA polymerase binding and Get1 binding to the UpkII promoter is more prominent in RT4 cells than in NHEK (Figure 8G). Furthermore, H3K9 acetylation, a marker of open chromatin, is increased on the *UpkII* promoter in RT4 cells, whereas H3K27 trimethylation, a marker of transcriptional repression, is more prominent in the NHEK cells (Figure 8G). Consistent with a role for histone deacetylation in repression of the *UpkII* gene in NHEK cells, treatment of these cells with the histone-deacetylation complex (HDAC) inhibitor trichostatin A (TSA) resulted in a 30-fold increase in the expression of *UpkII* in NHEK cells (Figure 8H). Together, these experiments suggest that the ability of Get1 to selectively activate the *UpkII* gene in uroepithelial cells is regulated at the level of DNA binding, which is most likely controlled by histone modifications and chromatin structure.

Discussion

In this paper, we carried out a genome-wide time-course gene-expression study of skin and bladder development to identify unique and common transcription programs. The Grainyhead transcription factor Get1 is among a small subset of transcription factors showing expression that correlates with differentiation-marker expression in both the skin and bladder. Gene deletion shows that *Get1* is critical for apical-



C

Expression of cell adhesion genes in *Get1* mutant bladder and skin at E18.5

	bladder		skin	
Gene symbol	Fold	*P*-value	Fold	*P*-value
Pcdh9	−2.47	0.0025	NC	>0.099
Evpl	−2.15	0.02	−1.3	0.04
Cldn9	−1.98	0.09	NS	
Cldn11	−1.91	0.01	NS	
Cldn5	−1.89	0.008	NC	>0.099
Cldn6	−1.85	0.015	NC	>0.099
Celsr1	−1.83	0.04	−1.3	0.06
Cldn8	−1.8	0.04	−1.48	0.09
Ocln	−1.31	0.2	−2.22	0.0022
Ppl	−1.15	0.31	−1.8	0.00009
Cldn23	−1.09	0.03	−3.01	0.0002
Cldn1	1.11	0.01	−2.31	0.0008
ZO1	1.28	0.07	NC	>0.099
Itga5	1.62	0.007	−1.04	0.07
Pkp2	1.79	0.05	NC	>0.099
Cdh1	1.8	0.0008	−1.23	0.09
Pvr	1.91	0.007	NC	>0.099
Dsc2	1.96	0.001	NC	>0.099
Cobl	2.02	0.008	−2	0.0002
Cldn7	2.19	0.0001	NC	>0.099
Cldn3	2.99	0.004	NS	
Cldn2	10.22	0.0006	NS	
Cdh17	12.03	0.0007	NS	
Cldn4	NC	>0.099	−2.2	0.0005
Dsc1	NC	>0.099	−1.54	0.03
Cdsn	NS		−1.49	0.01
Pkp2	1.85	0.003	−1.67	0.0024

Figure 6 Adhesion abnormalities in superficial urothelial cells in the *Get1*$^{-/-}$ bladders. (**A**, **B**) Transmission electron microscopy images of WT (A) and Get1 mutant (B) bladders at E18.5. The white arrows in panels A and B point to tight junctions. The red arrowheads in panel A point to normal cell–cell adhesions. The red arrowheads in panel B point to separated cell–cell adhesions. There are abundant fusiform vesicles in panel A, whereas only a few discoid vesicles are observed in panel B. Vesicles are indicated by the stars. Scale bar: 0.5 μM. The results are based on three WT and three *Get1*$^{-/-}$ E18.5 embryos. (**C**) The statistically significant differentially expressed cell-adhesion genes in *Get1*$^{-/-}$ bladder and skin. NC indicates no significant change; NS indicates not 'present' in the microarray data set.

membrane specialization of the urothelium and terminal differentiation of umbrella cells, as well as for barrier formation. A broad range of genes associated with urothelial differentiation are affected by the *Get1* deletion. Among the genes that were strikingly downregulated in *Get1*$^{-/-}$ mice are the prominent components of the specialized membrane, the uroplakins. The *UpkII* gene was identified as a direct target of Get1. The target-gene specificity in bladder epithelial cells is due to selective binding of Get1 to the *UpkII* promoter, which is mostly probably regulated by histone modification. These results suggest that *Get1* has analogous roles in regulation of terminal differentiation and barrier formation in

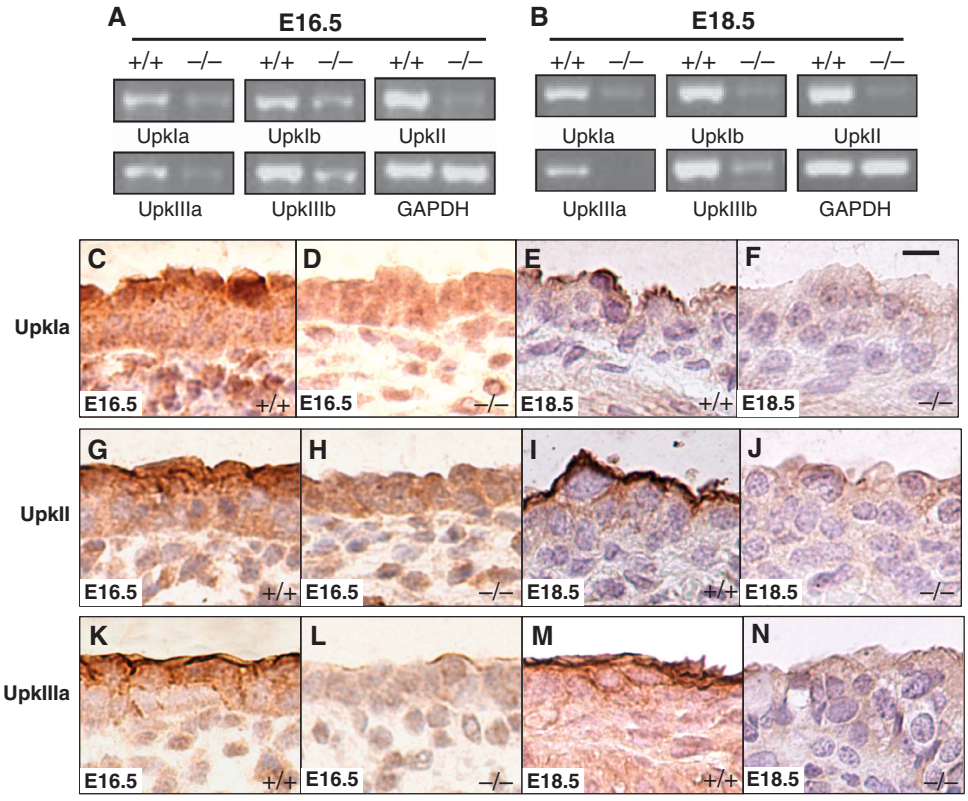


Figure 7 *Get1* is required for normal uroplakin expression in the bladder epithelium. (**A**, **B**) Semi-quantitative qRT–PCR analysis of UpkIa, UpkIb, UpkII, UpkIIIa, and UpkIIIb in WT and *Get1*$^{-/-}$ bladder epithelium at E16.5 (A) and E18.5 (B). Expression of all uroplakin transcripts is decreased in the *Get1*$^{-/-}$ bladders. (**C**–**N**) Immunostaining showing expression of UpkIa (C–F), UpkII (G–J), and UpkIIIa (K–N) in WT (C, E, G, I, K, M) and *Get1*$^{-/-}$ (D, F, H, J, L, N) bladder epithelium at the indicated stages. The bladder epithelial expression of UpkIa, UpkII, and UpkIIIa proteins is decreased in *Get1*$^{-/-}$ mice, based on four WT and four *Get1*$^{-/-}$ mice. Scale bar: (C–N) 12.5 μM.

different epithelia in the mouse by regulating distinct downstream targets.

Unique and shared transcriptional regulatory mechanism in bladder and skin development

The terminal differentiation programs in bladder and skin epithelium are distinct. In the mouse, the epidermis starts stratifying at E14.5, ultimately producing morphologically distinct layers, which are clearly identified at E18.5. The basal layer contains proliferating cells that undergo successive differentiation steps through the spinous, granular, and cornified layers as they move towards the surface (Candi *et al*, 2005). The cornified layer is composed of dead cells that contain a cellular envelope linked to a cytoskeleton of significant strength. Thus, in the epidermis, multiple cytoplasmic structural proteins, many encoded by the epidermal differentiation complex, as well as lipid-producing enzymes are critical for complete differentiation and barrier formation (Dai and Segre, 2004; Candi *et al*, 2005; Koster and Roop, 2007). In contrast, in the bladder epithelium, the uroplakin membrane proteins are critical for barrier formation (Hu *et al*, 2000; Kong *et al*, 2004). Furthermore, after the initial formation, cell renewal is much slower in the bladder epithelium than in the epidermis (Sun, 2006). In contrast to these differences, the barriers of the two epithelia have cell–cell adhesion molecules in common, in particular tight-junction complex mechanisms (Acharya *et al*, 2004; Niessen, 2007).

Consistent with these distinct differentiation programs, we found that 6.4 and 60.6% of the transcriptional regulators unique to the bladder and skin, respectively, were highly correlated with the expression of differentiation genes in each organ, whereas this was true for less than 1% of the transcriptional regulators common to the bladder and skin (Figure 1A). These data suggest that transcriptional programs specific for each organ are important for terminal differentiation. At the same time, our data also provide evidence for shared transcriptional mechanisms between these divergent epithelia, and Get1 is among the small number of transcriptional regulators showing expression that correlates with the differentiation programs in both organs (Figure 1C). Although previous work has established the role of Get1 in the regulation of epidermal differentiation and barrier formation in diverse species (Bray and Kafatos, 1991; Wilanowski *et al*, 2002; Venkatesan *et al*, 2003; Mace *et al*, 2005; Ting *et al*, 2005; Yu *et al*, 2006), this study shows that Get1 has a similar role in barrier formation in an internal epithelium. Interestingly, Get1 is expressed in other internal epithelia, including the gastrointestinal track (Kudryavtseva *et al*, 2003; Auden *et al*, 2006). In Get1$^{-/-}$ mice, the forestomach shows evidence of impaired differentiation and the intestine is dilated with blood in the lumen (Supplementary Figure S7A–D) (Yu *et al*, 2006), suggesting the possibility that *Get1* might have a more general role in maintaining internal epithelial integrity.

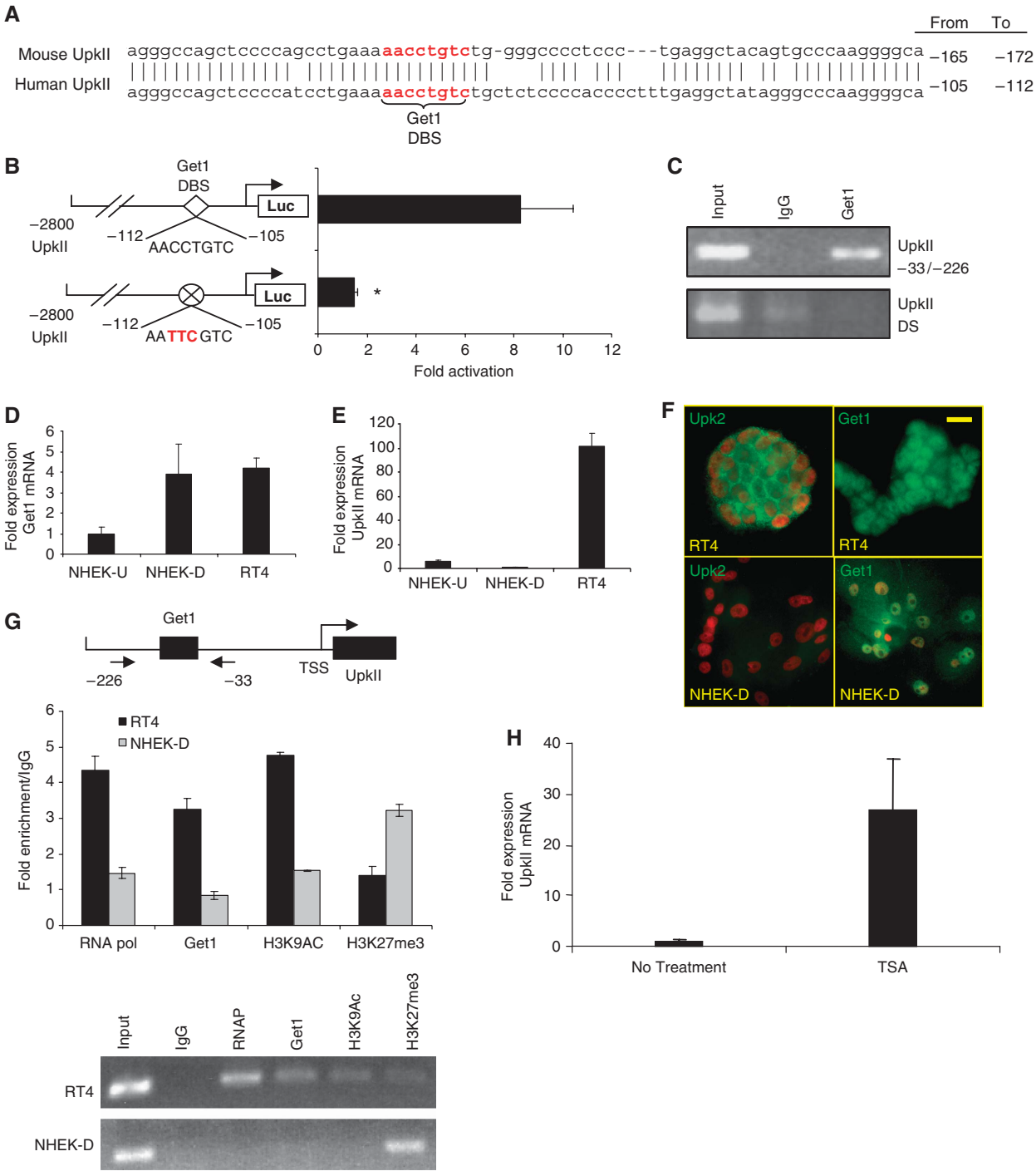


Figure 8 Get1 directly regulates Uroplakin II expression and is dependent on histone modifications. (**A**) Identification of an evolutionarily conserved Get1 DNA-binding site (DBS) in the *UpkII* promoter. Location of the binding site upstream of the transcriptional start site is indicated for both human and mouse *UpkII* genes. (**B**) WT and mutated −2800 *UpkII* luciferase-reporter constructs were transfected into RT4 bladder cells. Mutation of the Get1-binding site resulted in decreased *UpkII* promoter activity. (**C**) Chromatin immunoprecipitation (ChIP) assays carried out on RT4 cells using a Get1 antibody or IgG as a control, followed by PCR amplification of a region of the human *UpkII* promoter containing the Get1 site indicated in (A, upper panel) and a non-specific downstream (DS) region of the *UpkII* gene as a negative control (lower panel). Input DNA is unprecipitated chromatin from the IgG sample. Get1 binds specifically to the promoter region of the *UpkII* gene. (**D**, **E**) Quantitative real-time PCR of *Get1* (D) and *UpkII* (E) transcripts in undifferentiated (NHEK-U) and differentiated (NHEK-D) normal human epidermal keratinocytes, and RT4 cells. Standard error of the mean and mean fold differences in expression were calculated using three replicates normalized to *RPLPO*. (**F**) Get1 and UpkII protein expression in RT4 cells and NHEK-D detected by immunofluorescence staining. Although Get1 protein is expressed in both cell types, UpkII is selectively expressed in RT4 cells. Nuclear DAPI staining is red. (**G**) ChIP assays carried out on NHEK-D and RT4 cells using antibodies against RNA polymerase II, Get1, H3K9Ac, and H3K27me3, followed by PCR amplification of a 193-bp region of the human UpkII promoter containing the Get1 site in (A). Get1 and RNA polymerase-II binding, and histone modifications were quantified using quantitative PCR (upper) and also analyzed by direct inspection on agarose gels (lower). Black bars indicate binding in RT4 cells and gray bars indicate binding in NHEK-D cells. Get1 binding to the UpkII promoter is associated with increased RNA polymerase II binding and histone modifications associated with active gene expression. TSS indicates transcription start site. (**H**) Quantitative real-time PCR of *UpkII* in NHEK-D cells with and without TSA treatment. TSA treatment increased the expression of UpkII in human keratinocytes.

Get1 regulates uroplakin *gene expression, umbrella-cell differentiation, and barrier formation*

The most striking urothelial abnormality of the *Get1*$^{-/-}$ mice is defective formation of umbrella cells, suggesting that *Get1* is critical for the transition from intermediate to umbrella cells (Figure 3H). Decreased expression in the *Get1*$^{-/-}$ bladder of multiple genes that normally show urothelial differentiation-linked expression is also consistent with this conclusion (Figure 5). Thus, Grainyhead-like transcription factor Get1 is critical for the promotion of terminal differentiation of the bladder epithelium.

Among the differentially expressed urothelial differentiation genes, decreased uroplakin expression (Figure 7) is likely to be critical for this defect in *Get1*$^{-/-}$ mice. Uroplakins are the major subunits of the AUM plaques in the apical plasma membrane, which are generated after the formation of uroplakin heterodimers (UpkIa–II and UpkIb–III) in the endoplasmic reticulum and planar crystals in the post-Golgi compartment (Sun, 2006). Our results show that all uroplakins are moderately downregulated in *Get1*$^{-/-}$ urothelium at the mRNA level and strikingly at the protein level. Low expression of uroplakins leads to failure of plaque formation and loss of the typical rugged apical surface (Kong *et al*, 2004). In the *Get1*$^{-/-}$ urothelium, the normal plicated surface is replaced by numerous microvilli at the apical surface (Figure 3C and D). Therefore, the failure of AUM plaque assembly is probably the main cause of defective urinary bladder barrier formation in *Get1*$^{-/-}$ mice. Consistent with this idea, deletion of *UpkII* or *UpkIIIa* resulted in defective bladder barrier formation because AUM plaques were not well assembled in the umbrella-cell apical membrane (Hu *et al*, 2000; Kong *et al*, 2004). Both *UpkII*$^{-/-}$ and *UpkIIIa*$^{-/-}$ urothelium showed a similar phenotype as the *Get1*$^{-/-}$ urothelium. The superficial cells in *UpkII*$^{-/-}$ mice are smaller and devoid of rigid plaques at the apical membrane (Kong *et al*, 2004) secondary to the failed assembly of UpkIa/II heterodimers. The *UpkIIIa*$^{-/-}$ urothelium also had smaller superficial cells lacking mature fusiform vesicles, and the apical surface was covered with microvilli because of the assembly failure of the UpkIb–III heterodimer (Hu *et al*, 2000). The phenotypic similarity between *Get1*$^{-/-}$ on the one hand and *UpkII*$^{-/-}$ and *UpkIIIa*$^{-/-}$ urothelium on the other supports the idea that *Get1* affects barrier formation by regulating uroplakins and AUM assembly. However, *Get1* regulates additional terminal differentiation genes and these are likely to contribute to the phenotype as well.

Previous studies using *in vitro* differentiation systems have indicated that the nuclear receptor PPARγ is important for bladder epithelial differentiation. Activation of PPARγ induces the expression of *UpkII* and *UPKIa* and regulates the components of tight junction in bladder epithelial cells (Varley *et al*, 2004, 2006). Recent studies indicate that PPARγ acts upstream of transcription factors, FOXA1 and IRF-1, which may directly regulate *uroplakin* gene expression (Varley *et al*, 2009). Consistent with this idea, PPARγ shows increasing expression from E14.5 to E18.5 during bladder development (Figure 2). The effect of *PPARγ* gene deletion on bladder development has not been studied. In the epidermis, PPARγ is expressed at a relatively low level (Dahten *et al*, 2007), and *PPARγ*$^{-/-}$ mice did not show any abnormality (Mao-Qiang *et al*, 2004). Therefore, although PPARγ might be important for normal bladder epithelial differentiation, it seems to act differently from Get1.

The cell-specific regulation of Get1 on UpkII is dependent on histone modification

Our data indicate that Get1 transcriptionally regulates the expression of *UpkII* gene by directly binding to a high-affinity binding site in the *UpkII* promoter (Figure 8). Get1 might directly regulate other *uroplakin* genes as well, because several Get1-binding sites are found around all *uroplakin* genes (Supplementary Figure S6A and B). In addition to *UpkII*, the *UpkIb* and *UpkIIIa* genes contain conserved sites between human and mouse that bind Get1 in RT4 urothelial cells (Supplementary Figure S6C). Although the bladder epithelia of *Get1* and *UpkII* knockout mice show overlapping differentiation phenotypes, only the *UpkII* knockout mice show upregulation of other *uroplakin* genes, presumably because of compensatory mechanisms (Kong *et al*, 2004). In contrast, in *Get1*$^{-/-}$ mice, all uroplakins are downregulated, which further supports the primary role of Get1 in *uroplakin* gene regulation.

As *UpkII* is not expressed in the differentiated epidermis, we considered the reasons for selective actions of Get1 on the *UpkII* promoter in bladder epithelial cells. One possible model is that *UpkII* activation depends on a combinatorial action of Get1 with other transcription factors that are selectively expressed in bladder epithelium. Under this model, Get1 binds to the *UpkII* promoter in epidermal cells but is unable to activate the gene because the accessory factor is missing. An alternative model is that the *UpkII* gene is modified by chromatin alterations in the epidermis, thus preventing binding of Get1 in these cells. We tested these models by studying the binding of Get1 to the *UpkII* promoter in urothelial cells and differentiated epidermal keratinocytes. We found that Get1 binds selectively to the *UpkII* promoter in the urothelial cells, and that this binding is associated with binding of RNA polymerase (Figure 8G). In bladder epithelial cells, this region of the *UpkII* promoter has an enrichment of histone H3K9 acetylation, which has been shown to correlate with actively transcribed genes (Pokholok *et al*, 2005). In contrast, in epidermal keratinocytes, the *UpkII* gene has a marked decrease of Get1 and RNA polymerase binding, as well as decreased histone H3K9 acetylation. Instead, there is increased histone H3K27 trimethylation, a well-studied chromatin modification associated with silent genes (Zhao *et al*, 2007). Furthermore, differentiated epidermal keratinocytes treated with TSA, a potent Class I and II HDAC inhibitor, showed an increase in *UpkII* expression (Figure 8H), supporting the idea that the presence of an H3K9-acetylation mark is correlated with expression of *UpkII*. Therefore, our results suggest that epigenetic mechanisms could allow Get1 to selectively activate different gene targets in a cell-specific manner (Figure 9).

In conclusion, we have shown that the Grainyhead-like transcription factor Get1 has a critical function in regulating terminal differentiation and barrier function of the bladder epithelium, in addition to its previously described role in epidermal differentiation. During evolution, distinct gene sets involved in barrier formation in at least two different epithelia have acquired Get1-binding sites, thus bringing these genes under control of Get1. Epigenetic mechanisms could allow selective regulation of the appropriate set of genes for

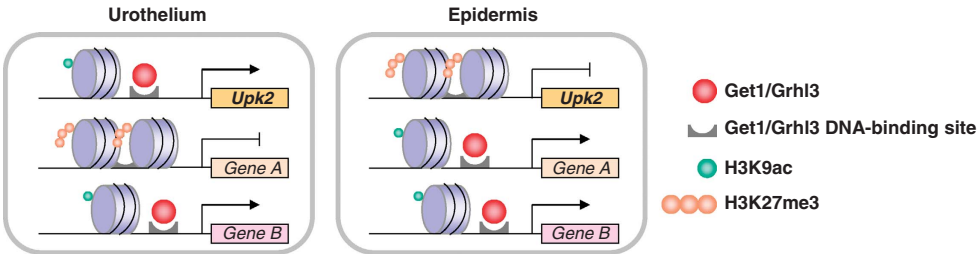


Figure 9 Model of UpkII regulation in the urothelium and epidermis. In the urothelium, the H3K9Ac mark is present and Get1 binds to the promoter and activates transcription of the *UpkII* gene. In contrast, in the epidermis, where the H3K9-acetylation mark is absent and an H3K27me3-repressive mark is enriched, Get1 does not bind as efficiently to the *UpkII* gene. We speculate that for some epidermal-specific genes (gene *A*) the reverse may true; Get1 activates selectively in epidermal cells. For yet other genes that are expressed in both the epidermis and urothelium (gene *B*), Get1 may bind and activate in both cell types.

each epithelium, and we speculate that such mechanisms might be widely used to allow cell-specific activity of a developmental transcription factor.

Materials and methods

Microarray analysis

Mouse backskin and bladder were dissected from WT and *Get1*$^{-/-}$ mice at E14.5, E16.5, and E18.5. RNA was isolated and microarray experiments were carried out as described previously (Yu *et al*, 2006). Both tissues contain both epithelial and mesenchymal components. The GC-robust multichip average method was used for background correction of the microarray data. Statistically differentially expressed transcriptional regulators during bladder and skin differentiation were identified by one-way ANOVA ($P<0.01$, false discovery rate within 10%) of the respective time-course profiles. Expression profiles of representative terminal differentiation markers of the bladder (uroplakins) and skin (claudin 4, plakophilin 3, periplakin, and envoplakin) were used to calculate Pearson product moment correlation coefficients. Correlation coefficients of at least 0.8 were used to define transcriptional regulators with expression highly correlated to terminal differentiation markers. Global similarity in gene expression between the dorsal skin and bladder at E14.5, E16.5, and E18.5 was determined by averaging the Pearson product moment correlation coefficients across all probe sets for each pair of sample types (tissue/stage). For example, the dorsal skin at E14.5 and bladder at E14.5 has an average correlation coefficient of 0.92 across all probe sets. Statistically differentially expressed genes (4101 probe sets) during bladder differentiation were identified by one-way ANOVA across time points ($P<0.01$, false discovery rate within 10%). After averaging the gene expression values across replicates for each time point, the time-course profiles were normalized to mean 0 and s.d. 1 for *k*-means clustering using Euclidean distance. Enrichment of functional categories (Gene Ontology Biological Process) within each cluster was found using the Functional Annotation Tool in DAVID 2008 (Dennis *et al*, 2003). As there are many redundant categories, only representative categories are shown in Figure 2. Transcriptional regulators were defined as probe sets with annotations of Gene Ontology Biological Process 'regulation of transcription, DNA-dependent' (GO ID 0006355) and Gene Ontology Cellular Component 'nucleus' (GO ID 0005634). Chromatin modifiers were defined as probe sets with annotations of Gene Ontology Biological Process 'establishment and/or maintenance of chromatin architecture' (GO ID 0006325). In order to account for the gene expression variability due to different scan dates in the Get1 wild-type and knockout microarrays, we removed the scan date batch effect using Partek Genomics Suite. Statistically differentially expressed genes (622 probe sets) between wild type and knockout were identified by one-way ANOVA across time points ($P<0.00138$, false discovery rate within 10%). This list was then filtered based on at least 1.5-fold up- or downregulation, resulting in 226 highly and significantly differentially expressed probe sets. The intersection between these genes and the normal bladder differentiation-regulated genes is 66 probe sets. Note that the microarray data have been submitted to http://www.ncbi.nlm.nih.gov/geo.

Histology, immunostaining, and in situ *hybridization*

Embryonic mouse urinary bladders were fixed in 10% formalin and paraffin embedded. Following sectioning, 5-μm sections were stained with hematoxylin and eosin. For immunohistochemistry staining, antigen retrieval was carried out by heating slides to 95°C for 10 min in 0.01-M citrate buffer (pH6) in a microwave oven. The sections were then immunostained by the ABC peroxidase method (Vector) using diaminobenzidine as the enzyme substrate and hematoxylin as a counterstain. We used the following primary antibodies: rabbit anti-mouse Get1 (Yu *et al*, 2008) and ARP33196 (Aviva Systems Biology); mouse monoclonal anti-human keratin 18 (Dako); mouse monoclonal anti-human cytokeratin 20 (Dako); rabbit anti-mouse keratin 6 (Covance); rabbit anti-phospho-histone H3 (Cell Signaling Technology); rabbit anti-mouse UpkIa (Santa Cruz Biotechnology); rabbit anti-mouse UpkII (Santa Cruz Biotechnology); and rabbit anti-mouse UpkIIIa (Santa Cruz Biotechnology). All the staining results were based on studies on at least three WT and three KO mice.

Scanning and transmission electron microscopy

For scanning electron microscopy, five WT and four *Get1*$^{-/-}$ bladders were dissected from E18.5 embryos and fixed in 10% formalin overnight. After a PBS wash, tissues were postfixed in 1% osmium for 1 h and dehydrated through graded-ethanol series and HMDS. After gold coating, tissues were observed with a scanning electron microscope as described previously. For transmission electron microscopy, three WT and three *Get1*$^{-/-}$ bladders were dissected from E18.5 embryos and fixed by immersion in 4% paraformaldehyde and 1% glutaraldehye in 0.1 M PBS (pH 7.4) at room temperature, followed by a PBS wash. The tissues were then postfixed with 0.2% osmium and dehydrated through graded-ethanol series, and embedded in Agar 100 resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate, and examined on a transmission electron microscope as described previously (Yu *et al*, 2008).

Methylene-blue penetration assay

We used a modification of a previously described method by Hu *et al* (2000). Four WT and four *Get1*$^{-/-}$ bladders were dissected from E18.5 embryos. A total of 20 μl of 0.1% methylene blue (Sigma–Aldrich) in 0.9% NaCl was injected to the bladder from the uretery side. The dye was removed 20 min later, and the bladders were extensively washed five times with 0.9% NaCl, after which the bladders were photographed by a Nikon Coolpix 995 camera. The post-sectioning was based on four WT and three *Get1*$^{-/-}$mice.

RT–PCR analysis

Semi-quantitative RT–PCR was carried out with cDNA generated using the High Capacity cDNA Archive Kit (Applied Biosystems), using total RNA prepared from the urinary bladder of WT and *Get-1*$^{-/-}$ embryos using Trizol Reagent (Invitrogen). Reactions were sampled after 25, 28, and 30 cycles at different PCR conditions to monitor product accumulation. We used the following primers: *UpkIa* forward: TCGGTACATGATCCTCACG; *UpkIa* reverse: GTCCGACGACAGCACAGTGG; *UpkIb* forward: GCCTCTTCTGCTTG

TCCGTTC; *UpkIb* reverse: CATCGTTATTCTCCACCCG; *UpkII* forward: CCACACTGCCTGTCCAGACCT; *UpkII* reverse: GGTTTGTCA CCTGATATGCG; *UpkIIIa* forward: TATGGTCAGATCCCATCCG; *UpkIIIa* reverse: CTTGCTGGAGAACACCTCTGC; *UpkIIIb* forward: AGATCCCGACCTCCCACAG; *UpkIIIb* reverse: AAGTTATGACGATCA-TACAGCCG; *Get1* forward: GAATTACAAGTCTGTGCCACCA; and *Get1* reverse: ATTTGCTGACCTTTTTCTGAGC. Quantitative RT–PCR was carried out using the following TaqMan Gene Expression Assays (Applied Biosystems): *UpkII* (Hs00171854_m1, Mm00447665_m1), *Get 1*(Grhl3, Hs00297962_m1, Mm01193339_m1), *UpkIa* (Mm01176593_m1), *UpkIb* (Mm00769504_m1), *UpkIIIa* (Mm00452321_m1), *UpkIIIb* (Mm00452321_m1), *Nfix* (Mm004777 96_m1), *Smarcd3* (Mm00491850_m1), *Snx31* (Mm00508591_m1), *Agr2* (Mm00507853_m1), *Fmo5* (Mm00515805), *Yeats4* (Mm0051 8302_m1), *Hadc2* (Mm00515108_m1), *Cldn2* (Mm00516703_s1), *Cldn5* (Mm00727012_s1), *Sprr1a* (Mm01962902_s1), *Reg3g* (Mm00441127_m1), human *RPLP0* and mouse *GAPDH* as endogenous controls.

Cell culture

RT4 cells were maintained in McCoy's 5A medium (Gibco) supplemented with 5% penicillin–streptomycin and 10% FBS. Neonatal human epidermal keratinocytes were purchased from Cascade and grown according to the manufacturer's instructions in EpiLife Medium (Cascade) supplemented with EDGS (Cascade) and 0.6 mM $CaCl_2$. Cells were grown for 5 days in a medium supplemented with a final concentration of 1.8 mM $CaCl_2$ to induce differentiation. For TSA experiments, NHEK cells were seeded into six-well plates, differentiated for 5 days, and then treated with 200 ng of TSA (Cell Signaling Technology). Cells were collected 40 h after treatment.

Site-directed mutagenesis and transient transfection reporter assays

A 2.8-kb mouse *UpkII* promoter sequence was cloned into the pGL3-basic luciferase reporter vector (Promega). The Get1 core-binding site was mutated to AATTCGTCT by site-directed mutagenesis, using the QuikChange® Site-Directed Mutagenesis Kit (Strategene) as per the manufacturer's instructions. RT4 cells were seeded into six-well plates 1 day before transfection. Luciferase reporter constructs (1 μg) were transfected by Expressfect (Denville Sci. Inc.) as per the manufacturer's instructions. To control for transfection efficiency, transfections were normalized to Renilla luciferase vector as described previously (Yu *et al*, 2006). Luciferase activity was measured 1 day after transfection. All experiments were carried out at least three times, each time in triplicate.

Gel mobility shift assays

Get1 protein expression and gel mobility shift assays were carried out as described previously (Yu *et al*, 2006). The sequences of oligonucleotides for binding were: consensus: TCCTGTTAAACC GGTTTTTCTAGT; consensus mut: CAGTGAGCAGAGATCACGCCA TTG; UpkIa (7.5*): CTTGCACAAACCAGTTCTGGTCCC; UpkIb (7.9*): CCAG GAGGAACAGGTTCATTTTCA; UpkIIIa (7.1*): TCTTCCTAAACCGGGTGTGGTGGC; UpkIIIa (7.5*): CAATGGCAAA CCAGTTTTGCCCTG; UpkIIIb (7.5*A): CTGCGTGAAACCAGTTACT GGTCT; UpkIIIb (7.5*B): GAGGAGAGAACCAGTTCTGGCAAG; human UpkIb: ACCAGTTGAACTGGTTATACATTC; human UpkIIIa: GACAGACAAACCAGTTTACTGCTG.

Chromatin immunoprecipitation assays

For the chromatin immunoprecipitation (ChIP) assays, we followed previously described methods (Rabinovich *et al*, 2008) with the following modifications. We used the following antibodies for immunoprecipitations: RNA polymerase II (Covance Cat#MMS-126R), IgG (Sigma Cat#15006-10MG), H3K9Ac (AbCam Cat# ab4441), H3K27me3 (AbCam Cat# ab6002), and Get1/Grhl3 (Aviva Cat# ARP33196). Primers used for amplification of ChIP DNA were: *RNApolII* forward, AGATGAAACCGTTGTCCAAACT; *RNApolII* reverse, AGGTTACGGCAGTTTGTCTCTC; *UpkII*-33/-226 forward, GGG TG GCTGATTTTGTCCT; *UpkII*-33/-226 reverse, GTGATTTCAAACCC CACCT; *UpkII*DS forward, TGGCCTCGTCTTTGTCTTTT; *UpkII*DS reverse, CATGATGAAACCCCGTCTCT; *UpkIb* forward, GGGTGAGG CAGAACATCACT; *UpkIb* reverse, TGGAGGGTTCATATCACAAAAG; *UpkIIIa* forward, CTGTTCAGGGGACAGGAAAG; *UpkIIIa* reverse, CCCAGGAGAAGTCCTGTGTC; *DHFR* forward, CTGATGTCCAGGAGG AGAAAAGG; *DHFR* reverse, AGCCCGACAATGTCAAGGACTG.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

This work was supported by NIH grants AR44882 (to BA) and NLM NRSA T15LM00744 (to KKL). We thank Xing Dai, Anand K Ganesan, Sergei A Grando, Steven Lipkin, and Elizabeth L Rugg for reading the paper; Francisco Espitia and William Gordon for technical help; the UC Irvine Microarray Core Facility for microarray experiments; and the Expression Analysis Core at UC Davis for ChIP training.

References

Acharya P, Beckel J, Ruiz WG, Wang E, Rojas R, Birder L, Apodaca G (2004) Distribution of the tight junction proteins ZO-1, occludin, and claudin-4, -8, and -12 in bladder epithelium. *Am J Physiol Renal Physiol* **287:** F305–F318

Andersen B, Weinberg WC, Rennekampff O, McEvilly RJ, Bermingham Jr JR, Hooshmand F, Vasilyev V, Hansbrough JF, Pittelkow MR, Yuspa SH, Rosenfeld MG (1997) Functions of the POU domain genes Skn-1a/i and Tst-1/Oct-6/SCIP in epidermal differentiation. *Genes Dev* **11:** 1873–1884

Auden A, Caddy J, Wilanowski T, Ting SB, Cunningham JM, Jane SM (2006) Spatial and temporal expression of the Grainyhead-like transcription factor family during murine development. *Gene Expr Patterns* **6:** 964–970

Baxter RM, Brissette JL (2002) Role of the nude gene in epithelial terminal differentiation. *J Invest Dermatol* **118:** 303–309

Bray SJ, Kafatos FC (1991) Developmental function of Elf-1: an essential transcription factor during embryogenesis in Drosophila. *Genes Dev* **5:** 1672–1683

Brissette JL, Li J, Kamimura J, Lee D, Dotto GP (1996) The product of the mouse nude locus, Whn, regulates the balance between epithelial cell growth and differentiation. *Genes Dev* **10:** 2212–2221

Candi E, Schmidt R, Melino G (2005) The cornified envelope: a model of cell death in the skin. *Nat Rev Mol Cell Biol* **6:** 328–340

Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T (2005) MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics* **21:** 2933–2942

Cullen PJ (2008) Endosomal sorting and signalling: an emerging role for sorting nexins. *Nat Rev Mol Cell Biol* **9:** 574–582

Dahten A, Mergemeier S, Worm M (2007) PPARgamma expression profile and its cytokine driven regulation in atopic dermatitis. *Allergy* **62:** 926–933

Dai X, Segre JA (2004) Transcriptional control of epidermal specification and differentiation. *Curr Opin Genet Dev* **14:** 485–491

Deng FM, Liang FX, Tu L, Resing KA, Hu P, Supino M, Hu CC, Zhou G, Ding M, Kreibich G, Sun TT (2002) Uroplakin IIIb, a urothelial differentiation marker, dimerises with uroplakin Ib as an early step of urothelial plaque assembly. *J Cell Biol* **159:** 685–694

Dennis Jr G, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA (2003) DAVID: database for annotation, visualisation, and integrated discovery. *Genome Biol* **4:** P3

Erman A, Veranic P, Psenicnik M, Jezernik K (2006) Superficial cell differentiation during embryonic and postnatal development of mouse urothelium. *Tissue Cell* **38:** 293–301

Garcia-Espana A, Chung PJ, Zhao X, Lee A, Pellicer A, Yu J, Sun TT, Desalle R (2006) Origin of the tetraspanin uroplakins and their co-evolution with associated proteins: implications for uroplakin structure and function. *Mol Phylogenet Evol* **41:** 355–367

Hislop NR, Caddy J, Ting SB, Auden A, Vasudevan S, King SL, Lindeman GJ, Visvader JE, Cunningham JM, Jane SM (2008) Grhl3 and Lmo4 play coordinate roles in epidermal migration. *Dev Biol* **321:** 263–272

Hu P, Deng FM, Liang FX, Hu CM, Auerbach AB, Shapiro E, Wu XR, Kachar B, Sun TT (2000) Ablation of uroplakin III gene results in small urothelial plaques, urothelial leakage, and vesicoureteral reflux. *J Cell Biol* **151:** 961–972

Hu P, Meyers S, Liang FX, Deng FM, Kachar B, Zeidel ML, Sun TT (2002) Role of membrane proteins in permeability barrier function: uroplakin ablation elevates urothelial permeability. *Am J Physiol Renal Physiol* **283:** F1200–F1207

Jezernik K, Romih R, Veranic P (2000) Urothelial cell detachment and differentiation in urinary bladder. *Pflugers Arch* **439:** R135–R136

Kong XT, Deng FM, Hu P, Liang FX, Zhou G, Auerbach AB, Genieser N, Nelson PK, Robbins ES, Shapiro E, Kachar B, Sun TT (2004) Roles of uroplakins in plaque formation, umbrella cell enlargement, and urinary tract diseases. *J Cell Biol* **167:** 1195–1204

Koster MI, Roop DR (2007) Mechanisms regulating epithelial stratification. *Annu Rev Cell Dev Biol* **23:** 93–113

Kudryavtseva EI, Sugihara TM, Wang N, Lasso RJ, Gudnason JF, Lipkin SM, Andersen B (2003) Identification and characterization of Grainyhead-like epithelial transactivator (GET-1), a novel mammalian Grainyhead-like factor. *Dev Dyn* **226:** 604–617

Lewis SA, de Moura JL (1982) Incorporation of cytoplasmic vesicles into apical membrane of mammalian urinary bladder epithelium. *Nature* **297:** 685–688

Mace KA, Pearson JC, McGinnis W (2005) An epidermal barrier wound repair pathway in Drosophila is mediated by grainy head. *Science* **308:** 381–385

Mao-Qiang M, Fowler AJ, Schmuth M, Lau P, Chang S, Brown BE, Moser AH, Michalik L, Desvergne B, Wahli W, Li M, Metzger D, Chambon PH, Elias PM, Feingold KR (2004) Peroxisome-proliferator-activated receptor (PPAR)-gamma activation stimulates keratinocyte differentiation. *J Invest Dermatol* **123:** 305–312

Min G, Wang H, Sun TT, Kong XP (2006) Structural basis for tetraspanin functions as revealed by the cryo-EM structure of uroplakin complexes at 6-A resolution. *J Cell Biol* **173:** 975–983

Morasso MI, Grinberg A, Robinson G, Sargent TD, Mahon KA (1999) Placental failure in mice lacking the homeobox gene Dlx3. *Proc Natl Acad Sci USA* **96:** 162–167

Negrete HO, Lavelle JP, Berg J, Lewis SA, Zeidel ML (1996) Permeability properties of the intact mammalian bladder epithelium. *Am J Physiol* **271:** F886–F894

Niessen CM (2007) Tight junctions/adherens junctions: basic structure and function. *J Invest Dermatol* **127:** 2525–2532

Oottamasathien S, Wang Y, Williams K, Franco OE, Wills ML, Thomas JC, Saba K, Sharif-Afshar AR, Makari JH, Bhowmick NA, DeMarco RT, Hipkens S, Magnuson M, Brock III JW, Hayward SW, Pope IV JC, Matusik RJ (2007) Directed differentiation of embryonic stem cells into bladder tissue. *Dev Biol* **304:** 556–566

Pokholok DK, Harbison CT, Levine S, Cole M, Hannett NM, Lee TI, Bell GW, Walker K, Rolfe PA, Herbolsheimer E, Zeitlinger J, Lewitter F, Gifford DK, Young RA (2005) Genome-wide map of nucleosome acetylation and methylation in yeast. *Cell* **122:** 517–527

Rabinovich A, Jin VX, Rabinovich R, Xu X, Farnham PJ (2008) E2F *in vivo* binding specificity: comparison of consensus versus nonconsensus binding sites. *Genome Res* **18:** 1763–1777

Segre J (2003) Complex redundancy to build a simple epidermal permeability barrier. *Curr Opin Cell Biol* **15:** 776–782

Segre JA, Bauer C, Fuchs E (1999) Klf4 is a transcription factor required for establishing the barrier function of the skin. *Nat Genet* **22:** 356–360

Staack A, Donjacour AA, Brody J, Cunha GR, Carroll P (2003) Mouse urogenital development: a practical approach. *Differentiation* **71:** 402–413

Sun TT (2006) Altered phenotype of cultured urothelial and other stratified epithelial cells: implications for wound healing. *Am J Physiol Renal Physiol* **291:** F9–F21

Sun TT, Zhao H, Provet J, Aebi U, Wu XR (1996) Formation of asymmetric unit membrane during urothelial differentiation. *Mol Biol Rep* **23:** 3–11

Ting SB, Caddy J, Hislop N, Wilanowski T, Auden A, Zhao LL, Ellis S, Kaur P, Uchida Y, Holleran WM, Elias PM, Cunningham JM, Jane SM (2005) A homologue of Drosophila grainy head is essential for epidermal integrity in mice. *Science* **308:** 411–413

Ting SB, Wilanowski T, Auden A, Hall M, Voss AK, Thomas T, Parekh V, Cunningham JM, Jane SM (2003) Inositol- and folate-resistant neural tube defects in mice lacking the epithelial-specific factor Grhl-3. *Nat Med* **9:** 1513–1519

Varley CL, Bacon EJ, Holder JC, Southgate J (2009) FOXA1 and IRF-1 intermediary transcriptional regulators of PPARgamma-induced urothelial cytodifferentiation. *Cell Death Differ* **16:** 103–114

Varley CL, Garthwaite MA, Cross W, Hinley J, Trejdosiewicz LK, Southgate J (2006) PPARgamma-regulated tight junction development during human urothelial cytodifferentiation. *J Cell Physiol* **208:** 407–417

Varley CL, Stahlschmidt J, Lee WC, Holder J, Diggle C, Selby PJ, Trejdosiewicz LK, Southgate J (2004) Role of PPARgamma and EGFR signalling in the urothelial terminal differentiation programme. *J Cell Sci* **117:** 2029–2036

Vega VB, Bangarusamy DK, Miller LD, Liu ET, Lin CY (2004) BEARR: batch extraction and analysis of cis-regulatory regions. *Nucleic Acids Res* **32:** W257–W260

Venkatesan K, McManus HR, Mello CC, Smith TF, Hansen U (2003) Functional conservation between members of an ancient duplicated transcription factor family, LSF/Grainyhead. *Nucleic Acids Res* **31:** 4304–4316

Wilanowski T, Tuckfield A, Cerruti L, O'Connell S, Saint R, Parekh V, Tao J, Cunningham JM, Jane SM (2002) A highly conserved novel family of mammalian developmental transcription factors related to Drosophila grainyhead. *Mech Dev* **114:** 37–50

Wu XR, Lin JH, Walz T, Haner M, Yu J, Aebi U, Sun TT (1994) Mammalian uroplakins. A group of highly conserved urothelial differentiation-related membrane proteins. *J Biol Chem* **269:** 13716–13724

Yu Z, Bhandari A, Mannik J, Pham T, Xu X, Andersen B (2008) Grainyhead-like factor Get1/Grhl3 regulates formation of the epidermal leading edge during eyelid closure. *Dev Biol* **319:** 56–67

Yu Z, Lin KK, Bhandari A, Spencer JA, Xu X, Wang N, Lu Z, Gill GN, Roop DR, Wertz P, Andersen B (2006) The Grainyhead-like epithelial transactivator Get-1/Grhl3 regulates epidermal terminal differentiation and interacts functionally with LMO4. *Dev Biol* **299:** 122–136

Zhao XD, Han X, Chew JL, Liu J, Chiu KP, Choo A, Orlov YL, Sung WK, Shahab A, Kuznetsov VA, Bourque G, Oh S, Ruan Y, Ng HH, Wei CL (2007) Whole-genome mapping of histone H3 Lys4 and 27 trimethylations reveals distinct genomic compartments in human embryonic stem cells. *Cell Stem Cell* **1:** 286–298