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## SNAI2 Controls the Undifferentiated State of Human Epidermal Progenitor Cells

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### Abstract

The transcription factor, SNAI2 is an inducer of the epithelial to mesenchymal transition (EMT) which mediates cell migration during development and tumor invasion. SNAI2 can also promote the generation of mammary epithelial stem cells from differentiated luminal cells when overexpressed. How SNAI2 regulates these critical and diverse functions is unclear. Here we show that the levels of SNAI2 expression are important for epidermal cell fate decisions. The expression of SNAI2 was found to be enriched in the basal layer of the interfollicular epidermis where progenitor cells reside and extinguished upon differentiation. Loss of SNAI2 resulted in premature differentiation whereas gain of SNAI2 expression inhibited differentiation. SNAI2 controls the differentiation status of epidermal progenitor cells by binding to and repressing the expression of differentiation genes with increased binding leading to further transcriptional silencing. Thus, the levels of SNAI2 binding to genomic targets determines the differentiation status of epithelial cells with increased levels triggering EMT and dedifferentiation, moderate (physiological) levels promoting epidermal progenitor function, and low levels leading to epidermal differentiation.

### Keywords

Stem Cell; Progenitor Cell; Differentiation; Epidermis; Skin Differentiation; SNAI2; SLUG; SNAIL; EMT; transcription factor; GRHL3; KLF4; metastasis; epithelium; ChIP-Seq; epidermal differentiation; EMT

### Introduction

Adult stem and progenitor cells replenish the tissue they reside in for the life of the animal[1]. These cells must balance proliferation and differentiation to maintain homeostasis as well as respond to injury. The molecular mechanisms underlying how adult stem cells maintain themselves in an undifferentiated state is still unclear and is an active area of research. The epidermis is an ideal tissue to decipher the mechanisms of self-renewal and differentiation since it is a tissue that has a rapid turnover as well as being able to regenerate the entire human epidermis in-vitro[2,3]. The basal layer which is the deepest layer of the

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epidermis contains stem and progenitor cells that upon differentiation migrate upwards to form the spinous layer. As differentiation proceeds, the cells continue to migrate upwards to form the granular layer, which is characterized by the assembly of the cornified envelope proteins underneath the plasma membrane. During the final stages of differentiation, transglutaminase is activated which crosslinks the cornified envelope proteins while lipids are extruded into the extracellular space. This insoluble sac of proteins and lipids forms the water impermeable barrier known as the stratum corneum[4]. We and others have recently shown that both transcriptional and post-transcriptional mechanisms are necessary for maintaining self-renewal of epidermal stem and progenitor cells. Transcriptional mechanisms include epigenetic factors such as DNMT1, EZH2, UHRF1, CBX4, and ACTL6A which act to suppress differentiation gene expression through epigenetic mechanisms[3,5-8]. Post-transcriptional mechanisms include RNA degradation enzymes such as the exosome complex which promotes self-renewal by targeting and degrading pro-differentiation mRNAs in progenitor cells[9]. The switch to a differentiated state requires the upregulation and actions of transcription factors such as grainy head-like 1 and 3 (GRHL1 and GRHL3), zinc finger protein (ZNF750), CCAAT/enhancer-binding protein (CEBPA and CEBPB), and kruppel-like factor 4 (KLF4) [10-15].

Recently, it was reported that overexpression of epithelial to mesenchymal transition transcription factors (EMT-TFs) could convert differentiated luminal cells into a mammary epithelial stem cell state[16,17]. The epithelial to mesenchymal transition is the process by which epithelial cells lose cell-cell adhesion and gain cell motility during the conversion to a mesenchymal state[18]. This process is mediated through a family of transcription factors including SNAIL, TWIST, and ZEB[19]. The SNAIL family of zinc-finger transcription factors contain a highly conserved carboxyl terminus containing between four to six C<sub>2</sub>H<sub>2</sub> zinc fingers which mediate specific binding to a consensus six base motif of CAGGTG (A subset of the E-box, CANNTG)[20,21]. Upon binding to the motif, SNAIL family members mediate transcriptional repression through the SNAG domain found in the amino terminus. The SNAIL family is composed of three members including SNAIL (SNAI1), SLUG (SNAI2), and SMUG (SNAI3). EMT-TFs have been shown to regulate development such as the ingression of the cells from the surface of the embryo to form organs[22]. In adults, SNAI2 has been shown to promote wound healing[23]. During cancer progression, EMT-TFs mediate the invasion and metastatic properties of epithelial tissues[24]. Because of EMT-TFs role in promoting cell motility and invasion, it was surprising that overexpression of EMT-TFs could promote both stem cell and cancer stem cell maintenance in the mammary epithelium[16,17]. The underlying mechanisms remain unclear. It is also not known whether promoting the stem cell state by EMT-TFs is unique to mammary stem cells or is applicable to other epithelial cells such as those found in the epidermis.

Here we investigate the function of the EMT-TF, SNAI2 in human epidermal cells. We find that the levels of SNAI2 expression dictate the differentiation status of the epidermis. High levels of SNAI2 leads to dedifferentiation and an EMT phenotype while moderate (physiological) levels of SNAI2 binding leads to sustained progenitor cell function. In contrast, low levels of SNAI2 seen during epidermal differentiation result in expression of differentiation genes due to loss of SNAI2 repressive activity at their promoters. These

results provide an explanation for how EMT-TFs can regulate both EMT and the differentiation status of epithelial cells.

## Results

### **SNAI2 expression is enriched in epidermal progenitor cells and downregulated during differentiation**

To identify EMT transcription factors important for maintaining progenitor cells in an undifferentiated state, we searched for genes whose transcripts levels were highly expressed in undifferentiated human epidermal keratinocytes. Of the SNAIL, TWIST, and ZEB family of EMT-TF, only SNAI2 was highly expressed while the others were undetectable or minimally expressed (Fig. 1A-B). Interestingly, SNAI2 was also downregulated on the transcript as well as protein level during epidermal differentiation (Fig. 1C-E). In adult human epidermis, SNAI2 protein expression was confined to the nucleus of the basal layer cells and diminished in the outer differentiated layers of the epidermis that is marked by the differentiation protein keratin 10: K10 (Fig. 1F). This suggests SNAI2 may play a role in preventing the differentiation of the progenitor cells residing in the basal layer.

### **The expression levels of SNAI2 determine the differentiation status of epidermal tissue and cultured cells**

To test for a potential functional role for SNAI2 in the epidermis, SNAI2 was constitutively expressed through a retrovirus in organotypic human epidermal tissue. This regenerated human tissue recapitulates epidermal stratification and differentiation in a three dimensional context through the use of primary human epidermal keratinocytes and intact extracellular matrix in the form of human dermis[2,3]. Ectopic expression of SNAI2 prevented the expression of differentiation protein K10 as well as differentiation genes *KRT1*, *FLG*, *TGM1*, *SPRR1A*, *GRHL3*, and *KLF4* (Fig. 2A-B). Overexpressed SNAI2 could be seen throughout the epidermis whereas endogenous SNAI2 was mainly localized to the basal layer (Supporting Information Fig. S1). Increased expression of SNAI2 in cultured primary epidermal progenitor cells resulted in an EMT phenotype with the cells acquiring a spindle shaped appearance and downregulation of epithelial adhesion genes such as *CDH1*, *CLDN1*, *CLDN7*, *GJB6* and upregulation of mesenchymal genes such as *VIM* (Supporting Information Fig. S2A-B) [19]. The progenitor cells also became dedifferentiated due to decreased expression of basal levels of *KLF4* and *GRHL3* (Supporting Information Fig. S2B). Conversely, depletion of SNAI2 using shRNAs resulted in faster induction and more robust expression of differentiation protein K10 during the time course of epidermal tissue regeneration (Fig. 2C). Importantly, the basal layer was much smaller in the SNAI2i tissue with at most 1 cell layer whereas in control tissue there were several layers of undifferentiated basal layer cells (Fig. 2C). The knockdown of SNAI2 was validated with the absence of SNAI2 staining in the basal layer of SNAI2i epidermis (Supporting Information Fig. S1). SNAI2 depletion in cultured cells resulted in premature expression of differentiation protein TGM1, increased cell adhesion and differentiation gene expression similar to cells undergoing calcium induced differentiation (Fig. 2D-F and Supporting Information Fig. S2C-D). These results suggest that the levels of SNAI2 are critical for the

differentiation status of epidermal cells with higher levels inhibiting and lower levels promoting differentiation.

### **SNAI2 controls a gene expression program that represses differentiation and cell adhesion**

To determine how the levels of SNAI2 impacts the differentiation status of epidermal cells, global gene expression profiling was performed on control and SNAI2 knockdown cells. In keratinocytes cultured in growth medium (GM), SNAI2 knockdown altered the expression of 801 genes (2 fold change; 5% FDR) with 490 genes upregulated and 311 genes downregulated (Fig. 3A and Supporting Information Table S1A). Comparison with our previously generated data set of genes that changed during calcium-induced differentiation (DM: differentiation medium) revealed a significant overlap of 558 genes (~70%, Fig. 3A and Supporting Information Table S1B-C)[5]. The vast majority (76%: 424/558) of the genes in the overlapped SNAI2i gene signature (SNAI2i GM) were regulated in the same direction as cells undergoing calcium induced differentiation (CTL DM) (Fig. 3A). 273 genes were upregulated in differentiated and SNAI2i cells with enriched gene ontology (GO) terms such as cell adhesion, cornified envelope, keratinization, and keratinocyte differentiation (Fig. 3A-B). 151 co-downregulated genes were enriched in terms such as cell motion, cell migration, and extracellular matrix (Fig. 3A and 3C). These results suggest that loss of SNAI2 mimics calcium induced epidermal differentiation through the increased expression of cell adhesion and differentiation genes and the downregulation of cell motility genes. To determine if increased levels of SNAI2 had the opposite effect as SNAI2 depletion, global gene expression profiling was performed on control LACZ and SNAI2 overexpressing epidermal progenitor cells. Increased SNAI2 expression resulted in the differential expression of 978 genes with 517 genes upregulated and 461 genes downregulated (Fig. 3D and Supporting Information Table S2A). 449 genes overlapped with the differentiation gene expression signature (Fig. 3D and Supporting Information Table S2B). Interestingly a majority of the overlapped genes (65%: 294/449) were oppositely regulated as the differentiation signature (Fig. 3D). 128 genes were upregulated in SNAI2 overexpressing keratinocytes while being downregulated during calcium induced differentiation (Fig. 3D). These genes were enriched in GO terms such as cell motion, cell migration, and extracellular matrix (Fig. 3E). 166 genes were downregulated in SNAI2 overexpressing cells and upregulated during differentiation with cornified envelope, cell-cell junction, and keratinocyte differentiation GO terms (Fig. 3D and 3F). This suggests that high levels of SNAI2 promote dedifferentiation and increased cell motility. To identify the genes that are the most susceptible to the levels of SNAI2 as well as potentially being direct targets, the SNAI2 depleted and overexpressed signatures were overlapped with each other. 248 genes overlapped with over 90% (225/248) of the genes oppositely regulated between the two data sets (Fig. 3G and Supporting Information Table S2C). 144 genes were downregulated in SNAI2 overexpressing cells and upregulated in SNAI2i cells with epidermal differentiation GO terms (Fig. 3G-H). These genes included differentiation induced structural (*LCE3D*, *SCEL*, *SPRR1B*), cell adhesion (*DSC2*, *CLDN1*, *CLDN4*) and transcription factor (*GRHL3*, *KLF4*) genes (Fig. 3J). 81 genes enriched for cell motion and cell migration were downregulated in SNAI2 depleted cells and upregulated in SNAI2 overexpressing cells (Fig. 3G and 3I). These include genes downregulated during

differentiation such as *VIM* and *VCAN* (Fig. 3J). These results suggest that the levels of *SNAI2* are critical to the differentiation status of epidermal cells. Decreased levels of *SNAI2* lead to increased differentiation due to higher cell adhesion, keratinization, and cornified envelope gene expression while increased levels of *SNAI2* promote cell motility and dedifferentiation.

### **SNAI2 expression dictates the levels of SNAI2 binding across the genome**

To determine the direct targets of *SNAI2*, we performed chromatin immunoprecipitations (ChIP) combined with deep sequencing (ChIP-Seq) using a *SNAI2* antibody on progenitor cells overexpressing *LACZ* or *SNAI2* and differentiated cells overexpressing *LACZ* or *SNAI2*. ChIP-Seq was also performed on control shRNA and *SNAI2i* progenitor cells to determine the specificity of the antibody. Depletion of *SNAI2* by shRNAs resulted in a dramatic loss of *SNAI2* binding on the genomic level as well as on single genes such as *GRHL1*, *CDH1*, and *ITGA3* (Supporting Information Fig. S3A-D). Validation by qPCR also showed that knockdown of *SNAI2* resulted in a dramatic loss of *SNAI2* binding to genes such as *GRHL1* and *ITGA3* as well as known target *CDH1* [25] (Supporting Information Fig. S3E). We identified 7,873 *SNAI2* bound peaks in control *LACZ* expressing progenitor cells which were reduced to 1,550 peaks in differentiated *LACZ* epidermal cells (Fig. 4A and Supporting Information Table S3A-B). This suggests that during differentiation, 80% of the *SNAI2* binding sites disappear due to the downregulation of *SNAI2* (Fig. 4A and Fig. 1D-E). Overexpression of *SNAI2* during differentiation (which blocks epidermal differentiation) restored the *SNAI2* binding sites to 10,298 peaks, which is similar to the 11,912 peaks found in progenitor cells overexpressing *SNAI2* (Fig. 4A and Supporting Information Table S3C-D). 50-55% of the *SNAI2* bound peaks centered in regions around the transcriptional start site (TSS)(Fig. 4A-B). This included the promoter (35-39%) and 5' UTR (15-17%) sequence of genes for all 4 conditions tested (Fig. 4A). A heat map of *SNAI2* bound regions covering -5KB to +5KB from the TSS of genes revealed that higher expression levels of *SNAI2* correlated with higher levels of binding to each site (Fig. 4B-C). *SNAI2* overexpression in progenitor cells (LZRS-*SNAI2* GM) had the highest levels of binding across all bound genes (Fig. 4B-C). Physiological levels of *SNAI2* expression in progenitor cells (LZRS-*LACZ* GM) had moderate levels of *SNAI2* binding which decreased further upon differentiation (LZRS-*LACZ* DM) (Fig. 4B-C). Remarkably, overexpression of *SNAI2* in differentiation conditions (LZRS-*SNAI2* DM) restored the binding of *SNAI2* to similar levels as LZRS-*SNAI2* GM cells (Fig. 4B-C). These results suggest that the expression levels of *SNAI2* correlate with the amount of *SNAI2* binding across the TSS regions of genes (Fig. 4B-C). To investigate whether the levels of *SNAI2* expression resulted in changes to its binding motif, de novo motif search was used for each of the four conditions. The consensus core motif of CAGGTG was identified in all conditions which is identical to the motif previously reported for *SNAI2* (discovered by in-vitro selection methods and transfection experiments) [26] (Fig. 4D). To define the genes that *SNAI2* directly regulates, the 7,873 *SNAI2* bound peaks in control *LACZ* progenitor cells (LZRS-*LACZ* GM) were mapped back to 4,924 genes (Supporting Information Table S4A). These genes were enriched for GO terms such as regulation of epidermal cell differentiation, negative regulation of cell differentiation, cell adhesion, and cell junction which correlates

with SNAI2's role in preventing epidermal differentiation and promoting cell motility (Fig. 4E).

### The levels of SNAI2 binding to differentiation genes determine the differentiation status of epidermal cells

Since SNAI2 is a known transcriptional repressor, we compared SNAI2 bound genes with the 490 genes upregulated in SNAI2i cells because these are likely to be the genes directly repressed by SNAI2 binding[21]. 215 genes were found in the overlap which are likely to be directly regulated by SNAI2 binding levels (Fig. 5A and Supporting Information Table S4B). These genes were enriched for GO terms such as epidermis development and cell junction (Fig. 5B). Examination of the gene list showed that SNAI2 directly bound to the genomic regions of differentiation associated cell adhesion genes such as *CLDN1* and *GJB6* as well as differentiation promoting transcription factors such as *KLF4* and *GRHL3* (Fig. 5C-D and Supporting Information Fig. S4A-B). Knockdown of SNAI2 decreased binding to those regions demonstrating the specificity of SNAI2 binding to *KLF4*, *GRHL3*, *CLDN1*, and *GJB6* genomic regions (Supporting Information Fig. S3F). The levels of SNAI2 binding to the transcriptional start site (+/- 2Kb) of these genes dictate the expression of these mRNAs (Fig. 5E and Supporting Information Fig. S4C). The higher the levels of SNAI2 binding to these genes the lower the expression level. SNAI2 overexpressing progenitor cells (LZRS-SNAI2 GM) had the most binding which correlated with the lowest expression of these genes (Fig. 5C-E and Supporting Information Fig. S4A-C). Physiological levels of SNAI2 binding in progenitor cells (LZRS-LACZ GM) resulted in moderate levels of expression. During differentiation (LZRS-LACZ DM) where SNAI2 binding to the TSS regions of these genes was decreased due to the downregulation of SNAI2 protein, the expression of these genes were increased to their highest levels (Fig. 4C, 5C-E and Supporting Information Fig. S4A-C). However, overexpression of SNAI2 in differentiated cells (LZRS-SNAI2 DM) restored the binding and blocked the expression of those genes (Fig. 5C-E and Supporting Information S4A-C).

Our results suggests that SNAI2 may directly regulate up to 43.9% (215/490) of the induced genes in SNAI2i cells. The other 56.1% may potentially be regulated indirectly by SNAI2 through direct regulation of other transcription factors. Candidate factors include *KLF4* and *GRHL3* which have been shown to be necessary for the transcriptional activation of the epidermal differentiation program[11,12]. To determine if the premature differentiation phenotype seen in SNAI2i cells is mediated in part by increased *KLF4* or *GRHL3* levels, SNAI2 and *KLF4* or SNAI2 and *GRHL3* were simultaneously knocked down. The mRNA levels of differentiation induced genes *SPRR1A*, *IVL*, and *TGM1* were increased in SNAI2i cells but were restored similar to control levels in *KLF4* and SNAI2 or *GRHL3* and SNAI2 double knockdown cells (Fig. 5F). Furthermore both *GRHL3* and *KLF4* had specific target differentiation genes as double knockdown of *GRHL3* and SNAI2 prevented the increase in *SBSN*, *S100P* and *LCE3D* gene expression levels seen in SNAI2i cells alone; whereas, *KLF4* and SNAI2 knockdown had minimal impacts (Fig. 5F). Similarly double knockdown of *KLF4* and SNAI2 prevented the increases in *KLK7* and *SCEL* differentiation gene levels while *GRHL3* and SNAI2 knockdown had no impact (Fig. 5F). These results suggest that while SNAI2 directly blocks the expression of a portion of the differentiation induced genes,

it also prevents premature differentiation of progenitor cells by repressing the expression of *KLF4* and *GRHL3*.

*SNAI2*'s regulation of *KLF4* and *GRHL3* suggest that it may regulate other differentiation promoting transcription factors. Supporting this, *SNAI2* binding was found in the TSS of both *CEBPA* and *GRHL1* (Supporting Information S5A-B). *CEBPA* is necessary for the commitment to epidermal differentiation and *GRHL1* regulates the expression of desmosomes during the differentiation process[10,14,15]. The amount of *SNAI2* binding correlated with the expression levels of both genes with decreased binding leading to higher gene expression levels (Supporting Information S5A-C). These results suggest that *SNAI2* regulates a subset of the differentiation program through differentiation associated transcription factors.

## Discussion

Prior studies on the function of *SNAI2* in the skin have revealed a role for *SNAI2* in mediating epidermal homeostasis[21]. Microarray analysis on *SNAI2* knockout mouse epidermis revealed alterations in epidermal differentiation, adhesion, motility, and angiogenesis although which genes are direct targets of *SNAI2* remain unknown[27]. Studies in human epidermal keratinocytes have shown that conditional activation of *SNAI2* can repress the expression of cell adhesion genes such as  $\alpha 3$  and  $\beta 1$  integrins[28]. Furthermore *SNAI2* is necessary for epidermal growth factor mediated re-epithelialization[29]. It is clear that *SNAI2* has a functional role in epidermal differentiation, adhesion, motility, and wound repair but the underlying mechanisms of how *SNAI2* controls these functions is unclear. Here, we report the mechanism by which the EMT-TF, *SNAI2*, controls the differentiation and adhesion status of epidermal cells. In the epidermis, *SNAI2* expression is restricted to progenitor cells and its expression is extinguished upon induction of differentiation. Constitutive expression of *SNAI2* in regenerated human epidermis inhibits differentiation. Overexpression of *SNAI2* in cultured epidermal progenitor cells resulted in dedifferentiation and decreased cell adhesion. Inhibition of *SNAI2* had the opposite effect and resulted in faster kinetics of differentiation and premature differentiation of the basal layer of the epidermis. Global gene expression profiling showed that cells with decreased *SNAI2* expression resembled cells undergoing calcium induced differentiation (~70% overlap with the differentiation gene expression signature) with increases in differentiation associated cell adhesion, keratinization, and cornified envelope formation. In contrast, increased expression of *SNAI2* served to dedifferentiate the cells as *SNAI2* overexpression still overlapped significantly with the differentiation signature but with the genes regulated in the opposite direction.

Genome-wide mapping of *SNAI2* binding sites showed that approximately half of *SNAI2* bound peaks were found in the promoter or 5' UTR of genes. Analysis of the *SNAI2* binding sites within +/- 5KB of TSS showed that the levels of *SNAI2* expression correlated with the amount of binding to those regions. Physiological levels of *SNAI2* in progenitor cells led to moderate amounts of binding allowing for maintenance of progenitor function. Upon differentiation where *SNAI2* expression is downregulated, the binding decreased (7,873 peaks were identified in progenitor cells which decreased to 1,550 peaks upon

differentiation) which resulted in expression of differentiation associated genes. Overexpression of SNAI2 in differentiation conditions restored the binding sites and blocked differentiation. Increased SNAI2 binding to the same sites due to SNAI2 overexpression in progenitor cells lead to dedifferentiation due to further downregulation of SNAI2 target genes. This suggests that the levels of SNAI2 binding determine the levels of differentiation gene expression. This phenomenon is similar to what has been recently described for c-Myc. In tumor cells elevated expression of c-Myc led to increased binding at promoter regions of active genes and resulted in further increases in gene expression which the authors coined as transcriptional amplification[30,31]. Our results show the opposite effect in which increasing levels of SNAI2 binding to canonical CAGGTG sites leads to further transcriptional silencing.

Many of the SNAI2 bound genes were cell adhesion and differentiation genes. 215 epidermal differentiation genes are likely to be directly regulated by SNAI2 since loss of SNAI2 expression lead to increased expression of these SNAI2 bound genes. These include differentiation induced cell adhesion genes such as *GJB6* and *CLDN1* as well as transcription factors such as *GRHL3*, *KLF4*, *CEBPA*, and *GRHL1*. Interestingly, double knockdown of either *GRHL3* or *KLF4* with SNAI2 can prevent the increases in differentiation gene expression seen in SNAI2 knockdown cells. These results suggest that at least part of the differentiation program (differentiation genes not directly regulated by SNAI2) is mediated through SNAI2 control of *KLF4* and *GRHL3* expression. Normal physiological levels of SNAI2 allow moderate levels of binding to its target genes (ie: *KLF4*, *GRHL3*, *GRHL1*, *CEBPA*, *GJB6*, and *CLDN1*) which maintains progenitor function by suppressing the differentiation program. This allows just the right amount of expression of cell adhesion and differentiation genes to maintain progenitor status (Fig. 5G). Upon induction of differentiation, SNAI2 is downregulated which causes the loss of SNAI2 binding. This alleviates SNAI2 mediated repression of target genes allowing increased cell adhesion, keratinization, and cornified envelope formation all features associated with differentiation (Fig. 5G). In contrast, elevated expression of SNAI2 which has been reported in numerous tumors as well as during RAS mediated epidermal tumorigenesis may result in additional binding to the same genes (ie *KLF4*, *GRHL3*, *GRHL1*, *CEBPA*, and cell adhesion genes)[32,33]. This increased binding results in transcriptional silencing amplification which causes an EMT phenotype and dedifferentiation due to further suppression of cell adhesion and differentiation genes. Similarly, SNAI2 expression is increased in epidermal cells bordering wounds which promotes a transient EMT phenotype to allow migration to the wound to promote reepithelialization[23]. Supporting this, wound healing is reduced in SNAI2 knockout mice[23]. In summary, the levels of SNAI2 binding to target genes are crucial in determining epidermal cell fate.

## Conclusion

In conclusion, our study provides an explanation for how EMT-TFs mediate EMT, progenitor cell maintenance, and differentiation. The levels of SNAI2 expression determine the amount of SNAI2 binding to differentiation and adhesion genes across the genome. This in turn dictates the expression levels of those genes. High levels of expression as seen in cancer cells leads to increased SNAI2 binding to differentiation and adhesion genes leading



to an EMT phenotype. Moderate (physiological) levels of binding promote the right amount of differentiation and cell adhesion gene expression to maintain progenitor function. Finally, low levels of SNAI2 binding as seen during epidermal differentiation results in the alleviation of SNAI2 mediated gene repression leading to full expression of the differentiation program.

## Materials and Methods

The Supplemental Materials and Methods section which includes a list of retroviral constructs, siRNAs, antibodies, and primer sequences is available online.

### Tissue culture

Primary human epidermal cells (keratinocytes) were derived from newborn foreskin. Cells were cultured in growth medium (GM) composed of KSF-M (Life Technologies) containing epidermal growth factor (EGF) and bovine pituitary extract (BPE). Cells cultured in growth medium are also referred to as progenitor cells. Cells were induced to differentiate (DM) by the addition of 1.2 mM calcium for 3 days in full confluence. Amphotrophic phoenix cells were maintained in DMEM and 10% fetal bovine serum.

### Gene transfer

Amphotrophic phoenix cells were transfected with 3 ug of each retroviral construct to either knockdown or overexpress genes. Transfections were done in 6 well plates using Fugene 6 (Roche). Viral supernatants were collected 48 hours post transfection. These supernatants were placed on primary human keratinocytes and centrifuged for 1 hour at 1000rpm with polybrene (5ug/ml). Cells were transduced a total of 2 times and selected using puromycin (2ug/ml) after the last transduction with shRNA retroviral constructs. Cells were transduced once for LZRS retroviral overexpression constructs.

### Gene knockdown and overexpression

ShRNA retroviral constructs were generated by cloning oligos into the pSuper Retro vector[34]. The full-length open reading frame of SNAI2 was cloned into the LZRS retroviral vector.

### Western blotting and immunofluorescence

40 ug of the cell lysates were used for immunoblotting. For immunofluorescence experiments, 7  $\mu$ m thick epidermal sections from adult human skin or organotypic cultures were used.

### Quantitative reverse transcriptase-PCR analysis

Total RNA from cells was extracted using the GeneJET RNA purification kit (Thermo Scientific). One ug of total RNA was reverse transcribed and quantitative PCR was performed. Samples were normalized to GAPDH.

### Gene expression profiling

Cells overexpressing SNAI2 or control LACZ were harvested 7 days post transduction. Cells knocked down for SNAI2 or control were harvested one week after the last infection. Microarray analysis using Affymetrix HG-U133 2.0 plus arrays was performed on duplicate samples. Significantly changed genes were identified as previously described[5]. Data was deposited in GEO with accession number: GSE55269.

### Regenerated human epidermis

1 million control or SNAI2 knockdown cells were seeded on devitalized human dermis and raised to the air/liquid interface in order to induce differentiation and stratification. This method of regenerating human epidermis was previously described [3]. Tissue was harvested at the indicated time points in the figures.

### Chromatin Immunoprecipitation Sequencing (ChIP-Seq)

ChIP was performed as previously described[3]. 10 million cells were used for ChIP for each antibody used. 4ug of antibody was used for each pull down experiment. Experiments were performed in triplicates. QPCR Results were represented as a percent of input DNA. For ChIP-Seq, the ChIP DNA library was prepared using the TruSeq sample prep (Illumina). Experiments were performed in duplicates. Sequencing was done on a Hi-Seq System (Illumina) using single 1×100 reads. Reads were mapped back to the human genome assembly (GRCh37/hg19). Peaks were called using the Partek Genomics Suite (Partek Incorporated) with FDR = 0.001. Data was deposited in GEO with accession number: GSE55421.

### RNA Seq Data Analysis

RNA seq data from the ENCODE project (ENCBS563ENC: CSHL RNA seq) for human epidermal keratinocytes was used to determine expression levels of EMT-TFs[35]. Reads were mapped back to the human genome assembly (GRCh37/hg19) and expression generated using TopHat and Cufflinks[36,37].

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

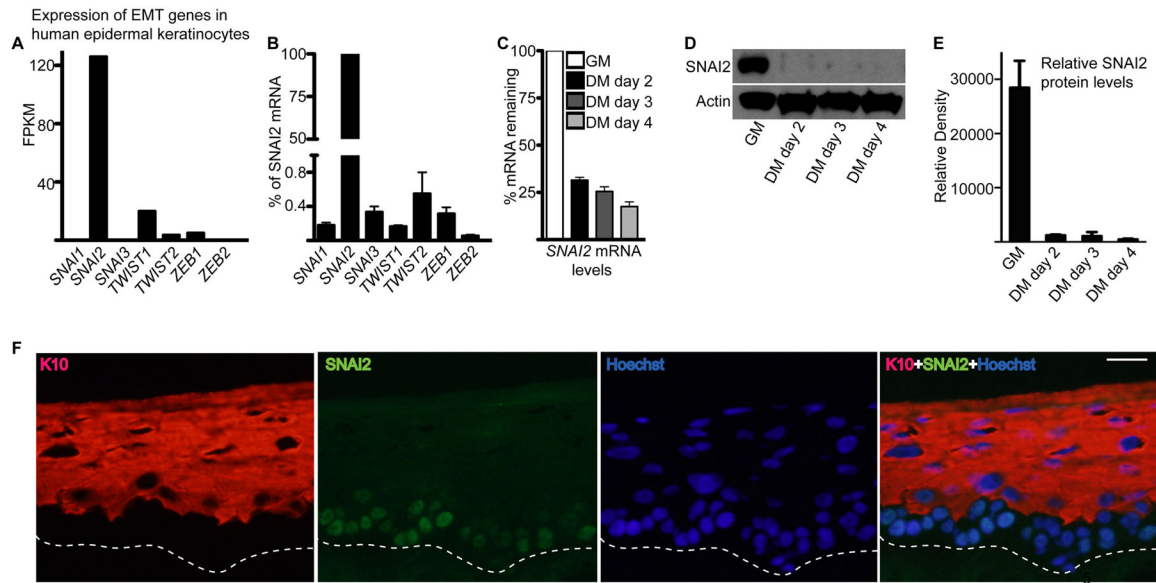
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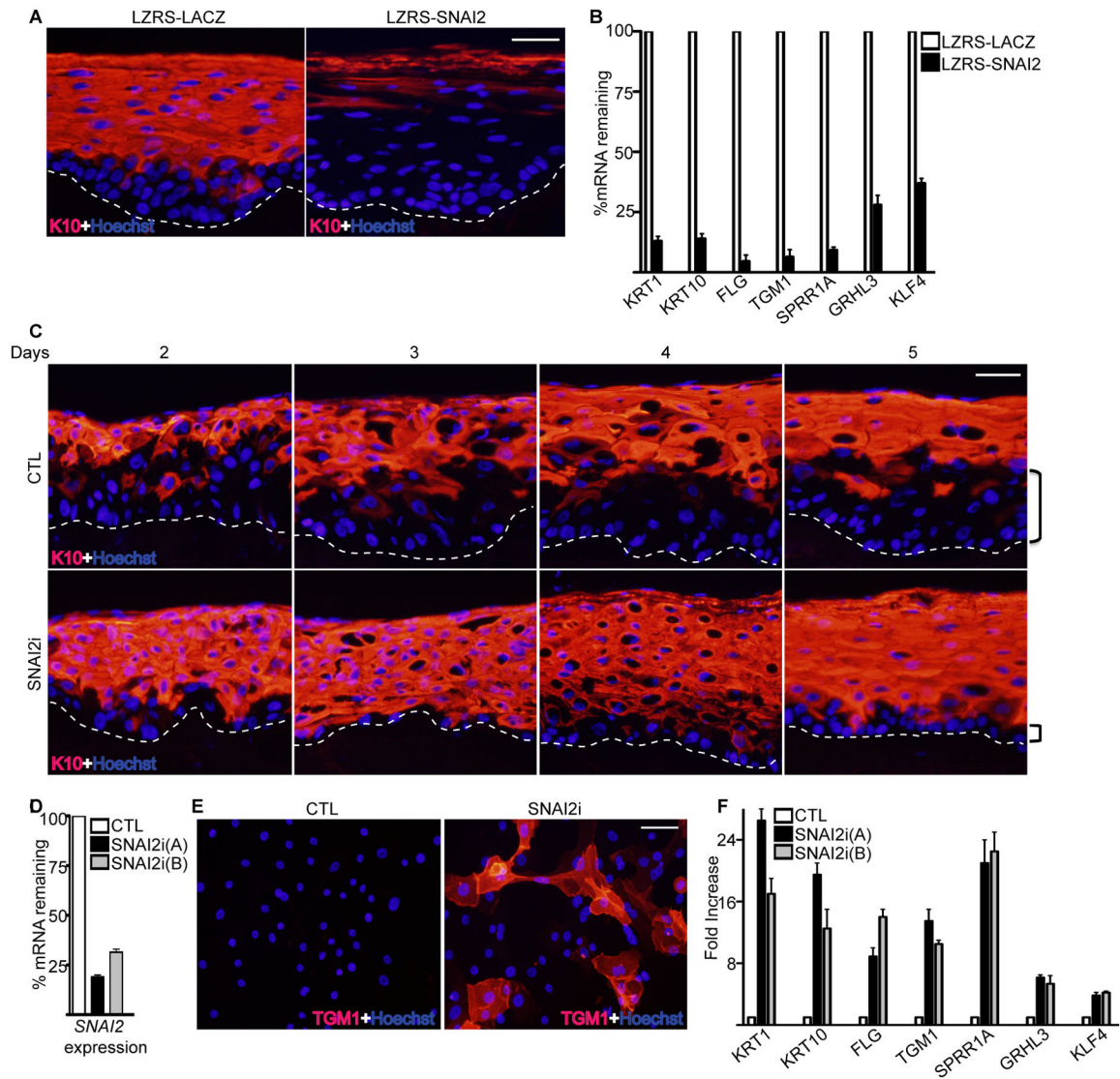
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**Figure 1. EMT-TF, SNAI2 is highly expressed in epidermal progenitor cells and downregulated during differentiation**

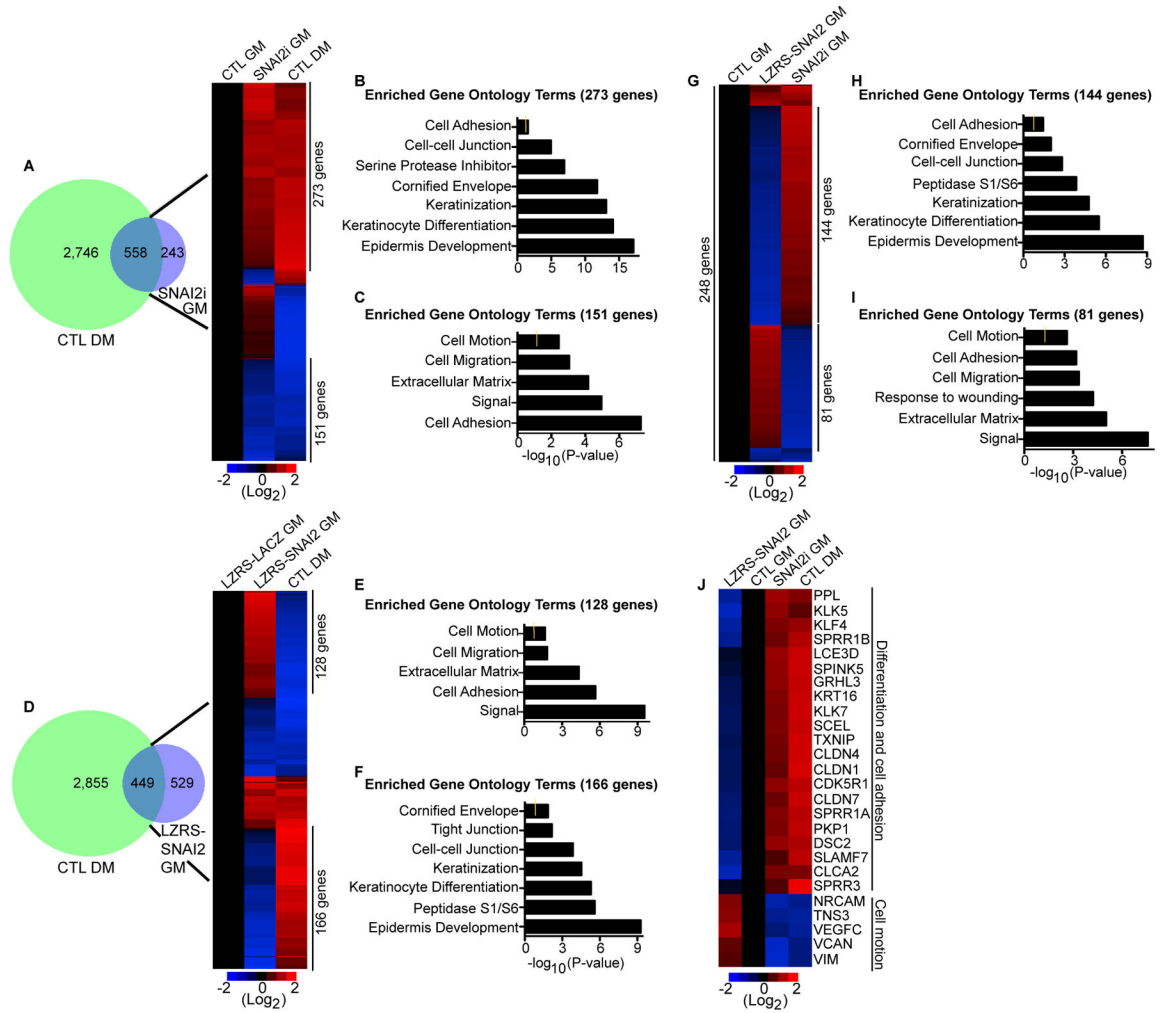
(A) RNA-seq analysis on the expression of EMT-TF family members in epidermal keratinocytes. Expression levels are represented as reads per kilobase per million (RPKM). Data was derived from the ENCODE RNA seq analysis of human epidermal keratinocytes. (B) RT-qPCR for expression of EMT genes. Due to the high levels of *SNAI2* expression, the expression of all other EMT genes were calculated as a percentage of *SNAI2* expression. (C) RT-qPCR for expression of *SNAI2* in progenitor cells (cultured in growth medium: GM) and differentiated cells (cultured in differentiation medium: DM). Expression levels were normalized to *GADPH*. Error bars=mean with SEM. (D) Western blot for protein levels of *SNAI2* in progenitor (GM) and differentiated cells (DM). Actin was used as a loading control. (E) Quantitation of Western blots shown in (D). Signals were normalized to actin loading control and quantitated using ImageJ. N=3 independent experiments. (F) *SNAI2* staining in adult human epidermis. *SNAI2* staining is shown in green and differentiation protein keratin 10 (K10) is shown in red. Hoechst staining in blue marks the nuclei. Scale bar=25 $\mu$ m; dashed lines denote basement membrane zone.



**Figure 2. The levels of SNAI2 controls epidermal differentiation**

(A) Epidermal progenitor cells transduced with the LZRS retrovirus encoding either LACZ controls (LZRS-LACZ) or SNAI2 (LZRS-SNAI2) were used to regenerate human epidermis by placing the cells on devitalized human dermis. Keratin 10 (K10) staining shown in red marks the differentiated epidermal layers. Hoechst staining in blue marks the nuclei. The dashed lines denote basement membrane zone (Scale bar=40 $\mu$ m; n=3 regenerated human epidermis per group). (B) RT-qPCR for expression of differentiation genes from samples isolated from (A). Expression levels were normalized to *GADPH*. Error bars=mean with SEM. (C) Epidermal cells were transduced with retroviruses expressing shRNAs targeting either control (CTL) or SNAI2 (SNAI2i) and used to regenerate human epidermis. Tissue was harvested days 2-5 after placement of cells onto dermis to determine the kinetics of epidermal differentiation with SNAI2 loss. K10 staining is shown in red. Brackets denote size of undifferentiated basal layer. Scale bar=40 $\mu$ m; n=3 regenerated human epidermis per group. (D) RT-qPCR for expression of SNAI2. Cells were knocked down for SNAI2 using

two distinct shRNAs. **(E)** Control and SNAI2i cultured primary epidermal progenitor cells were stained for differentiation protein transglutaminase I (TGM1: shown in red). Scale bar=20 $\mu$ m. **(F)** RT-qPCR for expression of differentiation genes from CTL and SNAI2i cultured cells. Knockdown of SNAI2 using two distinct shRNAs [SNAI2i (A) and SNAI2i (B)] is shown. Expression levels were normalized to *GADPH*. Error bars=mean with SEM.

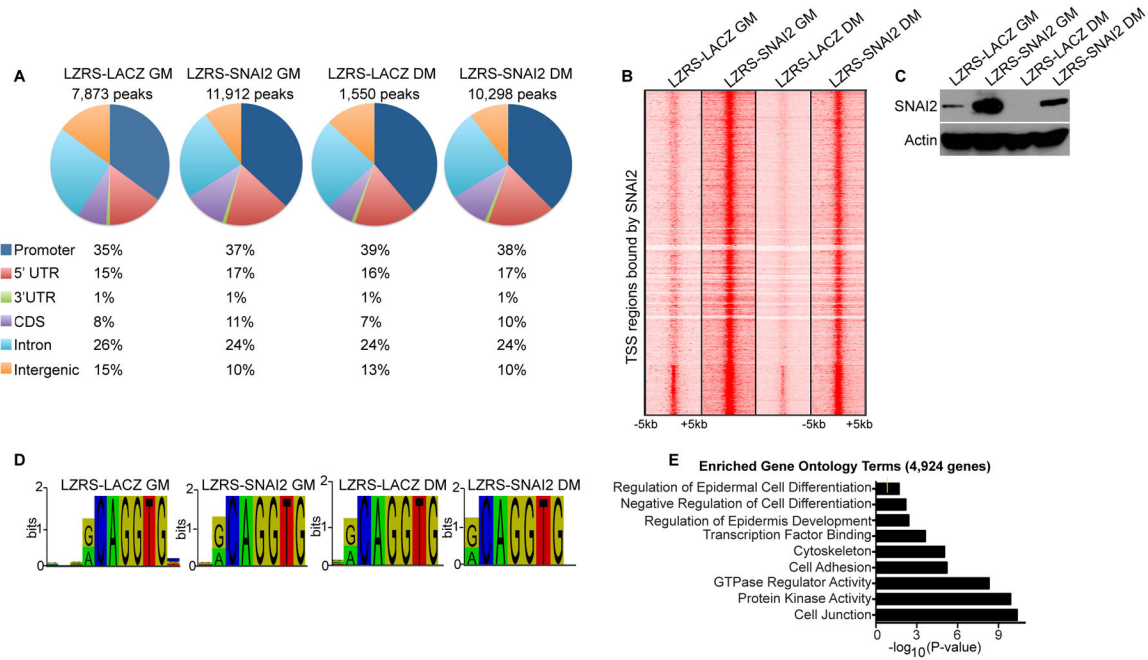


**Figure 3. SNAI2 represses the differentiation gene expression program**

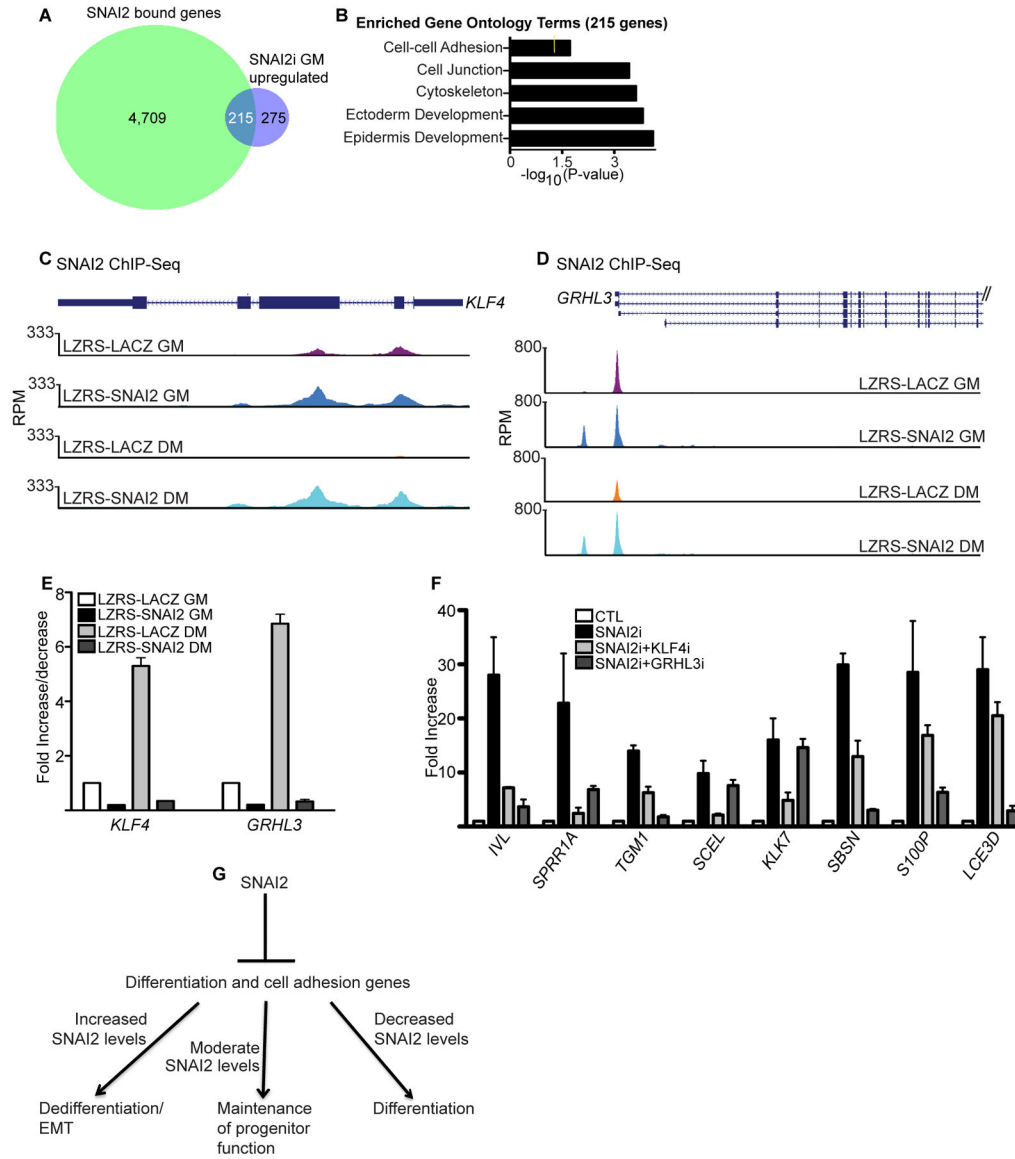
(A) Overlap (left panel) of the differentiation gene signature (CTL DM: 3,304 genes change) with the genes that change upon knockdown of SNAI2 in cells cultured in growth medium (SNAI2i GM: 801 genes change). The differentiation gene signature (DM) is the differentially expressed genes between cells grown in low calcium (growth medium:GM) to cells grown in high calcium (differentiation medium:DM). Heat map (right panel) of the 558 genes that overlap. Differentiated control samples (CTL DM) were compared to control (CTL GM) and SNAI2i (SNAI2i GM) samples. Heat map is shown in red (induced genes) and blue (repressed genes) on a log<sub>2</sub>-based scale. (B) Gene ontology analysis of genes with increased expression that are co-regulated by SNAI2i GM and CTL DM samples. Yellow mark in bar graphs demark p value=0.5. (C) Gene ontology analysis of co-regulated genes with decreased expression. (D) Overlap (left panel) of CTL DM with the genes that change upon overexpression of SNAI2. LZRS-SNAI2 cells were cultured in growth medium (LZRS-SNAI2 GM). Heat map (right panel) of the 449 genes that overlap. Differentiated samples (CTL DM) were compared to control LZRS-LACZ GM and LZRS-SNAI2 GM samples. (E-F) Gene ontology analysis of genes oppositely regulated between LZRS-SNAI2 GM and CTL DM samples. (G) Heat map of the 248 genes that overlap between LZRS-



SNAI2 GM and SNAI2i GM samples. **(H-I)** Gene ontology analysis of genes oppositely regulated between LZRS-SNAI2 GM and SNAI2i GM samples. **(J)** Heat map of differentiation, cell adhesion, and cell motility genes.



**Figure 4. SNAI2 expression correlates with the levels of SNAI2 binding across the genome** (A) Distribution of SNAI2 binding sites in epidermal progenitor and differentiated cells. Chromatin immunoprecipitation followed by high throughput sequencing (ChIP-Seq) was performed using a SNAI2 antibody on the following 4 samples: LZRS-SNAI2 GM and LZRS-LACZ GM (SNAI2 or control LACZ overexpressing cells cultured in growth medium); LZRS-LACZ DM and LZRS-SNAI2 DM (LACZ or SNAI2 overexpressing cells in differentiation medium). The total number of SNAI2 bound peaks were identified and their distributions across the genome calculated as a percentage for each sample. (B) Heat map of transcriptional start site (TSS) regions bound by SNAI2 in the four conditions tested. Each row shows the +/- 5kb centered regions around the TSS. (C) Western blot analysis for the expression of SNAI2 in the four conditions tested. Actin was used as a loading control. (D) SNAI2 binding motif found enriched under each condition. (E) Gene ontology analysis of the 4,924 genes that SNAI2 binds to in LACZ expressing progenitor cells (LZRS-LACZ GM). The 7,873 SNAI2 binding peaks identified in LZRS-LACZ GM samples were first mapped back to 4,924 genes and then gene ontology analysis performed. Yellow mark in bar graphs demark p value=0.5.



**Figure 5. The levels of SNAI2 binding to *KLF4* and *GRHL3* genomic regions determine the differentiation status of epidermal cells**

(A) Overlap of the 4,924 SNAI2 bound genes with the 490 genes that increase in expression upon SNAI2 knockdown. (B) Gene ontology analysis of the 215 genes found in the overlap. Yellow mark in bar graphs demark p value=0.5. (C-D) Gene tracks showing SNAI2 occupancy at regions around the TSS of *KLF4* and *GRHL3*. The x axis denotes genomic position and y axis shows signal strength (reads per million, RPM). Binding of SNAI2 is shown for four conditions: LZRS-SNAI2 GM and LZRS-LACZ GM (SNAI2 or control LACZ overexpressing cells cultured in growth medium); LZRS-LACZ DM and LZRS-SNAI2 DM (LACZ or SNAI2 overexpressing cells in differentiation medium). (E) RT-qPCR for expression of *KLF4* and *GRHL3* in the four conditions described in (C-D). (F) RT-qPCR for differentiation gene expression in control (CTL), SNAI2i, and cells knocked down for both SNAI2 and KLF4 or SNAI2 and GRHL3. Expression levels were normalized

to *GADPH*. Error bars=mean with SEM. **(G)** Model of SNAI2 mediated control of the differentiation status of epidermal cells.