

Dynamic Transcriptional and Epigenetic Regulation of Human Epidermal Keratinocyte Differentiation

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SUMMARY

Human skin is maintained by the differentiation and maturation of interfollicular stem and progenitor cells. We used DeepCAGE, genome-wide profiling of histone modifications and retroviral integration analysis, to map transcripts, promoters, enhancers, and super-enhancers (SEs) in prospectively isolated keratinocytes and transit-amplifying progenitors, and retrospectively defined keratinocyte stem cells. We show that >95% of the active promoters are in common and differentially regulated in progenitors and differentiated keratinocytes, while approximately half of the enhancers and SEs are stage specific and account for most of the epigenetic changes occurring during differentiation. Transcription factor (TF) motif identification and correlation with TF binding site maps allowed the identification of TF circuitries acting on enhancers and SEs during differentiation. Overall, our study provides a broad, genome-wide description of chromatin dynamics and differential enhancer and promoter usage during epithelial differentiation, and describes a novel approach to identify active regulatory elements in rare stem cell populations.

INTRODUCTION

The epidermis is a stratified epithelium differentiating from keratinocyte stem cells (KSCs) contained in the basal layer and in the bulge of hair follicles. Upon division, KSCs produce transit-amplifying (TA) progenitors that generate differentiated keratinocytes and other epithelial skin components. The available information on the molecular events underlying self-renewing and differentiation of KSCs comes from studies on the murine hair follicle (reviewed in [Blanpain et al., 2007](#)). Much less is known about human KSCs, which lack robust markers for prospective isolation and are defined only retrospectively by the nature of their progeny in cell culture or transplantation assays. Clonal analysis *in vitro* has defined three types of clonogenic cells, giving rise to the so-called holoclones, meroclones, and paraclones. Holoclone-forming cells have the highest self-renewing and proliferative capacity, and define in culture the KSCs of the epidermis or the corneal epithelium ([Pellegriani et al., 1999](#); [Rochat et al., 1994](#)). Meroclone- and paraclone-forming cells have proportionally less proliferative capacity and terminally differentiate into keratinocytes after 5–15 cell doublings, as expected for TA progenitors ([Barrandon and Green, 1987](#)). Few molecular markers are known for KSCs or TA progenitors: they include the p63, BMI1, CEBPs, MYC, and GATA-3 transcription factors (TFs), integrins, Wnt/β-catenin, NOTCH, HH, SGK3, and some bone morphogenetic pro-

teins ([Blanpain et al., 2007](#)). In particular, p63 is considered a master regulator of morphogenesis, identity, and regenerative capacity of stratified epithelia ([Pellegriani et al., 2001](#); [Yang et al., 1999](#)). Although some of the targets of p63 and other TFs involved in epidermal cell functions are known, little is known about the chromatin dynamics and the differential usage of promoters and enhancers driving the differentiation of human KSCs and TA progenitors.

Specific histone modifications are currently used to define chromatin regions with different regulatory functions. In particular, monomethylation of lysine 4 of histone 3 (H3K4me1) characterizes enhancer regions, whereas its trimethylation (H3K4me3) defines promoters ([Ernst et al., 2011](#); [Heintzman et al., 2009](#)). Acetylation of H3K27 defines transcriptionally active enhancers and large clusters of enhancers (super-enhancers [SEs]) involved in the definition of cell and tissue identity ([Hnisz et al., 2013](#)). In this study, we aimed to map transcriptional regulatory elements and define their usage during epithelial differentiation. By combining high-throughput identification of Pol-II-transcribed (capped) RNAs defined by Cap Analysis of Gene Expression (DeepCAGE) ([Carninci et al., 2006](#)) with genome-wide profiling of histone modifications determined by chromatin immunoprecipitation (ChIP-seq), we mapped active enhancer and SE elements in prospectively isolated TA progenitors and terminally differentiated keratinocytes. For KSCs, which



lack markers for prospective isolation, we exploited the integration characteristics of the Moloney murine leukemia retrovirus (MLV), which integrates in active promoters and enhancers (Biasco et al., 2011; Cattoglio et al., 2010; De Ravin et al., 2014) as a consequence of the direct binding of the viral integrase to the bromodomain and extra-terminal (BET) proteins BRD2, BRD3, and BRD4 that tether the pre-integration complex to acetylated chromatin regions (De Rijck et al., 2013; Gupta et al., 2013; Sharma et al., 2013). By using MLV vector integration clusters as surrogate genetic markers of active regulatory elements, we mapped a collection of putative enhancers and SEs active in bona fide KSCs, retrospectively defined by their capacity to maintain long-term keratinocyte cultures.

RESULTS

DeepCAGE Mapping of Active Promoters in Keratinocyte Progenitors and Differentiated Keratinocytes

To enrich keratinocyte progenitors (KPs) from a keratinocyte mass culture, we panned β 1 integrin-positive cells by adherence to collagen-IV-coated plates (Jones and Watt, 1993). Adhering cells were highly enriched in KPs, as determined by a clonogenic assay, and showed significantly increased expression of the progenitor-related markers *TP63* ($p < 0.05$), *LRIG1* ($p < 0.01$), *ITGB1*, *MCSP*, and *DLL1* ($p < 0.001$) by real-time qPCR, while the non-adhering fraction was depleted in colony-forming cells and expressed the differentiation markers *KRT1*, *IVL*, and *LOR* (Figures S1A–S1D). Differentiated keratinocytes (DKs) were obtained by in vitro differentiation in conditions of contact inhibition (Kouwenhoven et al., 2015; Shen et al., 2013), and showed residual colony-forming capacity and high expression of differentiation markers (Figures S1E and S1F).

To define global promoter usage, we used DeepCAGE on RNA extracted from KPs and re-analyzed an epidermal keratinocytes dataset available from ENCODE as a proxy of DKs. We identified a total of 15,283 CAGE promoters, 14,565 expressed in KPs and 15,027 in DKs. Most CAGE promoters mapped to known promoters (20%) or to immediately downstream 5' UTR regions (48.6%) or gene bodies (Figure 1A). We grouped CAGE promoters in three clusters based on the tag position with respect to transcription start sites (TSSs): promoters in cluster 3 showed a broad profile around TSSs and represented the majority of alternatively used promoters, cluster 2 represented canonical promoters with a sharp localization at TSSs, while cluster 1 exemplified pervasive transcription within genes (Figure 1B).

Epithelial Differentiation Is Characterized by Quantitative Regulation of a Large Set of Common Promoters

Most CAGE promoters (14,309) were active in both cell populations and represented 98.2% and 95.2% of KP and DK promoters, respectively. Only 256 and 718 promoters were strictly stage specific, the majority of which (>60%) represented uncharacterized TSSs or were associated with non-coding transcripts, mainly long non-coding RNAs (lncRNAs). Most of the changes in transcriptome associated with keratinocyte differentiation were therefore defined by quantitative changes in the expression of promoters active in both KPs and DKs. A total of 5,429 promoters were expressed at significantly different levels between KPs and DKs ($p < 0.001$, χ^2 test), with 1,838 promoters upregulated in KPs and 1,712 in DKs at a \log_2 fold change (FC) of ≥ 2 (Figure 1C). In KPs differentially expressed TSSs were more abundant in promoters and introns, while in DKs they were more abundant in introns and 3' UTRs (Figure 1D). qPCR analysis confirmed differential mRNA expression for 40 of the 46 randomly chosen promoters (Figure S2A). We detected alternative transcription initiation in 1,187 protein-coding genes, 455 of which underwent switch between alternative promoters during the KP-to-DK transition (Figure 1E). As an example, *PLEC1*, encoding six isoforms of the keratinocyte adhesion protein plectrin, is transcribed from different promoters predicting KP-specific, DK-specific, and common isoforms (Figures 1F and S2B).

We annotated all CAGE promoters in six classes on the basis of the combinatorial presence of TATA box and CpG islands, i.e., TATA⁺ or TATA⁻, and no-CpG (NCPs), low-CpG (LCPs), and high-CpG (HCPs). The majority (~75%) of the promoters fell in the HCP class and were mostly TATA⁻, a feature associated with housekeeping functions (Carninci et al., 2006; Schug et al., 2005). As expected, the proportion of LCP and NCP promoters progressively increased in the differentially expressed promoters at increasing FC values (Figure 1G).

Differential Promoter Usage Defines Stage-Specific Gene Expression Programs

Genes associated with differentially expressed CAGE promoters encoded known markers of follicular and interfollicular epidermal progenitors (i.e., *SOX9*, *LRIG1*, *BMI1*, *TCF3*, *TCF4*, *TP63*) and differentiating keratinocytes (*IVL*, *FLG*, *KRT1*, and genes belonging to the epidermal differentiation complex (EDC) on chromosome 1q21). To correlate differential promoter usage with gene-expression patterns, we carried out an RNA-sequencing (RNA-seq) analysis in KPs and DKs. The DK dataset showed a good correlation (Spearman's $r > 0.8$) with the RNA-seq data of human epidermal keratinocytes reported in ENCODE, demonstrating the similarity between the two populations and

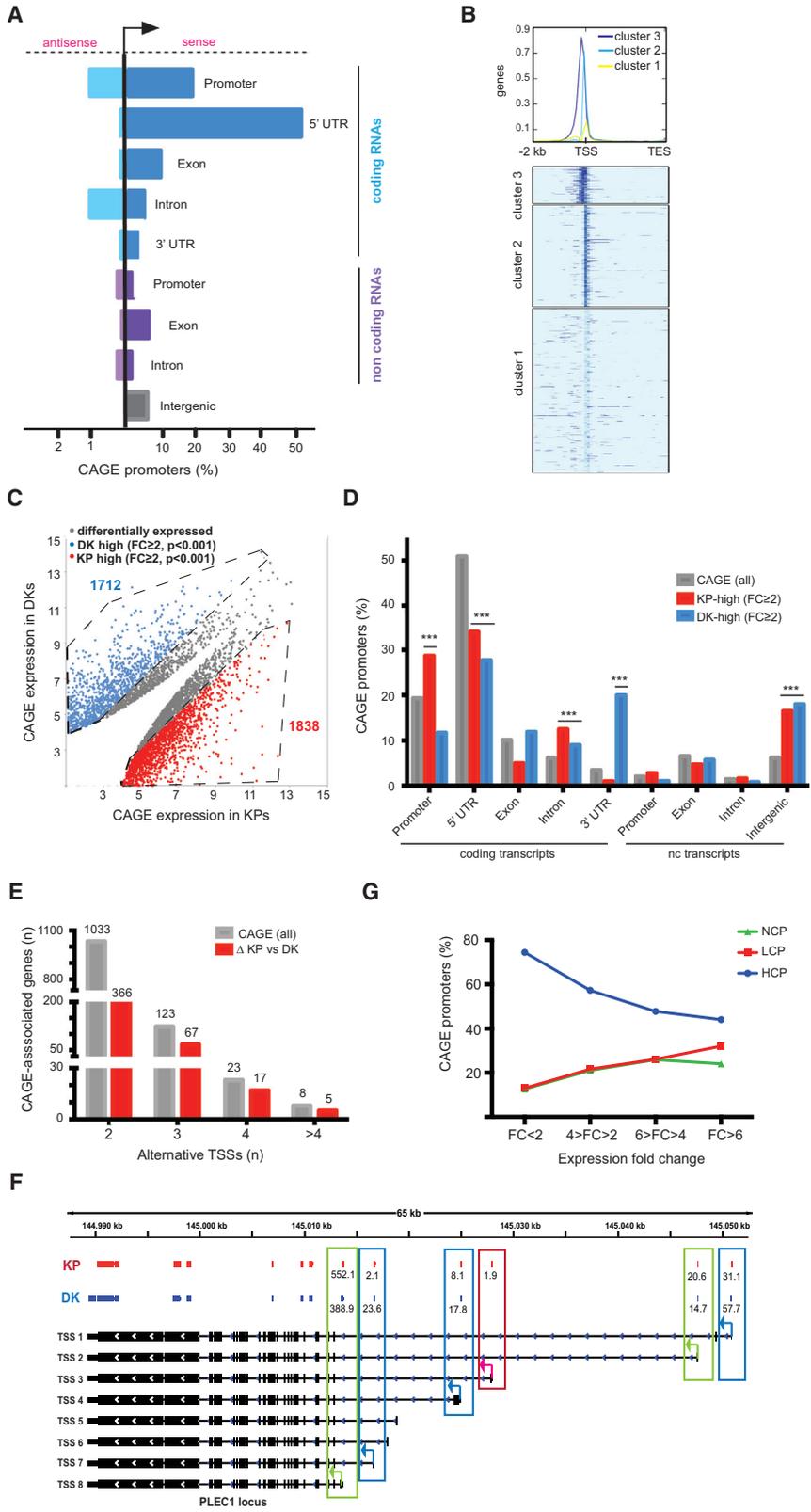


Figure 1. Transcriptome Analyses in Human Epidermal Differentiation by DeepCAGE

(A) Histogram depicting the proportion of CAGE tags aligned to promoters (defined as a 500-bp region upstream of TSS), 5' UTRs, exons and introns of coding and non-coding transcripts, and CAGE tags mapping to intergenic regions. Bars on the right side of the histograms represent CAGE tags on the same strand as the corresponding annotated transcript, while bars on the left represent tags on the opposite strand.

(B) CAGE tags distribution profile along the region spanning from 2,000 bp upstream of the TSS to the transcription end site (TES) of RefSeq genes. Gene bodies were stretched or shrunk to fit the same 1,000-bp length. CAGE promoters were grouped in three different clusters through k-means clustering, based on their tag distribution along the considered region.

(C) Scatterplot of gene expression profiling of KPs and DKs obtained from three biological replicates. Only genes that are differentially expressed ($p < 0.001$) are represented in the plot. Dashed lines indicate the 2-fold differential expression cut-off to define KP- or DK-high (genes upregulated in KPs or DKs with $FC \geq 2$, $p < 0.001$) genes. The numbers of differentially expressed genes with $FC \geq 2$ are indicated.

(D) Percentage of differentially expressed ($FC \geq 2$) and of all CAGE tags aligned to promoters, 5' UTRs, exons and introns of coding and non-coding transcripts, and in intergenic regions. The asterisks indicate the statistical significance in the level of enrichment of KP-high or DK-high CAGE tags in each category over all CAGE tags distribution (** $p < 0.001$, $n = 3$).

(E) Numbers of RefSeq genes using multiple TSSs (gray) and of CAGE promoters that are alternatively used in KPs and DKs (red).

(F) Genomic browser screenshot of alternative promoters usage for the *PLEC1* gene. CAGE promoters in KPs and DKs are represented together with their expression levels in transcripts per million (TPM). *PLEC1* TSSs used preferentially by DKs (blue box), KPs (red box), or equally in both cell types (green box) are shown.

(G) Proportion of differentially expressed CAGE promoters falling in the HCP, LCP, and NCP categories with respect to the fold change in expression.

See also [Figures S1 and S2](#).

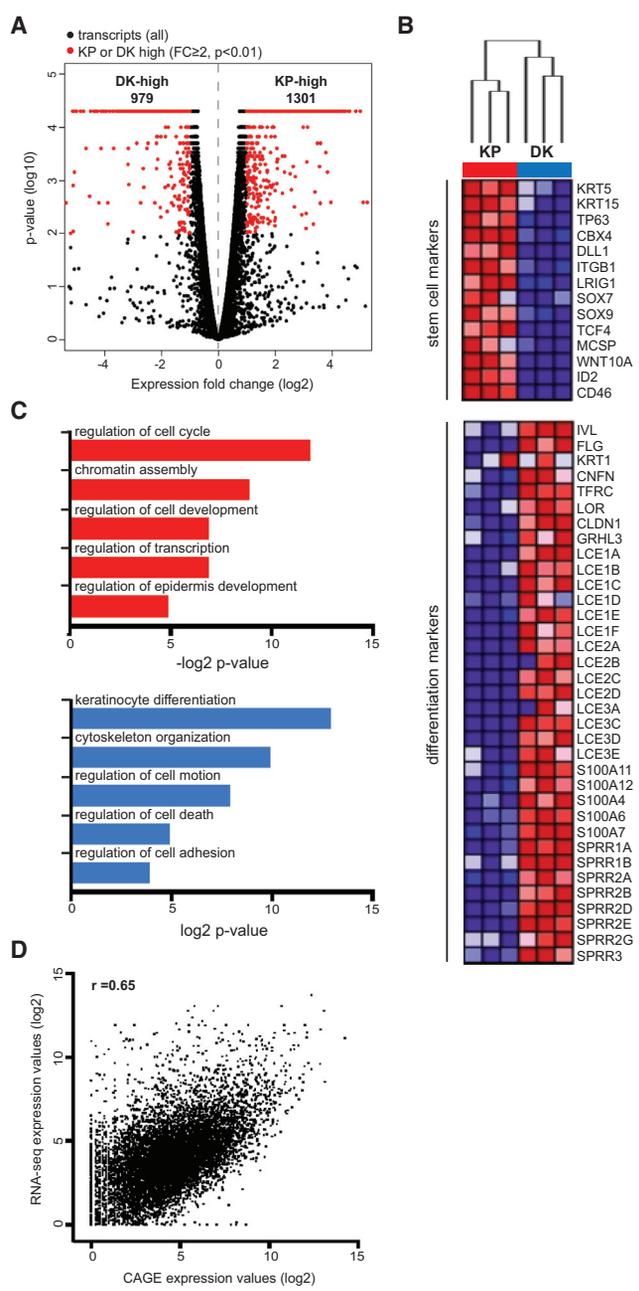


Figure 2. Transcriptome Analyses in Human Epidermal Differentiation by RNA-Seq

(A) Volcano plot of RNA-seq data from three biological replicates of KPs and DKs. Differentially expressed transcripts between KPs and DKs are highlighted in red and numbers are indicated.

(B) Heatmap and clustering of RNA expression profiles of manually selected genes relevant to stem cell or differentiation functions in epidermis.

(C) GO analysis of KP (red) and DK (blue) signature genes.

(D) Pearson's correlation plot of log₂-transformed expression values detected for differentially expressed transcripts/promoters by RNA-seq and DeepCAGE, respectively. See also Figure S3.

validating the use of the ENCODE CAGE data as a proxy of DKs (Figure S3).

RNA-seq analysis showed a substantially different transcriptome in KPs and DKs, with 2,280 differentially expressed transcripts (FC > 2, p < 0.01) (Figure 2A). Concordantly with CAGE promoter usage, DKs showed activation of well-known differentiation markers and a decreased expression of stem cell-related genes compared to KPs (Figure 2B). A gene ontology (GO) analysis showed statistically significant biases toward regulation of cell proliferation and epithelium morphogenesis in KPs, and epidermal differentiation and regulation of cell motility and apoptosis in DKs (Figure 2C). A correlation of RNA-seq and CAGE expression values for the same genes showed a statistically significant concordance between the two datasets (Pearson's r = 0.6) (Figure 2D).

Dynamic Epigenetic Changes in Active Promoter Regions during Epithelial Differentiation

ChIP-Seq analysis of histone modifications identified 22,813 and 15,440 promoter regions in KPs and DKs respectively, as defined by the H3K4me3⁺ and H3K4me1^{-/low} signature. The H3K27ac marker identified 8,557 and 11,341 "strong" promoters, respectively, >80% of which overlapped with CAGE promoters of the HCP class (Figures 3A and 3B). HCP and TATA⁺ promoters showed an H3K4me3⁺/H3K4me1^{-/low}/H3K27ac⁺ profile, while LCP and NCP elements were barely marked (Figure 3C).

Comparative analysis of ChIP-seq data showed no dramatic changes in chromatin configuration at the promoter level between KPs and DKs (Figure 3D). The 312 KP-specific active promoters were mainly annotated to ncRNAs (85%) and genes such as *RUNX1*, involved in the specification of hair follicle progenitors (Lee et al., 2014), and *CLDN1*, encoding an adhesion molecule (Figures 3D and 3E). Conversely, the 292 DK-specific promoters were annotated to genes in the EDC (*S100* and *SLC* gene clusters) or encoding suprabasal keratins (*KRT6*, *KRT75*), collagens, and the TF *SOX15* (Figures 3D and 3F). Despite the overall modest epigenetic changes, we observed a significant correlation between the intensity of H3K4me3 marking and CAGE expression levels. Promoters highly expressed in KPs were highly enriched in H3K4me3 with respect to DKs, and vice versa (Figure S4A). H3K4me3 levels were significantly increased in 1,363 KP and 458 DK promoters, the majority of which was linked to upregulated CAGE promoters and RNA-seq transcripts in the corresponding cells. In KPs, these regions included many regulators of skin and stem cells homeostasis (Figure S4B).

Analysis of the H3K27ac marker identified 1,119 KP-specific and 567 DK-specific promoters (Figure 3D) and a strong correlation between H3K27ac marking intensity and CAGE expression levels (Figure S4A). H3K4me3 and



H3K27ac intensities were directly correlated in both cell types.

We then looked at the H3K27me3 histone mark, which is associated with repression of gene expression during epidermal lineage transitions (Frye and Benitah, 2012). We found 7,255 promoters marked by H3K27me3 in DKs, the majority of which (72.9%) were driving genes not expressed in KPs or DKs by RNA-seq analysis and related to early embryonic functions such as morphogenesis, cell-fate commitment, neuroectoderm development, and homeobox TFs by GO analysis (Figure S4C). Interestingly, 1,932 promoters marked by H3K27me3 in DKs drove genes expressed in KPs and were downregulated in DKs: they were enriched in transcription regulators and chromatin remodelers, and regulators of cell cycle, ectoderm development, epidermal stem cell biology, and skin homeostasis (Figure S4D). Many of these genes harbored at least one repressed (H3K4me1⁺/H3K27me3⁺) enhancer within 100 kb from their repressed promoter (Figure S4D), suggesting epigenetic silencing of entire loci in differentiation.

Keratinocyte Differentiation Is Accompanied by Substantial Changes in Enhancer Usage

We defined enhancers as regions harboring an H3K4me1⁺/H3K4me13^{-/low} signature at a distance of >2.5 kb from any promoter. Enhancers were considered active when marked by H3K27ac (Ernst et al., 2011; Heintzman et al., 2009; Rada-Iglesias et al., 2011). We mapped 70,011 enhancers in KPs and 84,414 in DKs, located on average 43 kb away from any promoter, 14.5% and 21% of which were marked by H3K27ac. Overall, 1,000 intergenic or intronic H3K27ac⁺ enhancers were actively transcribed, as indicated by overlapping CAGE tags (Figure 3A). These were mainly cell-specific, CpG-poor CAGE clusters (Figure 3B) driving the expression of annotated ncRNAs.

More than 60% of the H3K4me1⁺ regions were uniquely mapped in either KPs or DKs, and among those mapped in both cell types, 24.4% were active (H3K27ac⁺) exclusively in KPs and 53.3% exclusively in DKs (Figure 3G). Interestingly, the intensity of H3K4me1 and H3K27ac deposition at enhancers correlated with the expression level of the closest CAGE promoter (Figure S4E). Functional annotation of KP- and DK-specific active enhancers using the GREAT tool showed their association with common epidermal cell functions, such as cell-junction organization, integrin and epidermal growth factor receptor pathways, as well as progenitor-specific processes such as homeostasis and wound healing (Figure 3H). The most abundant TF binding motifs present in KP-specific enhancers were those for SOX7 and TBX1, involved in stem cell and mouse hair follicle homeostasis (Chen et al., 2012; Tan et al., 2013), while DK-specific enhancers were highly enriched in binding motifs of differentiation-related

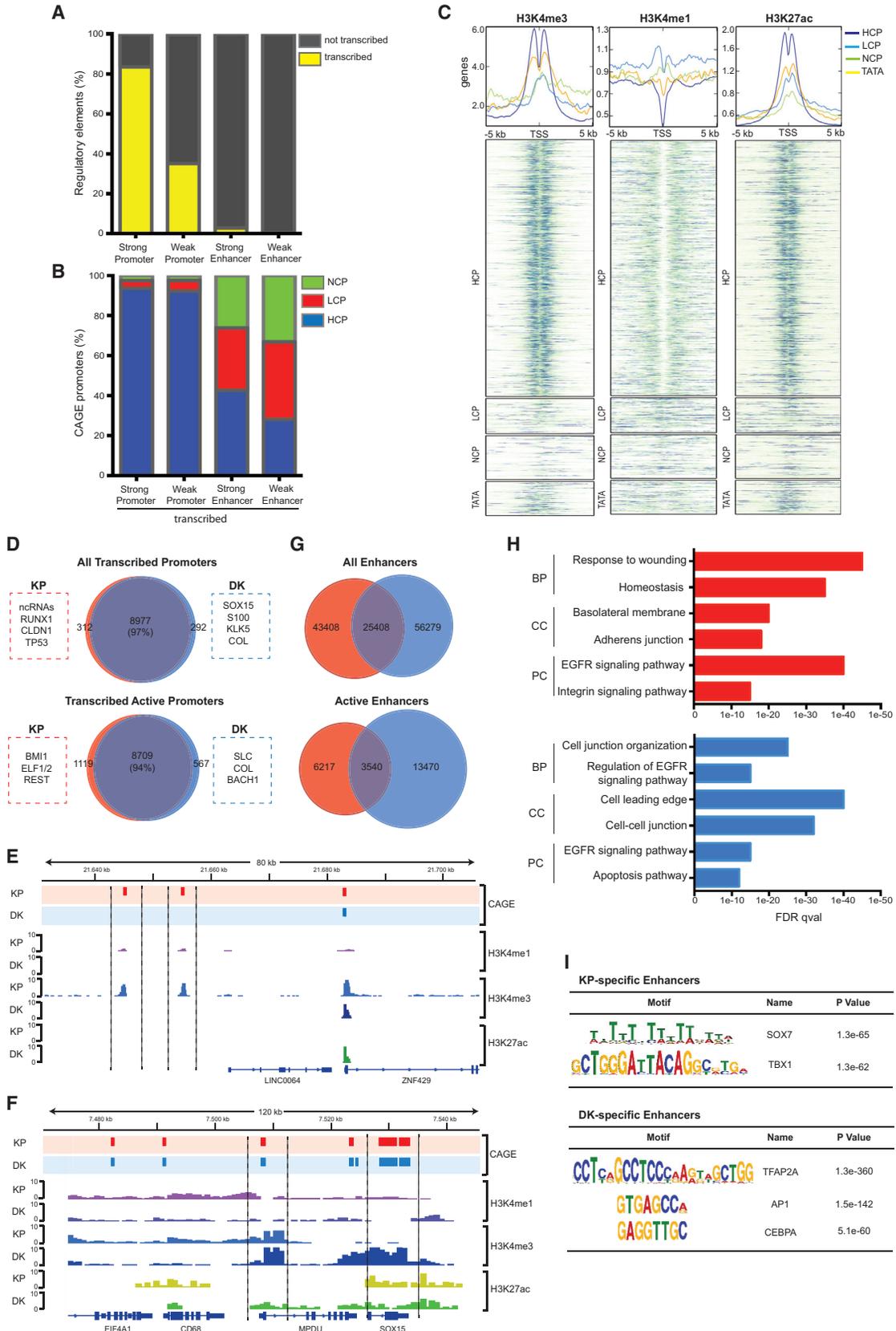
TFs such as TFAP2, AP1, and CEBPA (Fuchs, 2009; Lopez et al., 2009; McDade et al., 2012) (Figure 3I).

Super-Enhancers Define Core Transcriptional Regulatory Networks in Epithelial Differentiation

We used H3K27ac ChIP-seq data to identify SEs, i.e., large clusters of enhancers that drive the expression of genes essential for the definition of cell identity (Hnisz et al., 2013; Whyte et al., 2013). We retrieved 953 SEs in KPs and 1,090 in DKs (Figure 4A), a substantial portion of which (56% and 61%, respectively) was unique for each cell type and associated with cell-specific genes. SE-associated genes were expressed at higher levels than genes associated with typical enhancers ($p < 2.2 \times 10^{-16}$) (Figure 4B), and most of them encoded TFs and proteins necessary for key epidermal functions, such as laminins, keratins, cell-adhesion complexes, and components of the TGF, WNT, and SMAD signaling pathways. Among the >200 TF genes associated with SEs, we found fundamental regulators of skin and stem cell biology, such as *TP63*, *SOX9*, *SOX15*, *RUNX1*, *FOXP1*, *TCF4*, *TP53*, *MYC*, *KLF4*, and *TFAP2*. ncRNAs were strongly associated with SEs, including the keratinocyte-specific mir-203.

TF binding motifs for p63 and FOXP1 binding sites were significantly enriched in SEs of both KPs and DKs, while SMAD motifs were specifically enriched in KP-specific SEs and differentiation-related KLF5, AP1 and TFAP2C motifs in DK-specific SEs (Figure 4C). To validate the cues provided by TF-motif discovery, we mapped by ChIP-seq the p63 binding sites in our DK population: virtually all p63 sites overlapped with those previously identified in DKs (Kouwenhoven et al., 2015), validating the use of the latter dataset in our analyses (Figure S5A). Over 80% of the SEs in both KPs and DKs overlapped with at least one p63 binding site, a significantly higher proportion compared to the total enhancer population (35%) (Figure 4D). p63 binding sites were found in the SEs of the *TP63* gene itself (Figure 4E) and in SEs associated with genes encoding TFs enriched in keratinocyte-specific enhancers and SEs, such as *TFAP2A*, *RUNX1*, *SOX9*, *MYC*, *FOXP1*, *SMAD3*, and *KLF5*.

Interestingly, >50% of the SEs bound by p63 in KPs and DKs were cell-specific, indicating that p63 binds and controls cell-specific regulatory regions in both progenitors and differentiated cells. When we integrated genes associated with p63-bound SEs into molecular and transcriptional interaction networks, we observed that KP and DK networks barely overlapped, with only six nodes in common (*SOX9*, *SMAD7*, *LAMC2*, *RAD51B*, *GRHL3*, *EFNB1*) and different hubs. Genes in the KP network are involved in the developmental control of organ and epithelial tissue homeostasis (main hubs: *RUNX2*, *RUNX1*, *CEBPD*, *SOX9*, *JUNB*, *ETS*, *HMG2*, *STAT6*), while genes in the DK network are involved in signal transduction, cell communication,



(legend on next page)



cell size and apoptosis (main hubs: *TP53*, *CREB1*, *P21*, *YAP1*, *KLF4*, *KLF5*, *HES1*, *SOX9*, *ETS1*) (Figure S5B). When we analyzed all genes driven by p63-bound promoters, enhancers, or SEs, we found 825 KP-specific genes involved in the control of cell cycle and epidermal proliferation, and 591 DK-specific genes encoding lipoproteins and intermediate filament components involved in keratinization and epithelium differentiation (Figure S5C). These data indicate that p63 regulates distinct sets of genes at different differentiation stages through binding of stage-specific regulatory elements.

To gain insight into the combinatorial interactions among TFs operating on SEs, we looked at TF motifs in a 50-bp window around p63 binding sites, and discovered a specific enrichment of TCF4 and SMAD3 motifs in KP-specific SEs and of AP1 in DK-specific SEs. In particular, TCF4 seems to be uniquely enriched next to p63 binding sites in KP-specific SEs. When integrating genes associated with SEs enriched in these specific motifs into interaction networks, we found a tight and specific connection between the TFs and their target SEs in both KPs and DKs, with a significant overlap among target genes associated with SEs containing p63, SMAD3, and TCF4 motifs in progenitors and those containing p63 and AP1 motifs in differentiated cells (Figures S5D and S5E).

Discovering Transcriptional Active Regulatory Elements by Retroviral Integration Site Analysis

We decided to use retroviral integration sites to identify active regulatory elements retrospectively in cells maintaining epithelial cultures in vitro, a bona fide approximation of KSCs. Early-passage (P2) foreskin-derived primary keratinocytes were co-cultured on an NIH3T3-J2 feeder layer to maintain stem cell activity, and transduced with

a GFP-expressing MLV vector. Cells were subcultured for six passages (>35 cell doublings) to exhaust the populations of TA progenitors and enrich for the progeny of culture-maintaining KSCs (Figures S6A and S6B). A CFE assay indicated progressive decrease of clonogenic cells and increase of abortive colonies (Figures S6C and S6D). In parallel, we transduced a population of DKs that were collected 72 hr after infection (Figure S6A). Genomic DNA was extracted from the two transduced cell populations, and MLV integration sites mapped genome-wide as previously described (Cattoglio et al., 2010). We mapped 10,819 MLV integration sites in the progeny of KSCs and 9,815 in DKs, and identified 1,478 and 1,326 integration clusters, respectively, as defined by comparison with an adjusted random distribution (Cattoglio et al., 2010).

To validate retroviral scanning as a tool for the identification of regulatory elements, we analyzed the genomic characteristics of the 1,326 clusters mapped in DKs. All clusters overlapped with epigenetically defined active regulatory regions, and in particular 79% with strong enhancers and 19.7% with strong promoters ($p < 10^{-22}$ compared with random sites) (Figures 5A and 5B). Clusters associated with promoters mapped predominantly (82%) in a ± 2.5 -kb window around TSSs, while those associated with enhancers were in intergenic (50.9%) or intragenic (38.7%) locations >35 kb away from any TSS (Figures 5A and S5E). All clusters showed a strong preference for H3K27ac, conserved non-coding sequences (Figure 5C), and open chromatin regions identified by FAIRE (formaldehyde-assisted isolation of regulatory elements) sequencing and DNase sequencing in keratinocytes (Figure 5D). The average expression level of CAGE promoters in a ± 100 -kb window from MLV clusters was significantly higher in DKs than in an unrelated control cell population,

Figure 3. Promoter and Enhancer Regions Involved in the Regulation of Keratinocyte Differentiation

- Percentage of promoters and enhancers overlapping with sites of active transcription, as detected by DeepCAGE.
- Proportion of CAGE-defined TSSs overlapping with transcribed promoters and enhancers falling in the NCP, LCP, and HCP categories.
- ChIP-Seq density profiles and heat maps for the H3K4me1, H3K4me3, and H3K27ac histone marks within each CAGE promoter category (HCP, LCP, NCP, and TATA⁺).
- Venn diagrams showing genome-wide overlap of transcribed promoter regions between KPs and DKs. Promoters were defined as unique when present in only one dataset (in the "All Transcribed Promoters" category), and "unique active" when mapped in both datasets but active (H3K27ac⁺) only in one of them (in the "Transcribed Active Promoters" category).
- Genomic browser screenshot of KP-specific H3K4me3 peaks overlapping with KP-high CAGE promoters that map TSSs of transcripts with unknown function.
- Genomic browser screenshot of DK-specific promoters. The promoter of *SOX15* is marked with H3K4me3 uniquely in DKs, while the promoter of *MPDU* shows H3K4me3 signal in both KPs and DKs, but is active only in DKs. Both promoters are marked by DK-high CAGE tags.
- Venn diagrams showing genome-wide overlap of enhancers between KPs and DKs. Enhancers were defined as "unique" when present in only one dataset, and "unique active" when mapped in both datasets but active (H3K27ac⁺) only in one of them.
- Annotation of KP (red) and DK (blue) active enhancers using GREAT. Gene ontologies are listed by biological process (BP), cellular component (CC), and pathway common (PC) categories. The x axis values correspond to binomial false discovery rate (FDR) (corrected) q values.
- Selected TF binding sequence motifs enriched at KP- and DK-unique active enhancers.

See also Figure S4.

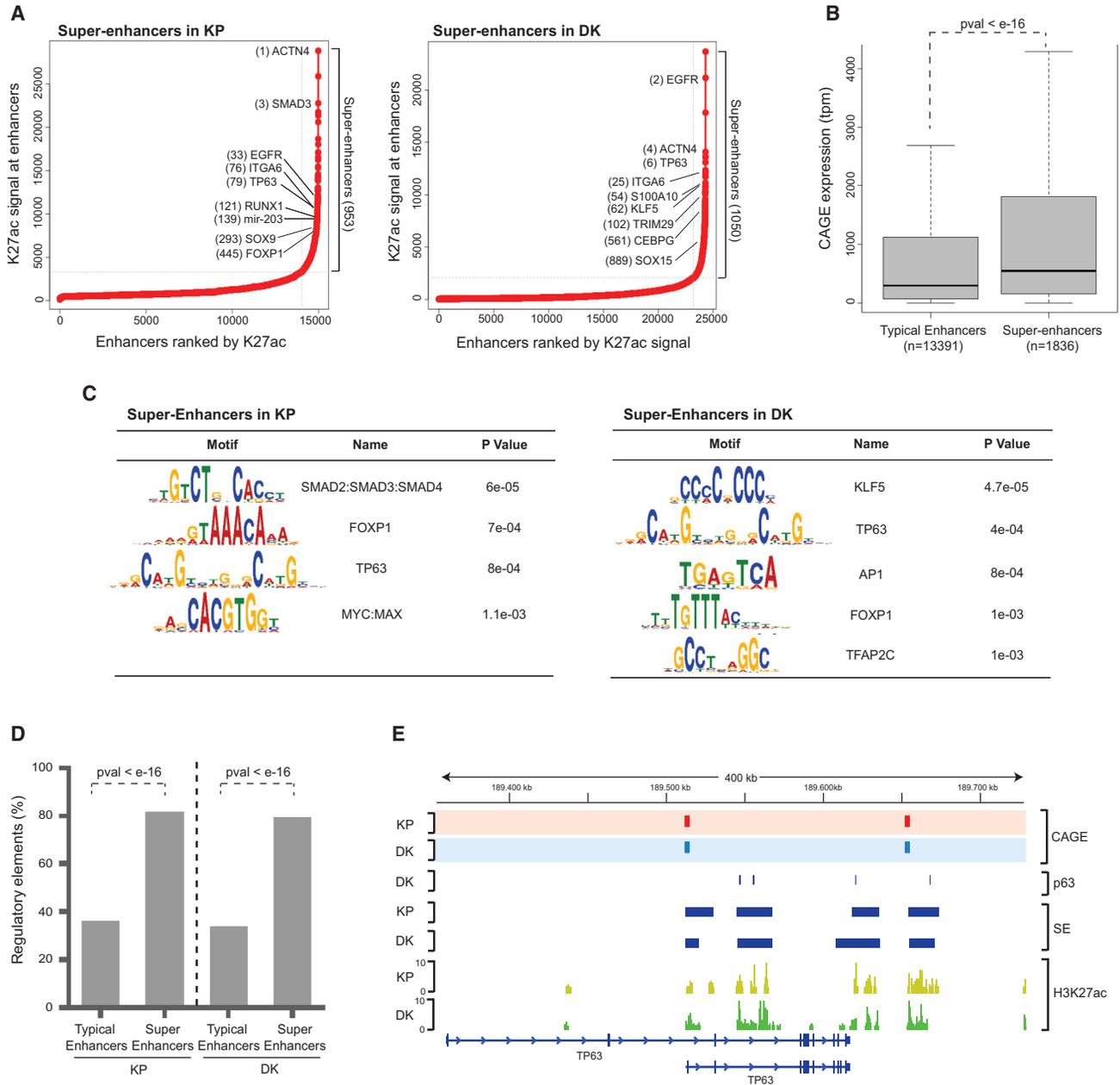


Figure 4. Super-Enhancers Define Specific Regulatory Networks in KPs and DKs

(A) Distribution of H3K27ac ChIP-seq signals across all the H3K27ac-containing enhancers (x axis), where enhancers are ranked by increasing H3K27ac ChIP-seq signal. Super-enhancers are found above the inflection point of the curve. Biologically relevant super-enhancers are highlighted together with their ranks and associated genes.

(B) Expression levels of CAGE promoters associated with typical enhancers and super-enhancers. Boxes show median line and quartiles, whiskers show the minimum and maximum boundary (1.5 times of the interquartile range from the first and third quartile) to define outliers. The p value was calculated using an unpaired Wilcoxon test.

(C) Selected TF binding sequence motifs enriched at enhancers in KPs and DKs.

(D) Percentages of typical enhancers and super-enhancers bound by p63. p Values were calculated using two-sample test for equality of proportion.

(E) Genome browser snapshot of KP- and DK-specific super-enhancers at the *TP63* locus. CAGE tags mark the TSS of the shortest *TP63* isoform and of an unknown transcript next to the 3' end of the gene. p63 binds to its own super-enhancers, as defined by p63 ChIP-seq. See also [Figure S5](#).



consistent with their enhancer function (Figure 5E). The 30% fraction of transcribed enhancers targeted by MLV integration was significantly more transcribed ($p < 10^{-5}$) than the average population (Figure 5F). Interestingly, 64% of the SEs were hit by at least one MLV integration compared with 7.8% of random sites ($p < 10^{-22}$). Functional annotation performed by GREAT showed a correlation between cluster-associated genes and differentiated cell functions, such as apoptosis, cholesterol biosynthesis, and FAS-, TAp63-, and TP53-linked pathways (Figure 5G).

To further validate the regulatory nature of the regions identified by the MLV clusters, we randomly chose 12 cluster regions and tested their transcriptional activity by a luciferase reporter assay in primary human keratinocytes: 6 of 12 regions scored positive for enhancer function and 2 of 12 for repressor function (Figure S6F).

Retroviral Scanning Uncovers Regulatory Regions Associated with Stem Cell Functions in Retrospectively Defined KSCs

MLV clusters mapped in the progeny of KSCs were intersected with those mapped in DKs to identify common and cell-specific regulatory regions. Less than 15% (195) of the KSC clusters overlapped for at least one base pair with any DK cluster, and <3% (41) overlapped completely, indicating that only a minority of the regulatory regions identified by MLV scanning was shared between the two populations. Only 28% of the remaining 1,283 KSC-specific clusters overlapped with enhancers epigenetically defined in KPs, indicating that MLV scanning identifies a set of potentially stem cell-specific regulatory elements.

KSC-specific clusters were associated with genes with stem cell-related functions, such as *LRIG1*, *ITGB1*, *ITGA6*, *YAP*, *MCSP*, and *WNT10A*. Functional annotation showed a clear correlation with developmentally regulated genes associated with regeneration, wound healing, anchoring and adherence junctions, and *ITGB1* and *TP63* signaling pathways (Figure 5G). No cluster mapped to the EDC or other genes associated with terminal differentiation functions. qPCR analysis showed that the expression of 9 out of 16 (56.3%) randomly chosen transcripts associated with KSC-specific clusters was higher in KPs than in DKs, indicating that putative stem cell-specific enhancers retain a higher activity in progenitors than in differentiated cells (Figure S6G). Expression of 7 out of 16 transcripts was barely detectable, suggesting that they represent stem cell-specific transcripts downregulated in both KPs and DKs.

KSC-Specific Regulatory Regions Are Characterized by a Unique Combination of Epithelial-Specific TF Binding Sites

A de novo search of TF binding motifs in a ± 1 -kb interval from KSC- and DK-specific clusters uncovered the same

motifs enriched in SEs, and particularly p63 binding sites (Figure 6A). p63 bound 47% of the sequences flanking MLV integration sites in DKs, and up to 73% when considering only SE-associated sites, a significant increase with respect to the 2.4% observed for random control sequences ($p < 10^{-16}$) (Figure 6B). Interestingly, the majority of the genes encoding TFs whose motifs are enriched in SEs and MLV clusters are in turn associated with SEs and MLV clusters (Figure 6C). Some of these genes, like *SMAD3*, *SOX9*, and *RUNX1*, were associated with KSC-specific MLV clusters overlapping KP-specific SEs (Figures 6C and 6D), and therefore identify TFs important for the execution of transcriptional programs in both stem and progenitor cells. Other TFs, such as *TCF4* and *SOX7* (Figures 6C and 6E), were associated with KSC-specific clusters but not KP- or DK-specific SEs, and may thus be involved in the execution of a more stem cell-specific program. These TFs were significantly more expressed in KPs than in DKs, as indicated by CAGE and RNA-seq analysis, and are known to play pivotal roles in the biology of murine hair follicle stem cells (Beck and Blanpain, 2012; Scheitz and Tumber, 2013).

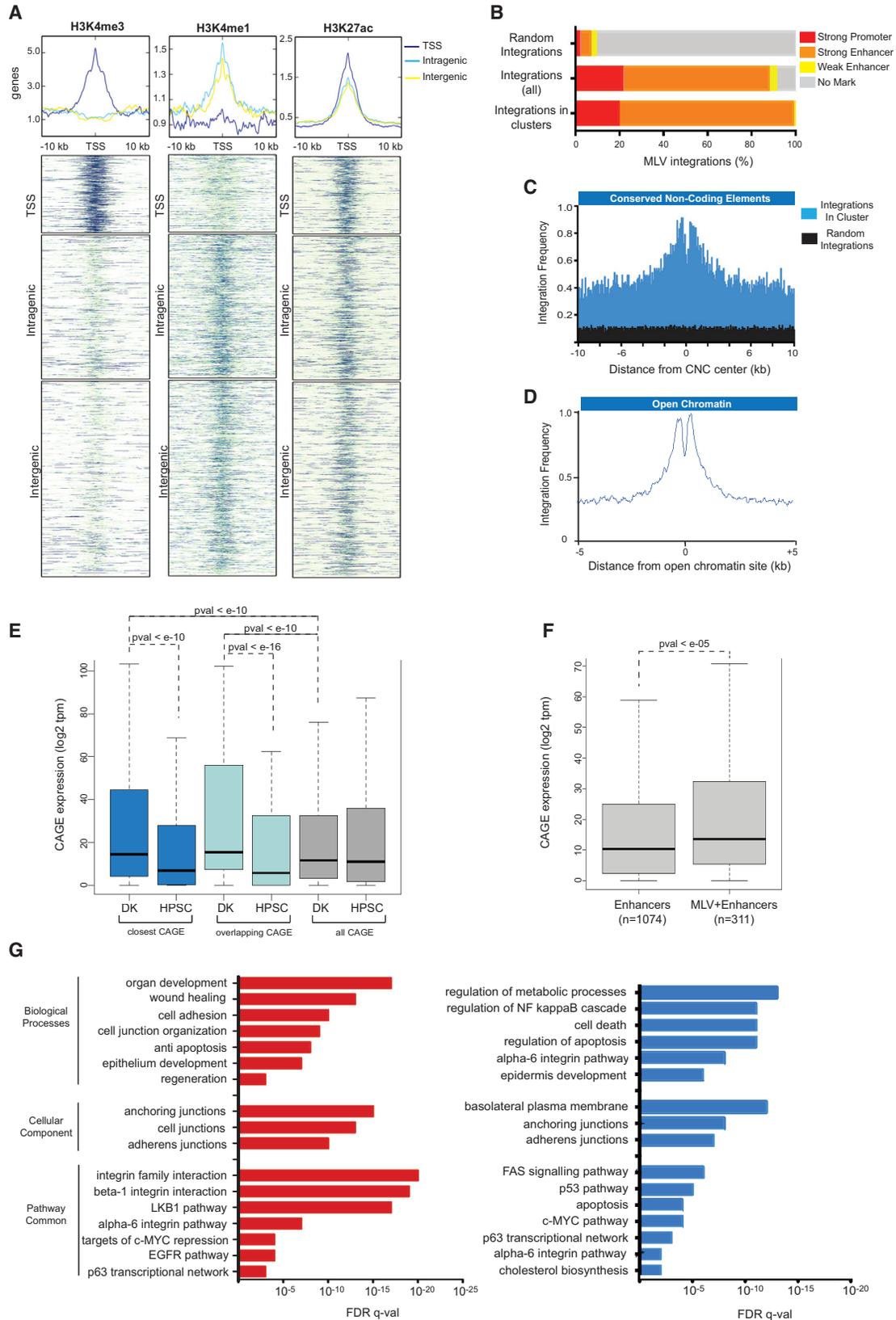
These analyses indicate that the regions uniquely identified by MLV scanning in retrospectively defined KSCs (see list in Table S1) represent bona fide stem cell-specific enhancers.

DISCUSSION

The hierarchy of keratinocyte stem and progenitor cells is defined by cell kinetics parameters, and is an ideal model to study transcriptional and chromatin dynamics driving differentiation of a human somatic stem cell. In this study, we mapped transcripts and transcriptional regulatory elements in prospectively isolated DKs and KPs, and retrospectively defined KSCs. We correlated CAGE promoter maps with epigenetic annotations of active promoters, enhancers, and SEs obtained by ChIP-seq, and integrated this information to discover shared or stage-specific regulatory elements.

Differentiation of Keratinocytes from Progenitors Is Determined by Quantitative Regulation of a Common Set of Promoters

The use of CAGE, ChIP-seq, and RNA-seq allowed the description of two different transcriptomes in KPs and DKs and a robust definition of promoters and their usage. We found that most of the >14,000 mapped promoters are shared between KPs and DKs and differentially expressed, indicating that the substantial transcriptome changes associated with differentiation are determined by quantitative regulation of promoters engaged in both



(legend on next page)



progenitors and differentiated cells rather than by the activation or silencing of stage-specific ones. The few, strictly stage-specific promoters were mostly unannotated or associated with non-coding transcripts and particularly lncRNAs, influential players in the control of lineage commitment and tissue identity. Three-quarters of the shared promoters showed housekeeping characteristics (TATA⁻ and high-CpG content), while the proportion of the TATA⁺/low-CpG promoters progressively increased in highly regulated and strictly cell-specific categories. Combining CAGE annotation with histone modification marks showed that the majority of the epigenetically defined “strong” (acetylated) promoters overlapped with CAGE promoters, while only one-fourth of the non-acetylated promoters were actually transcribed. The large overlap in promoter regions between KPs and DKs was found also at the epigenetic level, with just 300 regions specific for progenitors and just as few for differentiated cells. However, the intensity of the promoter-specific histone modifications differed in the two cell types and directly correlated with transcriptional activity. Transcriptional regulation is therefore accompanied by modest, essentially quantitative changes in histone modifications during keratinocyte differentiation, suggesting that the epigenetic landscape around promoters is already established at the progenitor state. Interestingly, silencing and downregulation of a large set of stem/progenitor cell-related genes in KPs and DKs was associated with H3K27 methylation of both promoters and enhancers, suggesting Polycomb-group-mediated repression as a mechanism for negative gene regulation in keratinocyte differentiation. Finally, CAGE analysis identified alternative transcripts in more than 1,100 protein-coding genes. Half of the alternative transcripts showed stage-specific changes in expression level, indicating that switching between alternative protein isoforms is an inherent part of the keratinocyte differentiation program.

Keratinocyte Differentiation Is Accompanied by Dramatic Changes in Enhancer Usage

Strikingly, enhancers were much more regulated than promoters during epithelial differentiation: more than 65% of the acetylated H3K4me1⁺ regions were strictly stage specific, indicating that enhancers are dramatically redefined during the KP-to-DK transition, and that differential enhancer usage is responsible for the quantitative regulation of promoter activity. Although the role of enhancers has been identified in other differentiation models (Creyghton et al., 2010; Heintzman et al., 2009; Rada-Iglesias et al., 2011), the difference between KPs and DKs is particularly striking given their developmental proximity. Functional annotation of active enhancers showed association with common epithelial pathways in both cells, but also cell-specific pathways such as wound healing in KPs and cell motility and apoptosis in DKs. We identified approximately 1,000 transcribed enhancers in both cell populations, which were mainly cell specific and drove the expression of annotated ncRNAs, consistent with previous reports (Andersson et al., 2014).

Super-Enhancers and TF Regulatory Circuits Play a Major Role in Epithelial Differentiation

SEs are large, highly acetylated clusters of transcriptional enhancers that drive the expression of cell-specific genes defining cell and tissue identity (Hnisz et al., 2013; Whyte et al., 2013). We mapped ~1,000 SEs in both KPs and DKs, the majority of which was cell-specific and associated with cell-specific genes playing key functions in epithelial homeostasis, as already shown in the murine hair follicle (Adam et al., 2015). These included laminins, keratins, cell-adhesion complexes, and components of the TGF, WNT, and SMAD signaling pathways, but also master regulators of skin and stem cell biology, such as p63, FOXF1, MYC, and KLF4, and ncRNAs such as *mir-203*, a suppressor of p63 and a key promoter of keratinocyte differentiation

Figure 5. MLV Integration Clusters Mark Regulatory Regions Associated with Cell-Specific Functions

- (A) ChIP-Seq density profiles and heatmaps are shown for the H3K4me1, H3K4me3, and H3K27ac histone marks within each MLV integration category (TSS-proximal, intragenic, and intergenic).
- (B) Percentages of randomly generated sites and clustered or unclustered MLV integration sites associated with epigenetically defined regulatory regions.
- (C and D) Distribution of the distance of MLV integration clusters from the midpoint of (C) conserved non-coding (CNC) elements in a 20-kb window, and of (D) open chromatin regions defined by FAIRE sequencing in a 10-kb window.
- (E) Differential expression levels (log₂ of CAGE TPM values) in DKs or hematopoietic stem-progenitor cells (HSPC) of CAGE promoters proximal to, or overlapping, MLV integration clusters in DKs.
- (F) Differential expression levels of total transcribed enhancers and transcribed enhancers marked by MLV integration sites. Boxplots in (E) and (F) show median line and quartiles, whiskers show the minimum and maximum boundary (1.5 times of the interquartile range from the first and third quartile) to define outliers. p Values were calculated using an unpaired Wilcoxon test.
- (G) Functional annotation of KSC-specific (red) and DK-specific (blue) enhancers identified by MLV clusters using GREAT. Gene ontologies are listed by biological process, cellular component, and pathway common categories. The x axis values correspond to binomial FDR (corrected) q values.

See also [Figure S6](#).

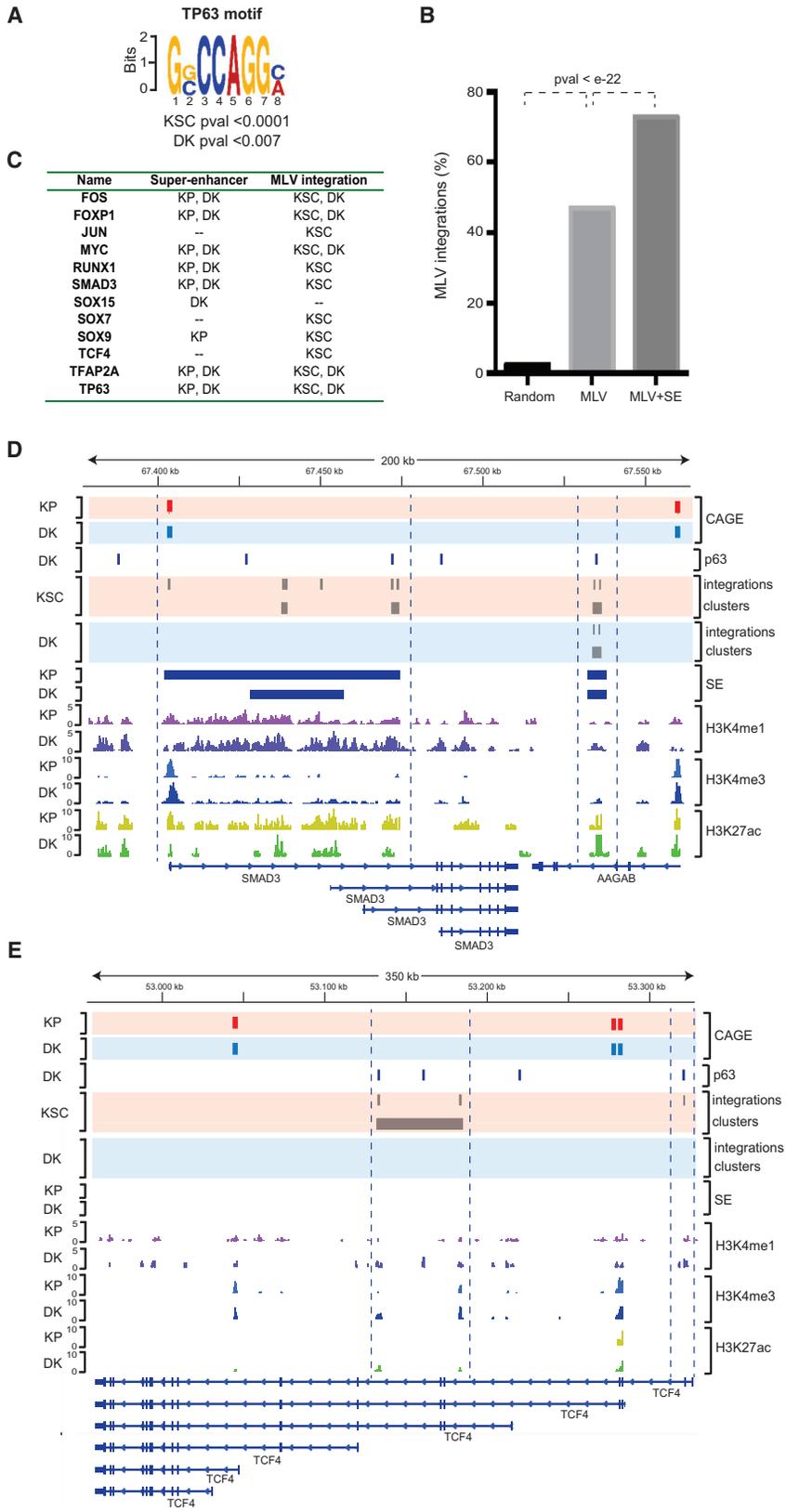


Figure 6. Retroviral Scanning Identifies Potential Regulatory Networks in Retro-spectively Defined KSC

(A) The TP63 motif found overrepresented in a 500-bp window around the center of MLV integration clusters in both KSCs and DKs.

(B) Percentages of randomly generated sites, MLV integration sites, and MLV integration sites mapping in SEs bound by p63 in DKs. p Values were calculated using a two-sample test for equality of proportion.

(C) List of key transcription factors associated with SEs and MLV integration sites in KPs, DKs, and KSCs.

(D) Genome browser snapshot of the *SMAD3* gene locus, harboring KSC-specific MLV integration clusters that overlap with p63 binding sites and with a SE in both KPs and DKs. *SMAD3* transcription (CAGE TPM values) is higher in KPs than in DKs. The expression of a close gene, *AAGAB*, is instead not significantly different between KPs and DKs, and its genomic locus is marked by the same MLV clusters and SEs in both cell types.

(E) Genome browser snapshot of the *TCF4* gene locus. The locus is barely marked by active histone modifications in both KPs and DKs, but harbors KSC-specific MLV clusters that represent putative KSC-specific regulatory regions.



(Yi et al., 2008). SEs in both KPs and DKs were particularly enriched in binding motifs for FOXP1, a regulator of hair follicle quiescence and activation (Leishman et al., 2013), and for the master regulator p63. Actual, ChIP-seq-mapped binding sites for p63 were highly enriched in SEs, validating the predictions provided by TF-motif discovery and indicating the pervasive role of p63 in the control of epithelial SE function. Interestingly, p63 binding sites were enriched in SEs associated with genes encoding p63 itself, FOXP1, and other key TFs binding to keratinocyte-specific enhancers and SEs, such as TFAP2A, RUNX1, SOX9, MYC, SMAD3, AP1, and KLF5. Finally, ChIP-seq and TF-motif analysis indicate that even though p63 is a master regulator throughout keratinocyte differentiation (Kouwenhoven et al., 2015), it regulates distinct sets of genes at each stage through binding of stage-specific promoters, enhancers, and SEs and in combination with stage-specific TFs.

Identification of Regulatory Networks in KSCs by Retrospective MLV Scanning

To identify enhancers and SEs in KSCs, a rare population which lacks robust markers for prospective isolation, we used MLV scanning as a technique for their retrospective identification: primary cultures were transduced by an MLV vector and integration sites were mapped in the progeny of long-term keratinocyte culture-maintaining cells, a characteristic that bona fide defines self-renewing KSCs. The MLV pre-integration complex specifically interacts through its integrase component with proteins (BET) that tether integration to highly acetylated, transcriptionally active regions (De Rijck et al., 2013; Gupta et al., 2013; Sharma et al., 2013). MLV integration clusters can therefore be used as surrogate markers of promoters, enhancers, and SEs, as previously reported in hematopoietic cells (Biasco et al., 2011; Cattoglio et al., 2010; De Ravin et al., 2014). We validated this concept also in DKs by correlating MLV integration clusters with CAGE and ChIP-seq data: MLV clusters were preferentially associated with SEs, probably due to their highly acetylated state, and genes associated with clusters included important regulators of epidermal differentiation and homeostasis such as p63, FOXP1, SOX9, SMAD3, KLF4, GATA3, GRHL3, and TFAP2.

More than 85% of the 1,327 MLV clusters mapped in KSCs were specific to these cells and showed no overlap with regulatory regions defined in KPs or DKs. Many of these KSC-specific regions were associated with genes known to play a role in epidermal stem cell functions, such as *LRIG1*, *ITGB1*, *ITGA6*, *YAP*, *MCSP*, or *WNT10A*, and none was associated with the EDC complex or genes necessary for differentiated cell functions. KSC-specific clusters showed an exceedingly high frequency of p63 binding sites, and binding motifs for other TFs identified

also in KP enhancers. Some of these genes, such as *SMAD3*, *SOX9*, and *RUNX1*, and *TP63* itself, were associated with KSC-specific clusters that overlapped to KP-specific SEs, identifying TFs important for the execution of transcriptional programs in both stem and progenitor cells. Other TFs, such as *TCF4* and *SOX7*, known to play pivotal roles in the biology of murine hair follicle stem cells (Beck and Blanpain, 2012; Scheitz and Tumber, 2013), were associated with KSC-specific clusters but not KP- or DK-specific SEs, and might thus represent TFs involved in the execution of a more stem cell-specific program. In general, the TF circuitries identified by KSC-specific clusters are in close agreement with previous studies in epidermal murine models, which demonstrated the importance of Sox, Ets, and the Wnt and Bmp signaling pathways—to which TCF4 and SMAD3 belong—in ectodermal and epidermal development, and the importance of MYC and GATA3 in keratinocyte differentiation (Fuchs, 2007). Moreover, TFAP2A, RUNX1, and AP1 were shown to cooperate with the epithelial master regulator p63 in the specification of the epidermal fate and differentiation programs (Kouwenhoven et al., 2015; McDade et al., 2012).

The analysis of SEs in progenitors and DKs, and of MLV clusters in stem cells, identify a complex regulatory and auto-regulatory TF network with p63 as the central player, which regulates the specification of the stem and progenitor cell identity and the execution of their differentiation program. In embryonic stem cells, TFs of the pluripotency module form an auto-regulatory loop whereby they cooperatively bind to their promoters and regulate their own expression as well as that of other TFs and ncRNAs, which form a core regulatory circuitry driven in large part by the activity of SEs (Whyte et al., 2013). Our data indicate that SE-mediated auto- and cross-regulatory TF circuitries play a key role in mediating identity and differentiation also in somatic cells, and particularly in the human epithelium.

EXPERIMENTAL PROCEDURES

Cell Culture

Human primary keratinocytes were obtained from foreskin biopsies of healthy donors and expanded on an NIH3T3-J2 cell feeder in FAD medium. KPs were obtained by collagen IV adherence assay. Keratinocyte differentiation was induced by cell-contact inhibition and by exclusion of several growth factors from the medium. See also [Supplemental Experimental Procedures](#).

DeepCAGE

RNA from three different KP selection experiments was isolated using an RNeasy Plus Mini kit (Qiagen) and pooled together. The DeepCAGE library was prepared by DNAFORM at RIKEN Omics Science Center, as described previously (Carninci et al., 2006). Samples were sequenced using the Illumina GA II sequencer,



with an average read length of 36 bases, and tags were extracted and mapped to the hg19 genome. See also [Supplemental Experimental Procedures](#).

Gene-Expression Analysis

Expression profiles were determined by RNA-seq analysis. RNA-seq libraries were prepared from 300 ng of RNA, and 75-bp single-end sequences were obtained on a NextSeq 500 Instrument (Illumina). Sequence tags were mapped to the hg19 genome using TopHat v2.0.6 and transcript levels were calculated using Cufflinks v2.0.2. See also [Supplemental Experimental Procedures](#).

ChIP-Seq

Chromatin was prepared from KPs and DKs and immunoprecipitated with antibodies against H3K4me1, H3K4me3, and H3K27ac, as previously described ([Cattoglio et al., 2010](#)). After Illumina sequencing, raw reads were mapped to the hg19 genome using Bowtie ([Langmead et al., 2009](#)) and ChIP-seq peaks were called using SICER default parameters ([Zang et al., 2009](#)) and using each INPUT data to model the background noise. See also [Supplemental Experimental Procedures](#).

Retroviral Scanning

The MLV-derived retroviral vector expressing GFP under a modified LTR control (MFG.GFP $_{mod}$) was used to transduce KSCs and DKs. Retroviral integration sites were mapped by linker-mediated PCR and Roche/454 pyrosequencing as previously described ([Cattoglio et al., 2007](#)). See also [Supplemental Experimental Procedures](#).

ACCESSION NUMBERS

The DeepCAGE, ChIP-seq, and microarray data were deposited in the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GEO: GSE64328. MLV integration sites sequencing data were deposited in the Sequence Read Archive (<http://www.ncbi.nlm.nih.gov/sra>) under accession number SRA: SRP051203.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2016.03.003>.

AUTHOR CONTRIBUTIONS

A.C. and F.M. conceived and designed the study; A.C., A.M., and O.R. obtained the data; A.C., L.P., G.M.T., M.S., and E.R. performed computational and statistical analyses under the supervision of C.P., G.D.B., and S.B.; A.C. and F.M. wrote the manuscript, with input from all authors.

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