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Mutant p63 Affects Epidermal Cell Identity through Rewiring the Enhancer Landscape

Graphical Abstract

Highlights
- Downregulated epidermal genes and upregulated non-epidermal genes in EEC keratinocytes
- A genome-wide redistribution of enhancers in EEC keratinocytes
- Gained enhancers are frequently bound by deregulated RUNX1 in EEC keratinocytes
- siRUNX1 partially rescues gene deregulation and the altered enhancer landscape

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In Brief
Using transcriptomics and epigenomics, Qu et al. elucidate how mutant p63 associated with EEC syndrome alters the enhancer landscape in skin keratinocytes. The genome-wide redistribution of enhancers leads to the downregulation of epidermal genes and upregulation of non-epidermal genes and affects the cell identity of skin keratinocytes.

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Mutant p63 Affects Epidermal Cell Identity through Rewiring the Enhancer Landscape

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INTRODUCTION

Transcription factor p63 is a key regulator of epidermal keratinocyte proliferation and differentiation. Mutations in the p63 DNA-binding domain are associated with ectrodactyly, ectodermal dysplasia, and cleft lip/palate (EEC) syndrome. However, the underlying molecular mechanism of these mutations remains unclear. Here, we characterized the transcriptome and epigenome of p63 mutant keratinocytes derived from EEC patients. The transcriptome of p63 mutant keratinocytes deviated from the normal epidermal cell identity. Epigenomic analyses showed an altered enhancer landscape in p63 mutant keratinocytes contributed by loss of p63-bound active enhancers and unexpected gain of enhancers. The gained enhancers were frequently bound by deregulated transcription factors such as RUNX1. Reversing RUNX1 overexpression partially rescued deregulated gene expression and the altered enhancer landscape. Our findings identify a disease mechanism whereby mutant p63 rewire the enhancer landscape and affects epidermal cell identity, consolidating the pivotal role of p63 in controlling the enhancer landscape of epidermal keratinocytes.

INTRODUCTION

The transcription factor (TF) p63 is an ancient member of the p53 gene family. Different from p53 that has a convincing function in tumor suppression, p63 is a key regulator of development of the epidermis, specifically in epidermal stem cell self-renewal, morphogenesis, and directing differentiation programs (Candi et al., 2008; Mills et al., 1999; Yang et al., 1999). Several p63 isoforms have been reported, and all isoforms contain the DNA-binding domain (Kouwenhoven et al., 2015b).

The role of p63 in epidermal development has been established by two independent p63 knockout mouse models (Mills et al., 1999; Yang et al., 1999). These p63-deficient mice do not have the epidermis and epidermal-related appendages. During embryonic development, p63-deficient mice develop a normal ectoderm with Krt8- and Krt18-positive simple epithelial cells. However, they fail to initiate embryonic stratification or produce mature Krt5- and Krt14-positive simple epithelial cells. These findings demonstrate that p63 is essential and required for the commitment to a proper epidermal cell fate during development.

In keratinocytes, p63 plays important roles in both proliferation and differentiation. The p63 protein, mainly the ΔNp63α isoform, is expressed at a high level in proliferating keratinocytes in the basal layer of the epidermis. Upon stratification, its expression level is reduced (Candi et al., 2007). Knockdown of p63 in keratinocytes affects proliferation and prevents cells from differentiating (Truong et al., 2006). At the molecular level, knockdown of p63 induces genes controlling cell-cycle arrest, such as p21 (CDKN1A) (LeBoeuf et al., 2010), and downregulates genes that are important for epidermal differentiation, such as PERP and KRT14 (Ihrie et al., 2005; Romano et al., 2007). These data show that p63 represses cell-cycle-arrest genes to promote proliferation and activates epidermal differentiation genes to induce differentiation.

In recent years, a number of epigenomic profiling studies established the master regulator role of p63 in the genome of keratinocytes, predominantly in controlling enhancers (Bao et al., 2015; Kouwenhoven et al., 2015a; Rinaldi et al., 2016). p63 bookmarks genomic loci and cooperates with specific TFs to activate epidermal genes via active enhancers. Consistently, transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) analysis showed that p63-binding sites are preferentially located in nucleosome-enriched regions in epidermal keratinocytes, and these sites are inaccessible in cell types where
p63 is not expressed (Bao et al., 2015). In keratinocytes, p63 cooperates with an ATP-dependent chromatin remodeling factor, BAF1, to make these regions accessible. It has also been shown that p63 directly regulates chromatin factors such as Satb1 and Brg1 that play roles in higher-order chromatin remodeling, covalent histone modifications, and nuclear assembly (Fessing et al., 2011; Mardaryev et al., 2014). These data suggest that p63 regulates epidermal cell fate determination and differentiation not only through direct target genes but also via modulating the chromatin landscape.

The key role of p63 in epidermal development has also been demonstrated in human disease models. Heterozygous mutations of TP63 encoding p63 cause a spectrum of developmental disorders. Among them, ectodactyly, ectodermal dysplasia, and cleft lip/palate (EEC) syndrome is caused by point mutations located in the p63 DNA-binding domain and manifests ectodermal dysplasia with defects in the epidermis and epidermal-related appendages, limb malformation, and cleft lip and/or palate (Rinne et al., 2007). Five hotspot mutations affecting amino acids, R204, R227, R279, R280, and R304, have been found in ~90% of the EEC population, and these EEC mutations were shown to disrupt p63 DNA binding and result in impaired transactivation activity (Browne et al., 2011; Celli et al., 1999). Therefore, these mutant p63 proteins have been proposed to have a dominant-negative effect on wild-type p63, probably by abolishing DNA binding as a result of tetramerization of wild-type and mutant proteins (Brunner et al., 2002). Furthermore, mouse genetic studies support the dominant-negative model. Heterozygous p63 knockout mice do not show any ectodermal phenotype (Mills et al., 1999; Yang et al., 1999), whereas heterozygous knockin mice carrying an EEC mutation resemble the human phenotype (Vernersson Lindahl et al., 2013).

Although the role of p63 in normal epidermal development and differentiation has been demonstrated, the molecular mechanism by which p63 mutations cause the epidermal phenotype in diseases is not yet understood. We previously reported that p63 mutant keratinocytes derived from EEC patients could not fully differentiate toward terminal stratification in both 2D and 3D cellular models (Shen et al., 2013). In this study, EEC patient keratinocytes carrying three hotspot mutations (R204W, R279H, and R304W) were assessed by transcriptomic and epigenomic analyses to identify the underlying molecular mechanism. Our data showed that deregulated gene expression accompanied by a rewired enhancer landscape leads to a less defined epidermal cell identity of p63 mutant keratinocytes, which potentially contributes to the pathogenic mechanism of EEC syndrome.

RESULTS

Loss of Characteristic Epidermal Expression Profiles in p63 Mutant Keratinocytes

Using an established in vitro differentiation model of epidermal keratinocytes (Kouwenhoven et al., 2015a), we characterized gene expression differences between keratinocytes derived from non-EEC individuals (control) and EEC patients carrying mutations in the DNA-binding domain of p63 (R204W, R279H, and R304W, p63 mutant) (Figures 1A and 1B). Similar to our previous report (Shen et al., 2013), p63 mutant keratinocytes retained largely unchanged morphology at the terminal stage of differentiation compared to the multilayer cell structures of control keratinocytes on day 7 (Figure S1A), indicating that they were unable to fully differentiate. To better characterize these mutant keratinocytes at the molecular level, we performed RNA-sequencing (RNA-seq) analyses. In principal-component analysis (PCA), gene expression of control keratinocytes from day 0 to day 7 moved along the principle component 1 (PC1) axis (51%) that probably defines the differentiation process, whereas mutant keratinocytes remained at the left side of PC1 (Figure 1C). Consistently, DAVID Gene Ontology (GO) annotation (Dennis et al., 2003) of the top 500 genes associated with PC1 showed terms related to epidermis development and keratinocyte differentiation (Tables S1A and S1B). Many deregulated genes were validated by qRT-PCR and at the protein level (Figure S1B). These molecular data confirmed the differentiation defect of p63 mutant keratinocytes.

We next analyzed differentially expressed (DE) genes (p < 0.05) between control and p63 mutant keratinocytes during differentiation. Overall, 3,373 genes were upregulated and 4,595 genes were downregulated in p63 mutant keratinocytes (Table S1D), which were distinguished into four clusters (Figure 1D; Table S1E). Among the upregulated genes (EC1), some were generally not expressed or expressed at a low level in control keratinocytes. There was an enrichment of genes involved in extracellular structure organization, actin cytoskeleton organization, and muscle cell function (e.g., ACTG1 and MYH10) (Table S1F). TF genes in this cluster include SOX4, TEAD2, and RUNX1, which are widely expressed in many cell types, and a number of Antp homeobox family members, such as HOX genes. Interestingly, TP63 was also detected in EC1, and ΔNp63 was the isoform detected in both control and mutant keratinocytes (Figure S1C). Compared to the decreased p63 expression during differentiation in control keratinocytes, an increased p63 expression at the proliferation stage was observed in p63 mutant keratinocytes, and this expression level stayed at a high level through differentiation (Figure S1C).

The 4,595 downregulated genes whose expression was dynamically induced during differentiation in control keratinocytes remained low and largely unchanged in p63 mutant keratinocytes. These genes were grouped into three clusters (EC2, EC3, and EC4) (Tables S1G–S1I). Genes in EC3 were highly expressed at the proliferation stage on day 0, and expression went down during differentiation. They were mainly involved in cell-cycle regulation (e.g., CDC20 and KIFC1). Finally, genes in EC4 showed a progressive upregulation in control keratinocytes, and many of them were involved in ectoderm development and keratinocyte differentiation. The TF genes in this cluster included OVOL1 and KLF4, and known p63 co-regulators, such as TFAP2A and NFE2L2. Of note, consistent with the morphological changes (Figure S1A), the deregulated gene expression was more evident in mutant keratinocytes carrying R204W and R304W than those carrying R279H (Figure 1D). Taken together, our RNA-seq analyses showed downregulation of epidermal
differentiation genes and upregulation of non-epidermal genes in p63 mutant keratinocytes, suggesting that p63 mutant keratinocytes have a less defined epidermal cell identity.

To better visualize the interaction between DE genes, we carried out weighted gene-coexpression correlation network analyses using the Cytoscape Network Analyzer (Tables S1J and S1K). Two significant coexpression modules were identified, of which many genes were involved in keratinization (e.g., LOR and FLG) and nucleosome assembly (Figures 1E and S2A; Table S1L). Most genes in both modules showed downregulated expression in p63 mutant keratinocytes. The upregulated genes in p63 mutant keratinocytes did not generate significant main modules but generated several small subnetwork modules. They likely played roles in extracellular matrix organization.

Figure 1. Transcriptome Dynamics of Control and p63 Mutant Keratinocytes during Differentiation
(A) EEC p63 mutant keratinocytes used in this study.
(B) The setup of in vitro differentiation of epidermal keratinocytes.
(C) Principal-component analysis (PCA) on RNA-seq data. Two independent control lines and three p63 mutant lines are indicated with different colors. Shapes indicate four stages.
(D) Hierarchical clustering of differentially expressed genes (p < 0.05). The Z score was calculated based on log10 (fragments per kilobase per million reads mapped (FPKM) + 0.01) of each gene. Enriched top two Gene Ontology (GO) terms of genes per gene expression cluster (EC) are shown.
(E) Coexpression network of deregulated genes in p63 mutant keratinocytes during differentiation. Interactions with connectivity weight > 0.1 were shown. Two main co-expression modules were labeled with corresponding GO terms. The node color indicates the expression fold change between mutant and control keratinocytes.

See also Figures S1 and S2 and Table S1.
(Table S1L). However, the higher inter-modal connectivity between keratinization, nucleosome assembly, and extracellular matrix organization modules suggests a biological relationship between these modules, indicating that changes in the chromatin landscape may contribute to gene deregulation. Consistent with this notion, many chromatin regulators were deregulated in p63 mutant keratinocytes (Figure S2B). These factors include KAT2B, which is a histone acetyltransferase, and SMYD3, which encodes a histone methyltransferase. The deregulation of these genes was confirmed with qRT-PCR (Figure S2C).

**p63 Orchestrates Enhancer Dynamics during Epidermal Differentiation**

Given the indicated relationship between p63 and the chromatin landscape, we first assessed the role of p63 in regulating the chromatin landscape during normal epidermal differentiation. We mapped histone modifications H3K27ac, H3K4me3, and H3K27me3, as well as p63 binding sites (BSs) of control keratinocytes, to open chromatin regions detected by DNase I hypersensitivity sites (DHSs) in normal human epidermal keratinocytes (NHEK) reported by ENCODE (Table S2). Two clusters of active enhancers (C3 and C4) were bound by p63 (Figures S3A–S3C). Regions in C3 showed higher p63-binding signals. GO annotation using the Genomic Regions Enrichment of Annotation Tool (GREAT), which permits functional interpretation of cis-regulatory regions (McLean et al., 2010), showed that nearby genes were involved in apoptosis and epidermis development. Regions in C4 had relatively lower p63-binding signals, and nearby genes were involved in keratinocyte differentiation (Figure S3C). Furthermore, cluster C7 represents a small group of open chromatin regions with H3K27me3 signals but devoid of p63 binding (Figure S3A). Genes near these regions were associated with “pattern specification process,” such as neuron fate commitment (Figure S3C).

To quantify chromatin dynamics, we used ChromHMM (Ernst and Kellis, 2012) to analyze chromatin state transitions. With the combination of H3K27ac, H3K4me3, and H3K27me3, we obtained six classes of chromatin state: active enhancers, active promoters, weak promoters, heterochromatin regions, bivalent promoters, and “unmodified” regions that were not decorated with any of the three modifications (Figure 2A; Tables S2B and S2C). By pairwise comparison between two adjacent stages of differentiation, we observed major transitions between active enhancers and unmodified regions as well as between unmodified regions and heterochromatin regions (Figure 2B). As expected, we found that the transition from unmodified regions to active enhancers was generally associated with gene upregulation; vice versa, the transition from active enhancers to unmodified regions was associated with gene downregulation, at least at early differentiation stages (days 0–2 and days 2–4) (Figure 2C), such as regulation of LOR (Figure 2D) and KRT1 (Figure S3D). Furthermore, there were also transitions between active promoters and unmodified regions (Figure S3E). Interestingly, many genes that are known to be expressed in cells of mesodermal origin (e.g., PAX2) were heavily marked by H3K27me3 in proliferating keratinocytes (day 0). The repression was relieved at the end of the terminal differentiation of keratinocytes (Figure S3F).

Next, we asked whether specific TFs control enhancer dynamics during differentiation. Therefore, we performed motif analysis of dynamic enhancers using the HOMER package (Figure 2E). We observed that bZIP, p53 or p63, Zinc finger (Zf), and TEA motifs were enriched in regions being activated from unmodified regions on day 0 to active enhancers on day 2, while p53 or p63 was the only enriched motif in regions being activated from unmodified regions on day 2 to active enhancers on day 4. The bZIP motif was predominantly enriched in regions being activated from unmodified regions on day 4 to active enhancers on day 7, whereas the p53 or p63 motif was the only enriched motif in regions changing from active enhancers on day 4 to unmodified regions on day 7 (Figure 2E). The temporal enrichment of p63 motifs in dynamic enhancers underscores the key role of p63 in orchestrating the enhancer landscape during keratinocyte differentiation.

**Decreased Active Enhancers Associated with p63-Binding Deficiency in p63 Mutant Keratinocytes**

Based on the DNA-binding deficiency of EEC mutants shown by various studies and the dominant-negative model, we expected to detect DNA-binding loss in p63 mutant keratinocytes. To characterize this and evaluate the effect of p63 mutations on the enhancer landscape, we performed p63 chromatin immunoprecipitation sequencing (ChIP-seq) using a p63 antibody that is not affected by mutations in the p63 DNA-binding domain (Figure S4A) (Shen et al., 2013) and H3K27ac ChIP-seq in all three p63 mutant keratinocytes. A total number of 33,366 p63-binding sites (p63 BSs) detected in both control and mutant keratinocytes were analyzed, and we observed globally reduced p63 binding signals in p63 mutant keratinocytes as compared to the control keratinocytes (Figure 3A; Tables S3A–S3E). It should be noted that no clear increased p63 binding or de novo p63 BSs were observed in p63 mutant keratinocytes. K-means clustering analysis showed that p63 BSs could be clustered into three groups based on the binding signals. Clusters p63-C1 and p63-C2 had decreased p63 binding to a lesser extent, whereas loci in p63-C3 showed more dramatic to almost complete loss of p63-binding signals (Figure 3A). The p63-binding pattern did not change much in mutant keratinocytes during differentiation (Figure S4D). Accordingly, the difference of H3K27ac signals at p63-C1 and p63-C2 between control and p63 mutant keratinocytes was not obvious, whereas a decrease of H3K27ac signals was detected at p63 C3 in p63 mutant keratinocytes (Figures 3A and S4B).

Using GREAT GO annotation to assess nearby genes, all three clusters of p63 BSs were significantly enriched for genes involved in epidermis development (Figure 3B). We also performed human phenotype analyses to investigate the disease significance of these p63 BSs, and the disease terms detected were mainly related to ectodermal dysplasia, such as plantar hyperkeratosis, nail dystrophy, and alopecia (Figure S4C). We further explored whether a specific molecular mechanism controls the discordant p63-binding losses. We examined whether the cooperation with different co-regulating TFs contributes to the differential p63-binding loss by motif scanning but did not find significant differential p63 co-regulators that can potentially cause the discordant p63-binding loss (Tables S3H–S3J). Next,
using our previously established p63scan algorithm (Kouwenhoven et al., 2010), we found that p63-C3 BSs sites had the lowest percentage of BSs with the p63 motif (85%) and the lowest average motif score (mean motif score of 8.3) compared to p63-C1 (98%; mean motif score of 9.5) and p63-C2 (93%; mean motif score of 8.8) BSs (Figure 3C; Tables S3K–S3N). Our observations thus suggest that p63 motif strength determines the selectivity of the loss of p63 binding.

Lastly, we examined whether gene deregulation was associated with impaired p63 binding. Genes near p63 BSs (all three [p63-C1, p63-C2, and p63-C3]) had a significantly larger proportion of deregulated genes (34.8%, p = 0, hypergeometric test) when compared to all annotated genes (12.5%) (Figure 3C; Table S3F). A significant difference in the percentage of deregulated genes was also found in genes associated with p63-C3 BSs (35.4%, p = 0, hypergeometric test) compared to all genes with p63 BSs (Table S3G). These data indicate that impaired p63 binding significantly contributed to deregulated gene expression, for both up- and downregulation (Figure 3E).

In summary, we showed that EEC mutations can result in loss of p63 binding and loss of active enhancers. The loss of p63 binding is apparently motif-strength dependent. p63-binding loss can lead to gene deregulation and potentially contribute to ectodermal dysplasia phenotypes.

Redistribution of Enhancers in p63 Mutant Keratinocytes

Although a significant percentage of deregulated genes (~54%) were associated with impaired p63 binding, a large number of deregulated genes did not seem to be directly regulated by p63. Furthermore, we observed many enhancers with unexpected increased H3K27ac signals near deregulated genes in p63 mutant keratinocytes (Figure 4A, blue shaded regions). Therefore, we compared H3K27ac between control and p63 mutant keratinocytes with MAnorm (Shao et al., 2012). We identified 17,931 genomic regions that had significantly higher H3K27ac signals in mutant keratinocytes (Figure 4A, blue shaded regions).
keratinocytes (referred to as control-specific enhancers) (Figures S5A and S4B; Table S4). Two replicas of H3K27ac ChIP-seq in R304W mutant keratinocytes showed a high correlation (Pearson correlation coefficient of 0.94; Figure S5B), demonstrating the high reproducibility of these datasets. To validate these findings, H3K27ac ChIP-qPCR was performed on three

Figure 3. Decreased Active Enhancers Associated with p63-Binding Deficiency
(A) K-means clustering of p63-binding sites (BSs) that are merged from control and p63 mutant keratinocytes groups p63 BSs into 3 classes (k = 3, metric = Pearson). Heatmaps and band plots are shown in a 4-kb window with summits of p63 BSs in the middle. Color intensity in heatmaps represents normalized read counts. In the band plots, the median enrichment was visualized as the black line while 50% and 90% ranges were depicted in lighter color, respectively.
(B) GREAT-based GO biological process annotation of p63 BSs in each cluster.
(C) p63 motif strength determined the selectivity of the loss of p63 binding. Top: pie charts showing the percentage of p63 BSs with a p63 motif. Bottom: boxplot showing motif score distribution. Data are shown as mean ± SD; ***p < 0.001, one-way ANOVA.
(D) Percentage of deregulated genes associated with all p63 BSs and p63 BSs from p63-C3 (p63 C3) compared with all annotated genes. ***p < 0.001, hypergeometric test.
(E) ChIP-seq of p63 and RNA-seq data at the RUNX1, EHF, SMYD3, and KAT2B loci in control and p63 mutant keratinocytes. Red bars represent p63 BSs that were lost or decreased in p63 mutant keratinocytes. See also Figure S4 and Table S3.
control-specific and three mutant-specific enhancer loci (Figure S5C). Genes nearby control-specific enhancers were associated with keratinocyte differentiation, whereas those nearby mutant-specific enhancers were involved in cell-cycle regulation, migration, and non-epithelial processes (Figure 4C). Furthermore, a significantly larger proportion of deregulated genes had either control-specific or mutant-specific H3K27ac sites (Figure 4D). As expected, control-specific enhancers were associated with gene downregulation, whereas mutant-specific enhancers were associated with gene upregulation in p63 mutant keratinocytes (Figure 4E). Taken together, the observed genome-wide redistribution of enhancers marked by H3K27ac indicates that epigenome rewiring occurs in p63 mutant keratinocytes.

To investigate the underlying mechanisms of enhancer re-distribution, a de novo motif scan was performed. We detected the p53 or p63 motif family as the top enriched motif among control-specific enhancers (Figure 4F; Table S4I), consistent with the specific enhancers were associated with gene upregulation in p63 mutant keratinocytes (Figure 4E).
impaired p63 binding and loss of active enhancers in p63 mutant keratinocytes (Figure 3A). In contrast, motif analyses of mutant-specific enhancers captured motifs of bZIP, TEA, high-mobility group (HMG), and Runt family TFs (Table S4M). These data suggest that aberrant recruitment of TFs induces gain of enhancers.

One scenario of aberrant recruitment of TFs may result from the abnormal upregulation of TFs in mutant keratinocytes. To assess this possibility, we examined differential expression of TFs. Among 1,581 examined TFs (Saeed et al., 2014), 106 and 103 TFs were down- and upregulated, respectively, in p63 mutant keratinocytes (Figure S6B). Interestingly, most downregulated TFs had p63 BSs nearby and therefore are potential p63 direct targets. In contrast, fewer upregulated TFs had p63 BSs. To predict candidate TFs that are potentially bound to mutant-specific enhancers, we used two criteria: (1) TFs whose binding motifs were enriched in mutant-specific enhancements (Figure 4F), and (2) TFs that were upregulated in all three p63 mutant keratinocytes at the proliferation stage (day 0) (Figure S6A). Among TFs that were consistently upregulated in mutant keratinocytes, RUNX1 and SOX4 belong to the TF families whose motifs were enriched in mutant-specific enhancers. We performed qRT-PCR validation to confirm the higher expression of SOX4 in p63 mutant keratinocytes (Figure S6B). ChiP-qPCR of SOX4 also confirmed a number of SOX4 BSs with higher binding signals in R304W mutant keratinocytes compared to control keratinocytes (Figure S6C). Interestingly, one of the candidate TFs RUNX1 is a known p63 target (Masse et al., 2012) and a potential p63 co-regulator. RUNX1 had many lost p63 BSs in the gene locus and was consistently upregulated in p63 mutant keratinocytes (Figures 3E and S6A).

Deregulated p63 and RUNX1 Cooperation Contributes to Transcriptional Rewiring in Mutant p63 Keratinocytes

To characterize p63 and RUNX1 co-regulation, we performed RUNX1 ChIP-seq in control keratinocytes. K-means clustering of RUNX1 BSs in combination with p63 binding and histone modification profiles showed that RUNX1 and p63 preferentially co-bound in active enhancer regions (Figure 5A). RUNX1 bound more frequently to active promoters marked by H3K4me3 (RUNX1-C4) than p63 (RUNX1-C1 and RUNX1-C3) (Figure 5A; Table S5A). Genes near the co-regulated enhancers (RUNX1-C1 and RUNX1-C3 [e.g., ITGB1 and EGFR]) were mainly involved in epidermis development and programmed cell death, respectively (Figures 5B and 5C). The observed upregulation of RUNX1 expression (Figure 5D) and loss of p63 binding in the RUNX1 gene body in p63 mutant keratinocytes (Figure 3E) indicated that deregulation of RUNX1 expression is probably due to loss of p63 control. To further confirm this, we performed small interfering RNA (siRNA) knockdown of p63 in control keratinocytes. Similar to the upregulated RUNX1 expression in p63 mutant keratinocytes, RUNX1 expression was significantly increased in p63 knockdown keratinocytes (Figure 5E).

To assess whether upregulated RUNX1 expression leads to its aberrant recruitment to mutant-specific enhancers in p63 mutant keratinocytes, we compared RUNX1 binding in control keratinocytes and R304W mutant keratinocytes (Figure 6A; Tables S5B–S5D). Among all RUNX1 BSs, there were 7,918 RUNX1 BSs with higher binding signals and 7,888 sites with lower binding signals in R304W mutant keratinocytes (Tables S5B–S5F). RUNX1 BSs with increased binding signals in R304W mutant keratinocytes were more often located in active enhancer regions accompanied by increased H3K27ac signals (Figure 6B; Table S5E), while those with decreased RUNX1-binding signals were more often located in promoter regions (Figures S7A and S7B; Table S5F).

To test whether increased RUNX1 expression is responsible for deregulating gene expression in p63 mutant keratinocytes, we performed RUNX1 knockdown in R304W mutant keratinocytes. As the proper RUNX1 expression level is important for cell proliferation (Hoi et al., 2010; Masse et al., 2012), we carefully titrated RUNX1 siRNA oligonucleotides to achieve a level of RUNX1 expression similar to that in control keratinocytes. 

RNA-seq analyses showed that the overall gene expression of siRUNX1-treated R304W mutant keratinocytes was more similar to that of control keratinocytes in PCA (Figure S7C). Among the 3,294 upregulated genes in R304W mutant keratinocytes, 276 genes were significantly rescued downregulated upon siRUNX1 (Figure 6C; Tables S5H and S5I), such as KRT7 and HESS (Figures 6E and S7D). Many of these genes are important for epidermal development and keratinocyte differentiation. The number of rescued genes was significantly higher than random expectations (276/3,294, hypergeometric test p < 7.272e–102; 218/3,184, hypergeometric test p < 4.305e–54). Partial but significant rescues by siRUNX1 were expected, as deregulated genes caused by loss of p63 binding in p63 mutant keratinocytes could not be rescued simply by RUNX1 knockdown. qRT-PCR and western blotting experiments validated rescued gene expression of KRT17, SMYD3, and KRT18 in R304W mutant keratinocytes upon siRUNX1 (Figures 6F and 6G). To further investigate whether siRUNX1 can rescue the enhancer landscape in R304W mutant keratinocytes, we performed H3K27ac ChiP-seq in these cells. Indeed, we observed a clear decrease of H3K27ac signals at enhancers that had higher H3K27ac signals and bound by RUNX1 in p63 mutant keratinocytes (Figure 6B; n = 6,035) in two biological replicates (Figures S6H and S7E), such as enhancers near NRP1, which is involved in angiogenesis (Figure 6J). Taken together, our data suggest that reversing upregulated RUNX1 expression can rescue deregulated gene expression and the enhancer landscape in p63 mutant keratinocytes.

DISCUSSION

The master regulator role of p63 in epidermal development has been established by many studies using in vitro and in vivo models. However, it remains unclear how p63 mutations affect the chromatin landscape and gene expression that contribute to diseases. In this study, we used EEC-patient-derived skin keratinocytes carrying heterozygous p63 DNA-binding domain mutations as the cellular model to characterize the global gene regulatory alteration. We showed that the epidermal cell identity was compromised in p63 mutant keratinocytes, as indicated by

Cell Reports 25, 3490–3503, December 18, 2018 3497
downregulated epidermal genes and upregulated non-epithelial genes. In addition to loss of p63 binding leading to reduced p63-bound active enhancers, we unexpectedly observed abnormally induced active enhancers that were bound by upregulated p63 co-regulators such as RUNX1. Reversing RUNX1 upregulation in p63 mutant keratinocytes rescued a part of the deregulated gene expression and altered enhancer landscape. Our data suggest an intriguing model whereby rewiring of the enhancer landscape, contributed by both loss of p63-bound active enhancers and gain of active enhancers induced by overexpressed p63 co-regulators, gives rise to gene deregulation and phenotypes of EEC syndrome (Figure 7).

Many epidermal genes were downregulated in mutant keratinocytes, consolidating the key role of p63 in epidermal development. In addition, upregulated mesenchymal genes and neuronal genes in mutant keratinocytes (Figure 1D) suggest that these cells have less defined epidermal cell fate. For example, mesodermal related genes such as ACTA2 and COL4A1, which were upregulated during epidermal commitment of p63-depleted embryonic stem cells (Shalom-Feuerstein et al., 2011), were also upregulated in p63 mutant keratinocytes (Table S1E). In agreement, gene coexpression network analyses showed that the scattered upregulated genes involved in extracellular matrix organization were connected with two modules (keratinization and nucleosome assembly) associated with downregulated genes in mutant keratinocytes (Figure 1E). This suggests that gene expression in p63 mutant keratinocytes deviates from the proper epidermal cell fate to establish a new differentiation direction through chromatin remodeling processes. In addition to directly regulating chromatin remodeling factors (Figure S2B), we showed in this study that p63 motif was most significantly enriched in active enhancers at early differentiation.

Figure 5. RUNX1 Is a Co-regulator and Target Gene of p63
(A) K-means clustering of RUNX1 BSs in control keratinocytes. RUNX1-binding signals are shown in heatmaps in a 4-kb window with summits of RUNX1 BSs in the middle (k = 4, metric = Pearson). Color intensity represents normalized read counts.
(B) GREAT-based GO biological process annotation of RUNX1 BSs in RUNX1 C1 and C3.
(C) Representative example of RUNX1 and p63 co-regulated genes ITGB1 and EGFR.
(D) Validation of RUNX1 gene expression by qRT-PCR and western blotting. In qRT-PCR analysis, relative gene expression was normalized to the reference gene hARP. Data are shown as mean ± SD, technical replicates n = 2; NS, p > 0.05; ***p < 0.001, two-way ANOVA. Actin was used as a loading control for the quantification of RUNX1 protein levels (shown as percentage) in western blotting.
(E) Gene expression analysis by qRT-PCR of TP63 and RUNX1 expression in TP63 knockdown (siTP63) compared to non-targeting siRNA (siNT) in control keratinocytes. Gene expression was normalized to the reference gene hARP. Data are shown as mean ± SD, technical replicates n = 2; ***p < 0.001, two-way ANOVA.

See also Figure S6 and Table S5.
Figure 6. Increased RUNX1 Binding Contributes to Gene Deregulation

(A) K-means clustering of RUNX1 BSs from control and R304W mutant keratinocytes are shown in heatmaps in a 4-kb window with summits of merged RUNX1 BSs in the middle (k = 2, metric = Pearson). Color intensity represents normalized read counts.

(B) Zoom-in re-clustering of increased RUNX1 BSs in R304W mutant keratinocytes showing that the majority of RUNX1 BSs with increased signals are enhancers.

(C) 276 significantly upregulated genes in R304W mutant keratinocytes that were rescued with siRUNX1 (p < 0.05). The significance of the overlap was calculated using a hypergeometric test (p < 7.272e-102). Expression (RNA-seq FPKM) of rescued genes under different conditions is shown as mean \pm SD. ***p < 0.001, t test. DAVID-based GO biological process annotation is shown at the bottom.

(D) 218 significantly downregulated genes in R304W mutant keratinocytes that were rescued with siRUNX1 (p < 0.05). The significance of the overlap was calculated with hypergeometric test (p < 4.305e-54).

(E) UCSC genome browser screenshots of RNA-seq data at the gene loci of KRT1 and KRT8 are shown as examples of rescued genes by siRUNX1.

(F) Gene expression analyses by qRT-PCR of RUNX1, KRT1, and SMYD3. Relative gene expression of these genes was normalized to the reference gene hARP. Data are shown as mean \pm SD, technical replicates n = 2; *p < 0.1, **p < 0.01, ***p < 0.001, two-way ANOVA.

(legend continued on next page)
stages (Figure 2E). These results support the pivotal role of p63 in regulating enhancers to activate epidermal differentiation genes, especially at the initiation stage.

In addition to its activator role for epidermal genes, it has been shown that p63 can function as a repressor to repress p21 (CDKN1A) through recruiting histone deacetylase 1 (HDAC1) and HDAC2 (LeBoeuf et al., 2010; Ramsey et al., 2011). RUNX1, a co-regulator of p63, is regulated by p63 in a complex fashion. Depending on the differentiation state of keratinocytes, p63 activates or represses RUNX1 expression (Masse et al., 2012). In our analyses, we detected upregulation of RUNX1 in proliferating mutant keratinocytes (Figures 3E and 5D), consistent with the repressor role of p63 for these genes. However, p63-bound enhancers at the RUNX1 locus were active enhancers, marked by H3K27ac, in contrast to classically defined repressed regions that are marked by H3K27me3 and H3K9me3. Furthermore, no H3K27me3 or H3K9me3 repression mark was found at any p63 BS (Kouwenhoven et al., 2015a), indicating that p63-bound enhancers are not repressed by polycomb- or heterochromatin-related mechanisms. To reconcile the apparent contradiction that p63 binds to active enhancers to repress gene expression, we speculate that p63 binds to these active enhancers to fine-tune expression of these genes by recruiting repressors such as HDACs. When p63 expression or function is compromised, such as in p63 mutant keratinocytes or p63 knockdown cells (Figures 5D and 5E), the fine-tuning or repression mechanisms are relieved, and the expression of these genes is enhanced (Figure 7B).

In this study, we showed that p63-binding loss and loss of active enhancers occurs at a genome-wide scale in patient keratinocytes carrying heterozygous EEC mutations (Figure 3A). This indicates that the mutant p63 protein has a dominant-negative effect on DNA binding over the wild-type p63 that is also present in the cell, likely because the p63 protein is DNA-binding competent as a tetramer (Dötsch et al., 2010; Serber et al., 2002). These genome-wide findings corroborate the dominant-negative model of EEC p63 mutations that has been proposed by several previous studies using in vitro approaches and allele-specific knockdown of p63 mutant.

Unexpectedly, we observed a large number of gained active enhancers in all three p63 mutant keratinocytes (Figures 4A, 4B, and S5A). These mutant-specific enhancers were enriched for motifs of TFs that normally cooperate with p63 in keratinocytes (Figure 4F). Many of these TFs were deregulated in p63 mutant keratinocytes (Figure S6) and are direct p63 targets, such as RUNX1 (Figures 3E and 5D). This indicates that rewiring of the transcriptional program is caused not only by loss of p63-bound active enhancers but also by an indirect effect of altered expression of p63 co-regulating TFs. ChIP-seq analyses revealed an altered RUNX1-binding profile in p63 mutant cells.
keratinocytes (Figure 6A). The increased RUNX1 binding was associated with increased H3K27ac signals (Figure 6B). Intriguingly, reversing RUNX1 expression upon siRUNX1 could partially rescue gene deregulation and the enhancer landscape (Figures 6C–6I), indicating that overexpression of RUNX1 in mutant keratinocytes contributes to gene deregulation and is at least partially responsible for the differentiation defects. Therefore, mutations in the p63 DNA-binding domain can give rise to an indirect gain-of-function effect by inducing aberrant binding of deregulated TFs to genome-wide enhancers (Figure 7C).

It should be noted that this gain-of-function model of p63 EEC mutations is different from the gain-of-function mechanism of a p63 mutation leading to phenotypically distinct Acro-dermatungual-lacrimal-tooth (ADULT) syndrome (Rinne et al., 2007) and p53 mutations that give rise to cancers. It has been shown that some p53 mutants involved in cancer cannot bind to bona fide p53 targets, but cooperate with normal p53 co-regulators and bind to ectopic genomic sites to activate abnormal gene expression (Zhou et al., 2014; Zhu et al., 2015), a classical paradigm of gain of function. The most apparent difference between p53 and p63 EEC mutations is that EEC mutant p63 does not seemingly bind to ectopic genomic loci, as we did not observe any novel p63 BS in mutant keratinocytes (Figure 3A). The indirect gain-of-function action of p63 mutations is due to the overexpression of p63-co-regulating TFs, such as RUNX1, and their aberrant recruitment to mutant-specific enhancers.

In the siRUNX1 experiment, we did not expect a full rescue in p63 mutant keratinocytes, as p63-binding loss could not simply be rescued by RUNX1 downregulation. With the same rationale, we also do not expect that overexpression of RUNX1 would fully mimic p63 mutant keratinocyte phenotypes. However, it would be interesting to test whether overexpression of RUNX1 in control keratinocytes can induce any differentiation defects. Furthermore, it is also of interest to further test the effect of other overexpressed p63 co-regulators such as TFs of the bZIP, TEA, and HM3 families (SOX4) (Figure 4F) in the EEC disease mechanism. It is conceivable that knockdown of multiple such overexpressed TFs may rescue differentiation defects of p63 mutant keratinocytes to a better extent.

In conclusion, we identified a rewired enhancer landscape as a common mechanism in EEC p63 mutant keratinocytes. The enhancer rewiring includes loss of p63-bound active enhancers that regulate epidermal genes (Figure 7A) and gain of enhancers bound by overexpressed TFs that are normally fine-tuned by p63 (Figures 7B and 7C). It is conceivable that in EEC p63 mutant keratinocytes, the chromatin environment is less tightly controlled, and deregulated TFs can therefore bind to more exposed enhancers. The rewired enhancer landscape gives rise to gene deregulation that contributes to the less-defined epidermal cell fate and skin phenotypes of the disease. Taken together, enhancer landscape rewiring contributes to the disease mechanism of p63 mutation and may be common to many other diseases.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:


**STAR METHODS**

**KEY RESOURCES TABLE**

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Huiqing Zhou (j.zhou@science.ru.nl; jo.zhou@radboudumc.nl).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human primary keratinocyte
All procedures for establishing and maintaining human primary keratinocytes were approved by the ethical committee of the Radboud university medical center (“Commissie Mensgebonden Onderzoek Arnhem-Nijmegen”). Informed consent was obtained from all donors of a skin biopsy. Primary keratinocytes were established previously from skin biopsies of three EEC syndrome patients carrying heterozygous mutations in the p63 DNA-binding domain, R204W (van Bokhoven et al., 2001), R279H (van Bokhoven and Brunner, 2002), and R304W (Celli et al., 1999), as well as of two healthy volunteers (Dombi23 and PCK19, referred to as Control) (Rheinwald and Green, 1977). Sex of the control keratinocytes is unknown because they are derived from the anonymous donors.

METHOD DETAILS

Cell culture
Primary keratinocytes were cultured in Keratinocyte Basal Medium supplemented with 100 U/mL Penicillin/Streptomycin, 0.1 mM ethanolamine, 0.1 mM O-phosphoethanolamine, 0.4% (vol/vol) bovine pituitary extract, 0.5 μg/mL hydrocortisone, 5 μg/mL insulin and 10 ng/mL epidermal growth factor. Medium was refreshed every other day. When cells were more than 90% confluent (day 0), differentiation was induced by depletion of growth factors in addition to cell contact inhibition, as described previously (Van Ruissen et al., 1996). Cells were collected at four differentiation stages, proliferation (day 0), early differentiation (day 2), mid differentiation (day 4), and late differentiation (day 7) for subsequent experiment. No mycoplasma contamination is found during cell culture.

RNA extraction and quantitative real-time reverse transcription PCR (RT-qPCR)
Total RNA was isolated using the NucleoSpin RNA kit and quantified with NanoDrop. cDNA synthesis from 1 μg freshly prepared total RNA was carried out using the iScript cDNA synthesis kit. Reverse transcription quantitative PCR (RT-qPCR) primers were designed using Primer3 to obtain exon spanning primers wherever possible. Each primer set has been tested for its linear amplification dynamic range. RT-qPCRs were performed in the CFX96 Real-Time system (Bio-Rad) by using iQ SYBR® Green Supermix according to the manufacturer’s protocol. The human acidic ribosomal protein (hARP) or glucuronidase beta (GusB) was used as the housekeeping gene for normalization. Differences in the expression of each gene during differentiation (relative expression) were calculated by 2ΔΔCt method (Livak and Schmittgen, 2001). Sequences of all RT-qPCR primers were provided in Table S1C.

Western blotting
A total of 12.5 μg protein was loaded for each sample. The actin antibody (1:100,000) was used to control equal protein loading. Protein extracts were run on SDS-PAGE and transferred to PVDF membranes using the NuPAGE system (Life Technologies). LumiGLO (Cell Signaling Technology Inc.) was used for chemiluminescent detection by the Bio-Rad Universal Hood Gel Imager (Bio-Rad Laboratories). Antibodies used in this study include LOR (1:2500), RUNX1 (1:50), K18 (1:500). All the original blots were provided in Table S6.
**RNA-seq and analysis pipeline**

RNA-seq experiment was performed as described previously (Kouwenhoven et al., 2015a) with the starting material of 500 ng total RNA, to obtain double-strand cDNA (ds-cDNA). After purification with the MinElute Reaction Cleanup Kit, 3 ng ds-cDNA was processed for library construction using KAPA Hyper Prep Kit according to the standard protocol except that a 15-minute USER enzyme incubation step was added before library amplification. The prepared libraries were quantified with the KAPA Library Quantification Kit, and then sequenced in a paired-ended manner using the NextSeq 500 (Illumina) according to standard Illumina protocols.

Sequencing reads were aligned to human genome assembly hg19 (NCBI version 37) using STAR 2.5.0 (Dobin et al., 2013) with default options. A detailed summary of RNA-seq data generated in this study was shown in Table S6A. For data visualization, wig-ToBigWig from the UCSC genome browser tools was used to generate bigwig files and uploaded to UCSC genome browser. Genes with the mean of DESeq2-normalized counts (“baseMean”) > 10 were considered to be expressed. Differential gene expression (adjusted P value < 0.05) and principal-component analysis were performed with the R package DESeq2 using read counts per gene (Love et al., 2014). Hierarchical clustering was performed based on log10 (FPKM+0.01). Functional annotation of genes was performed with DAVID (Huang da et al., 2009). For Weighted Gene Co-expression Network Analysis (WGCNA (Langfelder and Horvath, 2008)), only high variance genes (adjusted P value < 0.01, sum of baseMean > 100, 3162 genes) between control keratinocytes and p63 mutant keratinocytes during differentiation were included. WGCNA clustering within p63 mutant keratinocyte samples was performed using power of 26 and the minimum module size of 15. A total of 16 co-expression modules were identified based on gene co-expression patterns (Table S1J). To visualize the gene network organization, only nodes (genes) with connectivity weight > 0.1 were kept (Table S1K). Cytoscape (Smoot et al., 2011) was used for gene network visualization.

**ChIP-seq and analysis pipeline**

Chromatin for ChIP was prepared as previously described(Kouwenhoven et al., 2010). ChIP assays were performed following a standard protocol (Novakovic et al., 2016) with minor modifications. Antibodies against H3K27ac (1.2 µg), H3K4me3 (1 µg), H3K27me3 (1.5 µg), p63 (1 µg, recognizing the C-terminal z tail of p63) and RUNX1 (4 µg) were used in each ChIP assay. Resulted DNA fragments from four independent ChIP assays were purified and subjected to a ChIP-qPCR quality check. Afterward 5ng DNA fragments were pooled and proceeded on with library construction using KAPA Hyper Prep Kit according to the standard protocol. The prepared libraries were then sequenced using the NextSeq 500 (Illumina) according to standard Illumina protocols.

Sequencing reads were aligned to human genome assembly hg19 (NCBI version 37) using BWA (Li and Durbin, 2009). Mapped reads were filtered for quality, and duplicates were removed for further analysis. A detailed summary of ChIP-seq data generated in this study was shown in Table S6B. In addition, the bamCoverage script was used to generate and normalize bigwig files with the RPKM formula. The peak calling was performed with the MACS2 (Zhang et al., 2008) against a reference input sample from the same cell line with standard settings and a q value of 0.05. Only peaks with a P value < 10e-5 were used for differential analysis with MAAnorm (Shao et al., 2012). Association of peaks to genes and associated GO annotation were performed with GREAT (McLean et al., 2010). P values were computed with a hypergeometric distribution with FDR correction. k-means clustering and heatmap and band plot generation were carried out with a Python package fluff (Georgiou and van Heeringen, 2016). H3K27ac ChIP-seq analyses of control and mutant keratinocytes, including those performed in siRUNX1 experiments, were performed in duplicates. The ‘plot-Correlation’ function from the deepTools package was used and the Pearson correlation coefficient was calculated accordingly (Ramirez et al., 2014).

**ChromHMM analysis**

Chromatin states were characterized using ChromHMM v1.11 (Ernst and Kellis, 2012). The peak files from three tracks (H3K27me3, H3K4me3, and H3K27ac) across four stages were used as input. The six-emission state model was determined to be optimal which for the developmental stages (N = 4). Each element x (m, n) represents the chromatin state of interval m at stage n. For each chromatin group, occurrences were counted per stage N (Table S2C). The changes between stage N and N+1 were plotted pairwise using Sankey diagrams (http://sankeymatic.com/).

**Motif analysis**

Previously described p63scan algorithm was used for p63 motif evaluation(Kouwenhoven et al., 2010). HOMER (http://homer.ucsd.edu/homer/motif/) was used for motif scan against corresponding background sequences.

**siRNA nucleofection**

Nucleofection was performed as described previously (Mulder et al., 2012) using the Amaza 96-well shuttle system (Lonza, program FF113). In short, keratinocytes were harvested with Accutase® solution and resuspended in nucleofector buffer SF (Lonza). Each 20 µL transfection reaction contained 200,000 cells mixed with 2 µM validated siRNA (Silencer Select siRNAs, Ambion/Applied Biosystems). In siRUNX1 experiments, siRUNX1-1 oligo was used for the RNA-seq experiment and siRUNX1-2 oligo was used for the western blot experiment. Both siRUNX1 oligoes were used in the RT-qPCR and H3K27ac ChIP-seq experiments. After transfection,
the samples were incubated for 10 minutes at room temperature before resuspension in KBM and seeding at 50,000 cells per well (12 well plate). Medium was refreshed each day for the indicated periods till the end of experiments.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Data are expressed as mean ± standard deviation error of the mean unless otherwise specified. Dataset statistics were analyzed using the GraphPad Prism software. Differences under \( p < 0.05 \) were considered statistically significant, NS \( P \) value > 0.05, * \( P \) value < 0.05, ** \( P \) value < 0.01, *** \( P \) value < 0.001. Gene expression analysis by RT-qPCR was performed in biological duplicates (n ≥ 2); data are shown as mean ± standard deviation, two-way ANOVA. The comparison of gene expression (fold change) was analyzed with the unpaired t test. Hypergeometric test was performed with the online tool GeneProf. The comparison of gene expression after siRNA knockdown was performed with t test. Other statistical methods used in this study were specified in the Figure legends.

**DATA AND SOFTWARE AVAILABILITY**

To review dataset of control cells, go to GEO database (accession GSE98483) (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE98483). To review our complete dataset in Genome Browser, please go to https://genome.ucsc.edu/cgi-bin/hgTracks?hgS_doOtherUser=submit&hgS_otherUserName=Jieqiong%20QU&hgS_otherUserSessionName=hg19_p63_RUNX1_Jieqiong


All data supporting the findings of the study and in-house codes are available on request.