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Regulation of the dynamic chromatin architecture of the Epidermal Differentiation Complex is mediated by a c-Jun/AP-1-modulated enhancer

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Abstract

The Epidermal Differentiation Complex (EDC) locus comprises a syntenic and linear cluster of genes whose concomitant expression is a hallmark feature of differentiation in the developing skin epidermis. Many of the EDC proteins are cross-linked together to form the cornified envelope, an essential and discrete unit of the mammalian skin barrier. The mechanism underlying coordinate transcriptional activation of the EDC is unknown. Within the human EDC, we identified an epidermal-specific regulatory enhancer, 923, that responded to the developmental and spatio-temporal cues at the onset of epidermal differentiation in the mouse embryo. Comparative chromosomal conformation capture (3C) assays in proliferating and differentiated primary mouse keratinocytes revealed multiple chromatin interactions that were physiologically sensitive between the 923 enhancer and EDC gene promoters and thus depict the dynamic, chromatin topology of the EDC. We elucidate a mechanistic link between c-Jun/AP-1 and 923, whereby AP-1 and 923-mediated EDC chromatin remodeling is required for functional EDC gene activation. Thus, we identify a critical enhancer/transcription factor axis governing the dynamic regulation of the EDC chromatin architecture and gene expression and provide a framework for future studies towards understanding gene regulation in cutaneous diseases.

INTRODUCTION

The epidermis lies at the surface of the skin and provides the first line of defense against the external environment (Koster and Roop, 2007; Fuchs, 2009; Kubo *et al.*, 2012). Protecting against infection and inflammation, the epidermis comprises stratified layers of epidermal cells or keratinocytes that are individually surrounded by a cornified envelope and function as one of the essential core units of the skin barrier. To build the epidermal barrier akin to a “bricks-and-mortar” architecture, a basal keratinocyte in the innermost layer of the

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CONFLICT OF INTEREST

The authors state no conflict of interest.

epidermis receives an inductive cue to differentiate and orients its mitotic spindle perpendicularly to the basement membrane (Lechler and Fuchs, 2005). In doing so, an asymmetric cell division gives rise to a basal cell and a suprabasal daughter cell that is committed to terminal differentiation. As the keratinocyte completes the differentiation process, it is pushed upward and sequentially through the spinous and granular layers and finally to the outermost stratum corneum.

A hallmark feature for the execution of the terminal epidermal differentiation program is the expression of genes encoded by the Epidermal Differentiation Complex (EDC) locus (Mischke *et al.*, 1996; Zhao and Elder, 1997; Marshall *et al.*, 2001; de Guzman Strong *et al.*, 2010). The EDC (located on human 1q21 and mouse 3q) consists of 4 gene families that are associated with skin barrier formation: Small Proline Rich Region (*SPRR*), Late Cornified Envelope (*LCE*), filaggrin (*FLG*) and filaggrin-like (*FLG*-like), and *S100* genes. Genes encoded in the EDC are coordinately activated during embryonic epidermal differentiation (de Guzman Strong *et al.*, 2010). Exciting and recent studies in mice have identified a role for epigenetics in the regulation of the EDC during skin development (reviewed in Botchkarev *et al.*, 2012). Epidermal-specific loss of *Ezh2*, an essential component of the Polycomb repressor complex for histone modification, resulted in early epidermal differentiation owing to precocious recruitment of AP-1 transcription factor to the EDC for gene expression (Ezhkova *et al.*, 2009). Furthermore, *Satb1*, a higher-order genome organizer, was recently identified as a p63 target and binds to the EDC (Fessing *et al.*, 2011). *Satb1*^{-/-} mice exhibited alterations in the chromatin conformation of the EDC resulting in defects in keratinocyte-specific and EDC gene expression and hence abnormal epidermal morphology and further demonstrated a requirement for the proper establishment of higher order EDC chromatin structure and coordinated gene expression. This was further supported by confocal microscopy and computational modeling that identified distinct and active remodeling of the nuclear architecture associated with gene expression specifically in the terminally differentiated keratinocyte (Gdula *et al.*, 2013). Comprehensive studies of the β -globin locus control region and the X-inactivation center further support evidence for causality of chromatin folding and 3D genome organization with respect to gene regulation (Deng *et al.*, 2012; Nora *et al.*, 2012). However, despite these studies, the molecular mechanism(s) that underlie activation and coordinate regulation of the EDC genes at the nucleotide level are unknown. The synteny and linearity of the EDC across a wide range of mammalian species suggests a molecular mechanism originating at the proximal genomic level.

One plausible model is the activation of critical EDC expression by cis-regulatory elements during skin barrier formation. Comparative genomics and the Encyclopedia of DNA Elements (ENCODE) consortium that has annotated 80% of the genome attributed to function have greatly facilitated identification of regulatory elements (Dunham *et al.*, 2012). We previously identified many conserved non-coding elements (CNEs) within the human EDC that could synergistically or independently coordinate EDC gene expression (de Guzman Strong *et al.*, 2010). Approximately 50% of them exhibit regulatory activity. CNE 923 (approximately 923 kb from the transcriptional start site of *S100A10*, the most 5' EDC gene) displayed the strongest enhancer activity in proliferating and differentiated

keratinocytes in our functional screen. This result corroborated with transgenic reporter mice that demonstrated epidermal-specific enhancer activity for CNE 923 *in vivo*. This led us to hypothesize a role for 923 in the coordinate transcriptional activation of the EDC.

Here, we tracked the activity of CNE 923 during development in transgenic mice and identified spatio-temporal sensitivity for 923 that coincides with the onset and patterning of epidermal differentiation. Chromatin conformation capture (3C) studies were employed to determine the physical interactions between 923 and EDC gene promoters and revealed multiple chromatin spatial interactions surrounding 923. Comparative 3C analyses between proliferating and differentiated primary keratinocytes revealed a dynamic 923-centric EDC chromatin domain associated with concomitant EDC gene expression. Comparative genomics and genetic studies identified an AP-1 transcription factor binding site within 923 that was required for enhancer activity. We determine that the AP-1 binding site in 923 is functionally relevant, as pharmacological inhibition of AP-1 in calcium-induced keratinocytes repressed EDC gene expression and was associated with aberrant chromatin remodeling and loss of c-Jun/AP-1 binding to 923. Thus, our results provide a framework to examining molecular mechanisms that link DNA sequence to chromatin architecture and biological functions relevant to development and disease.

RESULTS

923 is an epidermal-specific enhancer responsive to the spatial and temporal cues in the developing mouse epidermis

We previously observed that human CNE 923 exhibited epidermal-specific enhancer activity, driving *lacZ* expression in G0 transgenic mice (923-*hsp68-lacZ*) analyzed only at mouse embryonic day (E)16.5 (de Guzman Strong *et al.*, 2010). However, the onset and the spatial and temporal patterning for 923 during mouse embryonic development were unclear. To address this, we generated additional 923-*hsp68-lacZ* transgenic mice. 923 enhancer activity (as measured by *lacZ* transcript levels) was detected as early as E15.5 in the developing mouse epidermis (Figure 1e), and coincided with the onset of early epidermal differentiation as demonstrated by positive Keratin 1 (K1) expression (Figure 1d) and activation of involucrin (*Ivl*), *Flg*, and loricrin (*Lor*) expression (Figure 1e). X-galactose reactivity (blue) was not detectable at E15.5 in whole-mount or cross-sections of the epidermis (Figure 1a, 1b) owing to the lack of β -galactosidase protein expression. At E16.5 and E17.5, we observed expression of 923 enhancer activity correlating with the patterning of barrier acquisition (dorsal to ventral migration pattern (Hardman *et al.*, 1998)) (Figure 1a). 923 β -galactosidase enhancer activity was localized to the spinous to stratum corneum layers of the dorsal epidermis at E16.5 and E17.5 (Figure 1b). Failure to detect β -galactosidase activity on the dorsal epidermis of E17.5 whole-mount embryos is consistent with barrier acquisition that precludes substrate penetration to detect β -galactosidase activity. Together, the data supports the responsiveness of 923 enhancer activity to the spatio-temporal cues of the developing mouse epidermis.

The dynamic chromatin architecture of the EDC

We previously identified DNaseI hypersensitivity for 923 (de Guzman Strong *et al.*, 2010) and note ENCODE-annotated H3K4me1 histone modification mark in proliferating primary human keratinocytes that independently tags functional enhancers (Ernst *et al.*, 2011) (Figure 3a). Enhancers are known to form long-range physical interactions with target gene promoters for activation (Tolhuis *et al.*, 2002). Given these observations and the spatio-temporal sensitivity in the developing mouse epidermis, we hypothesized a role for 923 in mediating the chromatin conformation of the EDC.

To test this hypothesis, 3C assays coupled with quantitative PCR (qPCR) (Hagege *et al.*, 2007) were employed in proliferating and differentiated primary mouse keratinocytes to detect physical chromatin interactions at the sub-megabase level between the endogenous mouse 923 ortholog and the EDC genes. 923 formed multiple interactions with EDC gene promoters (9 out of 46 tested queries, *Sprrr2a1*, *Sprrr2d*, *Sprrr2f*, *Sprrr1b*, *Sprrr3*, *Ivl*, *Lce1b*, *Lce1a2*, and *Crct1* [cysteine-rich C-terminal 1]) in proliferating keratinocytes despite the lack of EDC gene expression relative to the differentiated keratinocytes (Figure 2a, 2b). In differentiated keratinocytes, a reconfiguration of the EDC chromatin state was identified and was associated with eleven 923-mediated chromatin spatial interactions between a HindIII fragment 5' of *Lce3b* and *S100a6*, *Sprrr2a1*, *Sprrr2b*, *Sprrr3*, *Sprrr4*, *Ivl*, *Lce6a*, *Lce1b*, *Lce1e*, and *Crct1* gene promoters that was relatively consistent with their expression during terminal differentiation (Figure 2a, 2b). In comparison to the proliferating cells, the observed interactions in differentiated keratinocytes that were lost included *Sprrr2d*, *Sprrr2f*, and *Sprrr1b*, and *Lce1a2* (within 250 kb of 923) as well as a gain with 5' of *Lce3b*, *S100a6*, *Sprrr2b*, *Sprrr4*, *Lce6a* and *Lce1e*. Notably, the gain of 923's interaction with *S100a6* was located 2Mb away across a gene desert and observed higher frequencies of interactions with *Sprrr2a1* and *Sprrr2b* (both >250kb away from 923). All of the above genes with the exception of *Sprrr2f* are expressed by E15.5 as previously described in the newly differentiated dorsal epidermis of the developing mouse embryo (de Guzman Strong *et al.*, 2010). The genes for which there were gains of interactions in differentiated keratinocytes had similar if not increased levels of expression at E16.5 relative to E15.5. These observations further identify a longer range EDC chromatin topology in differentiated cells (Figure 2a) and are underestimated given the modest coverage associated with 3C methodology and as not all keratinocytes completely differentiated. In sum, our results support both shared and unique chromatin spatial interactions between the 923 epidermal-specific enhancer and EDC gene promoters in proliferating and differentiated primary mouse keratinocytes that represent the dynamic chromatin architecture of the EDC.

AP-1 transcription factor binding is required for 923 enhancer activity and EDC gene expression

To elucidate the molecular mechanism underlying 923 enhancer activity, we performed a bioinformatics search to identify core transcription factor binding sequences within 923 that are responsible for driving functional enhancer activity. Assessment of core enhancer functional activity in 4 PhastCons blocks were prioritized and represent highly conserved sequences between 28 vertebrate species and therefore likely to impart function (Siepel *et al.*, 2005; Alexander *et al.*, 2010) (Figure 3b). Deletions of blocks 1 and 4 at the 5' and 3'

ends of 923 significantly decreased luciferase activity (Figure 3c) thus demonstrating a functional role for these blocks for 923 enhancer activity. As deletion of block 1 resulted in the greatest and more significant decrease in enhancer activity, we prioritized a search for transcription factor binding motifs within block 1. We identified an AP-1 transcription factor binding site (Figure 4a) and hypothesized that AP-1 is required for 923 enhancer activity. Deletion of the AP-1 binding site by site-directed mutagenesis led to a significant decrease in 923 enhancer activity under proliferating and differentiated conditions (Figure 4b) thus demonstrating a functional role for AP-1 to mediate 923 enhancer activity in both physiological states.

To examine a role for AP-1 activity with respect to 923 enhancer activity and EDC gene activation, AP-1 binding was inhibited by Tanshinone IIA (TanIIA) treatment in primary mouse keratinocytes induced to differentiate (calcium induction) (Ezhkova *et al.*, 2009). Calcium-induced keratinocytes treated with TanIIA exhibited repressed EDC gene expression (Figure 5b). Moreover, chromatin immunoprecipitation for AP-1 (c-Jun) revealed that the repression was associated with the loss of AP-1 binding to block 1 in 923 in TanIIA-treated, calcium-induced keratinocytes compared to mock controls (Figure 4c). Together, the data demonstrates a requirement for AP-1 in activating EDC gene expression, specifically associated with functional AP-1 binding within the 923 enhancer *in vivo*.

The c-Jun/AP-1/923 axis regulates the EDC transcriptome by modulating the chromatin architecture

To identify the mechanism by which AP-1 inhibition represses EDC gene expression, we returned to 3C assays to examine the chromatin conformation of the EDC with respect to 923 in the context of AP-1 pharmacological inhibition. The chromatin conformation was assessed at 48 hours post-TanIIA treatment to best ascertain the direct effects of AP-1 inhibition as opposed to secondary effects beyond 48 hours. Although there was no significant differences in the number of 923-mediated chromatin interactions in TanIIA-treated vs. mock-treated differentiated keratinocytes (11 vs. 12), only 6 interactions were shared (*Sprr2a1*, *Sprr3*, *Ivl*, *Lce1d*, *Lce1e*, and 5' of *Lce3b*) and are close by (within 325 kb) (Figure 5a). Moreover, in TanIIA-treated keratinocytes, there was a loss of 923 interactions with *Sprr2b*, *Sprr2d*, *Sprr1b*, *Lce6a*, and *2310050C09Rik* and a gain of spatial interactions with *Sprr1a*, *Lce1b*, and *Lce1c* and at the relatively extreme 5' and 3' ends of the EDC reaching as far away as >2 Mb and 866 kb in *S100a13* and *Tchh* (trichohyalin), respectively. Despite the gain of chromatin interactions, no appreciable differences in gene expression for *Sprr1a*, *Lce1b*, *Lce1c*, *S100a13* and *Tchh* were observed. It appears that AP-1 pharmacological inhibition resulting in decreased c-Jun/AP-1 binding at 923 (Figure 4c) was not sufficient to completely abrogate all chromatin spatial interactions within the EDC that could be maintained by other transcription factors. However, our data supports a role for AP-1 in mediating proper 923-centric EDC chromatin conformation for EDC gene activation.

DISCUSSION

Although 3C assays and recent high-throughput genomic studies have enhanced our understanding of chromatin architecture and gene regulatory modules (de Wit and de Laat, 2012), the mechanisms governing chromosomal spatial interactions are poorly understood. Our studies identify a molecular mechanism describing transcription factor/enhancer modulation of a cluster of genes, namely AP-1 in the EDC architecture required for epidermal differentiation. We translate a “linear” interpretation (de Wit and de Laat, 2012) of the keratinocyte genome from our studies and ENCODE and prioritize functional studies on 923 to elucidate the 3D structure or chromatin interactions within the EDC. Our study demonstrates that 923 displays epidermal-specific enhancer activity that tracks with spatial and temporal patterns of epidermal differentiation and barrier formation during normal mouse development. We further elucidate an association of 923 with the coordinate activation of EDC genes during epidermal differentiation based on 3C assays that identified nearby chromatin spatial interactions between 923 and several EDC genes located as far as 2Mb away. Specifically, we observe a chromatin state of the EDC in proliferating keratinocytes that are marked by fewer cis-spatial interactions with 923 and do not express EDC genes. By contrast, the chromatin state of the EDC remodels in differentiated keratinocytes that express many EDC genes, as demonstrated by greater observed 923-mediated interactions with EDC gene promoters. Although *in vivo* knockout studies for 923 are beyond the scope of this study and would address the functional role of 923 as an intriguing locus control region (LCR) for the EDC during mouse development, our data nevertheless support a functional role for 923 in mediating the chromatin spatial interactions of the EDC. In support of this model, a recent study evaluating a ZF-mediated Ldb1/ β -globin LCR physical tethering to the β -globin promoter in GATA-1 deficient erythroid cells demonstrated causality of chromatin spatial interactions to gene transcription (Deng *et al.*, 2012). The requirement of the AP-1 binding site for 923 enhancer activity in both proliferating and differentiated states and the repression of EDC expression by pharmacological inhibition of AP-1, suggest that the AP-1/923 axis is an important mechanism to coordinate the EDC transcriptome. A bioinformatic analysis of transcription factor binding sites in 923 suggests additional putative transcription factor binding sites including CREB within PhastCons Block 4 that could likely contribute to 923 enhancer function.

It is interesting to note that even in a proliferative state, the loss of the AP-1 binding site in 923 led to a significant decrease in enhancer activity and chromatin interactions were observed between EDC gene promoters and 923. A majority of AP-1 members are expressed in basal keratinocytes with a more restricted expression of specific AP-1 members in the suprabasal layers (Jochum *et al.*, 2001). AP-1 is known to translate extracellular signals to a transcriptional response (Schonthaler *et al.*, 2011). Together, these observations and our data suggest a role for AP-1 (c-Jun) in modulating 923 activity in basal keratinocytes by folding the EDC chromatin state, and for which activation of EDC transcription in the terminally differentiated keratinocyte is driven by the specificity of an AP-1 homo/heterodimeric partner. Although the epidermis with targeted loss of c-Jun (Zenz *et al.*, 2003) and c-Jun/JunB (Guinea-Viniegra *et al.*, 2009) exhibited normal skin

morphology (that could be attributed to compensatory mechanisms to correct for skin barrier (Koch *et al.*, 2000; Huebner *et al.*, 2012)), the epidermal-specific c-Jun/JunB and JunB knockouts exhibited inflammatory defects owing to interleukin 6 (IL-6) and tumor necrosis factor α (TNF α expression respectively. Together, these observations lay the groundwork for investigations linking the role of chromatin architecture in skin barrier as well as inflammation.

A recent study revealed that chromatin architectural proteins may play a greater role than transcription factors in mediating promoter-enhancer interactions (Phillips-Cremins *et al.*, 2013). Genome-wide analysis of chromatin interactions lost during the differentiation of embryonic stem cells (ESCs) to neural progenitor cells (NPCs) unveiled a strong colocalization of the architectural proteins Mediator and cohesin to the ESC-specific interactions, a partial colocalization of the Oct4/Sox2/Nanog (OSN) transcription factors with Mediator and cohesin, and far fewer interactions that were enriched for only transcription factors. The roles of Mediator and cohesin in mediating enhancer-promoter interactions were validated by the abrogation of an interaction between *Olig1* and a putative ESC-specific enhancer in Mediator and cohesin knockdown cells. This data suggests that a proportion of AP-1-mediated 923 interactions may in fact be dependent on the presence of chromatin architectural proteins that are able to maintain these interactions even when AP-1 activity is inhibited, while a smaller proportion of interactions are mediated solely by AP-1.

A genomic study has recently elucidated the chromatin topologies of the human and mouse genome that are marked by distinguishing structural topological domains (Dixon *et al.*, 2012). These domains are stable, highly conserved, and often demarcated by boundaries enriched for CCCTC-binding factor (CTCF), housekeeping genes, tRNAs, and short interspersed nuclear element (SINE) transposons. That the EDC is also syntenic and linear across a wide range of metatherian genomes (de Guzman Strong *et al.*, 2010) suggests a model for the EDC as a single distinct topological domain. Of note, ENCODE-annotated CTCF elements flank the gene families within and just outside the EDC, suggesting a role for CTCF as boundary elements for a putative EDC topology (Ernst *et al.*, 2011). More high-throughput and higher resolution characterization of the EDC chromatin conformation using 4C or 5C methodology would certainly address this hypothesis as well as to depict additional chromatin interactions in an unbiased manner.

The EDC has been implicated in atopic dermatitis and psoriasis (Giardina *et al.*, 2006; Palmer *et al.*, 2006; Sandilands *et al.*, 2007; de Cid *et al.*, 2009; Esparza-Gordillo *et al.*, 2009; Hirota *et al.*, 2012; Paternoster *et al.*, 2012). Specifically, discovery of *FLG* mutations initially in ichthyosis vulgaris (Smith *et al.*, 2006) and particularly in atopic dermatitis (AD) (Palmer *et al.*, 2006) and other atopic diseases such as asthma and allergic rhinitis (Irvine *et al.*, 2011), highlights the importance of how even one of the EDC components broadly affects prevalent allergic diseases. Even at the exclusion of common *FLG* mutations, genetic association to the EDC continues to persist in atopic dermatitis suggesting additional genetic variants within the EDC (Morar *et al.*, 2007; Esparza-Gordillo *et al.*, 2009). Our analysis provides a genomic framework for which we can begin to interrogate regulatory element variants as causative in these diseases. Although discovery of causative variants is prioritized in genomic regions in linkage disequilibrium (LD) with genome-wide association

study (GWAS)-identified SNPs, our chromatin experiments suggest discovery of causative SNPs that are not in LD but are in “physical proximity” and *trans* to GWAS-identified SNPs.

MATERIALS AND METHODS

Mice

h923-*hsp68-lacZ* reporter FVB/N mice were housed in pathogen-free, barrier facilities at NIH (Bethesda, MD) and Washington University School of Medicine (St. Louis, MO). All animal procedures were approved by the NHGRI Animal Care and Use Committee and Washington University Division of Comparative Medicine Animal Studies Committee. All animal work was conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Morning observation of a vaginal plug was designated as embryonic day (E) 0.5.

LacZ staining and Immunohistochemistry

Whole-mount embryos and frozen OCT sections were stained overnight for β -galactosidase activity as previously described (de Guzman Strong *et al.*, 2010) and imaged on a Nikon SMZ 1500 Stereomicroscope and a Nikon Eclipse 80i brightfield microscope (Nikon, Tokyo, Japan), respectively. Primary antibodies used for immunofluorescence are rabbit K1 (17iKSCN, 1:500), rabbit FLG (5C-KSCN, 1:500) and chicken K14 (5560, 1:1000) (courtesy of J. Segre). Secondary antibodies used were goat anti-rabbit (Alexa Fluor 488, 1:500) and goat anti-chicken (Alexa Fluor 594, 1:1000) IgG antibodies (Life Technologies, Frederick, MD). Sections were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) prior to permeabilization with 0.1% Triton X-100 and subsequent antibody incubation. Sections were counterstained with SlowFade Gold antifade reagent with DAPI (Life Technologies, Frederick, MD) prior to fluorescent imaging on a Zeiss AxioImager Z1 and captured with AxioCam MRc and Axiovision software (Carl Zeiss, Stockholm, Sweden).

Chromosomal conformation capture (3C) assay

Primary keratinocytes were isolated from newborn mice as previously described (Lichti *et al.*, 2008) and plated under proliferating or differentiating (2.0 mM Ca^{2+}) conditions in custom keratinocyte media. 3C assays were performed as previously described (Hagege *et al.*, 2007). Briefly, approximately 10 million cells were harvested at 72 hours post-calcium treatment and cross-linked with 2% formaldehyde prior to overnight HindIII (New England Biolabs, Ipswich, MA) digestion. Each 3C library was assessed for efficient digestion efficiency by qPCR, and then further ligated with T4 DNA ligase (New England BioLabs, Ipswich, MA) overnight, decrosslinked using Proteinase K (IBI Scientific, Peosta, IA), and purified by phenol/chloroform (Life Technologies, Frederick, MD). Each putative physical interaction between 923 and an EDC gene promoter (detected by head-to-head [same strand] primer pair within 50–150 bp of a HindIII cut site and designed in NCBI37/mm9, Table S1) was detected by qPCR (Quantitect SYBR Green, Qiagen, Chatsworth, CA, ViiA7, Applied Biosystems, Foster City, CA) in triplicate in 3C libraries of equivalent concentrations. C_T values for each measured interaction were normalized against C_T values across an uncut

region and a pan-cell *Ercc3* chromatin interaction (Hagege *et al.*, 2007). Positive 3C interactions represent a minimum average of at least 1 replicate from 2 independent 3C libraries as a criteria to best exclude false positive and random events.

RNA isolation and analysis

RNA-seq—RNA isolation and analysis is described in supplementary methods. RNA-seq data have been deposited in the NCBI SRA under accession number PRJNA210793.

qRT-PCR—Real-time qPCR on cDNA (generated using SuperScript II reverse transcriptase (Life Technologies)) using SYBR Green was performed in triplicate (ABI ViiA7, Foster City, CA) and normalized to β_2 -microglobulin. Only C_T values with single peaks on melt-curve analyses were included. Primers are listed in Table S2.

Luciferase assay

923 deletion constructs were cloned synthetically (IDT) or by PCR amplification. AP-1 site deletion was generated by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Agilent, Palo Alto, CA). All clones were verified by Sanger sequencing. Dual luciferase assays were performed in duplicate as previously described (de Guzman Strong *et al.*, 2010).

Transcription factor binding prediction

Transcription factor binding sites were predicted by aligning each PhastCons block sequence (with a relative profile score threshold of 80%) against the JASPAR CORE database of transcription factor binding profiles (<http://jaspar.genereg.net/>) (Byrne *et al.*, 2008).

AP-1 binding inhibition assay

Primary keratinocytes grown under differentiating conditions (2.0mM Ca^{2+}) were treated with either 1.0mg/L Tanshinone IIA (Biomol, Plymouth Meeting, PA) or DMSO (mock) during calcium shifting 24 hours after plating. Cells were harvested at 2 days post-calcium treatment for 3C assays and RNA isolation for gene expression analysis by real-time qPCR.

Chromatin Immunoprecipitation

Chromatin (approximately 5×10^6 cells) was sonicated (Bioruptor XL [Diagenode, Denville, NJ]) prior to immunoprecipitation with rabbit antibodies: c-Jun (AP-1) (Abcam, Cambridge, MA; Cat. # ab31419) and IgG (Millipore, Billerica, MA; Cat. # 12-370) antibodies bound to Dynabeads Protein A (Life Technologies). ChIP Primers are listed in Table S4.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

EDC	Epidermal Differentiation Complex
3C	chromosomal conformation capture
SPRR	Small Proline Rich Region
LCE	Late Cornified Envelope
FLG	filaggrin
ENCODE	Encyclopedia of DNA Elements
CNEs	conserved non-coding elements
E	embryonic day
qPCR	quantitative PCR
TanIIA	Tanshinone IIA

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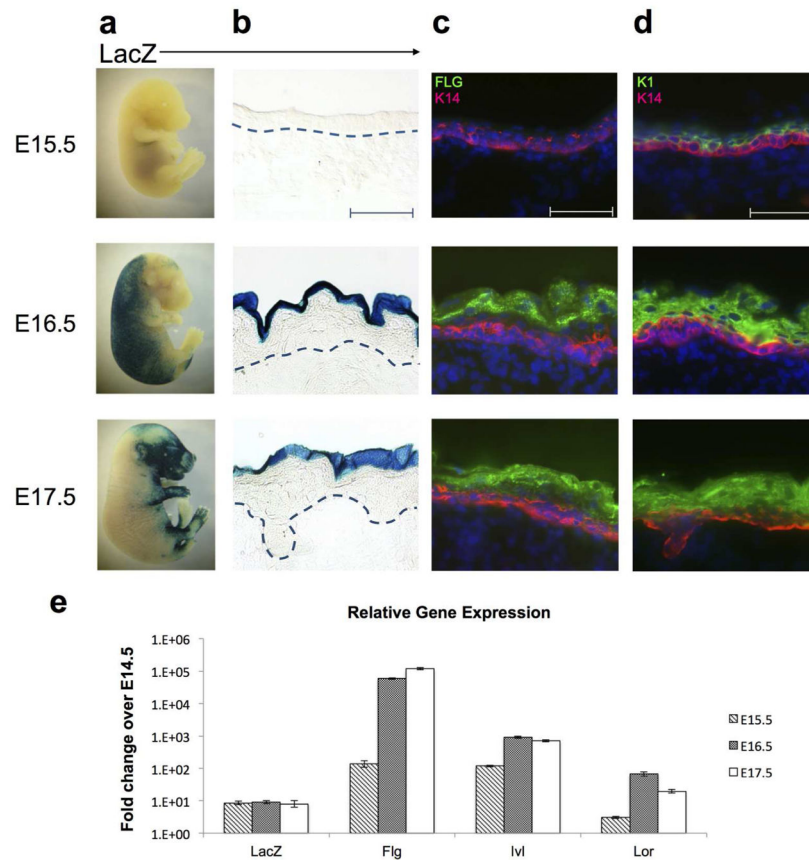


Figure 1. 923 is sensitive to spatio-temporal cues during mouse embryonic epidermal development

(a) Whole mount lacZ staining of 923-*hsp68-lacZ* mice demonstrates 923 activity (β -galactosidase/X-gal blue reactivity) following spatio-temporal patterns of epidermal barrier formation in the developing embryo by initial observation of activity at E16.5 (dorsal) that migrates ventrally by E17.5. (b) 923 activity localizes to granular and spinous layers of embryonic dorsal epidermis with corresponding (c) filaggrin (FLG) and (d) keratin1 (K1) immunofluorescent staining (green). Keratin14, K14 (red) marks basal keratinocytes, 20X. Dotted lines, basement membrane. Experiments observed in 2 independent mice. (e) 923 activity (qPCR, lacZ transcript) is noted at E15.5, E16.5, and E17.5 dorsal epidermis, concomitant with *Flg*, involucrin (*Ivl*), and loricrin (*Lor*) transcription relative to E14.5. Error bars represent mean \pm SD. Scale bar = 50 μ m.

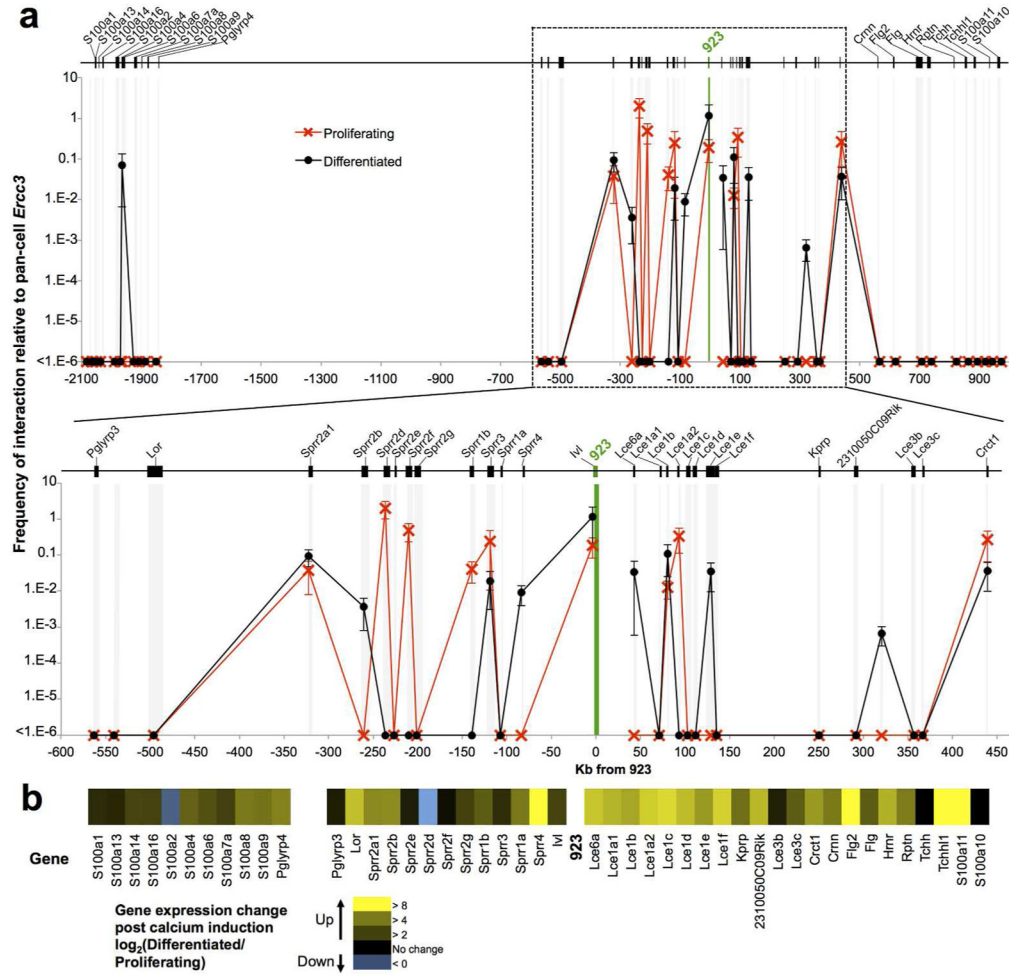


Figure 2. The Chromatin State of the Mouse EDC is Dynamic

(a) Semi-quantitative chromosomal conformation capture (3C-qPCR) assays were performed on primary mouse keratinocytes (proliferating and differentiated). Peaks = frequencies of physical interactions observed between HindIII restriction fragments containing 923 (green bar) and queried restriction fragments containing/neighboring EDC gene promoters (black bars + gray lines) or proximal sequences (gray lines) relative to a cell-ubiquitous *Ercc3* control. HindIII fragment (*5'* of *Lce3b*) represents a chromatin interaction that was not enhancer-promoter specific. Peaks, average of at least 2 biological replicates. Error bars represent the mean \pm SEM. (b) EDC gene expression heatmap (fold change) in differentiated vs. proliferating keratinocytes based on analysis of 3 pairwise RNA-seq libraries, green/yellow = upregulated, blue = downregulated, black = no change, (Table S3).

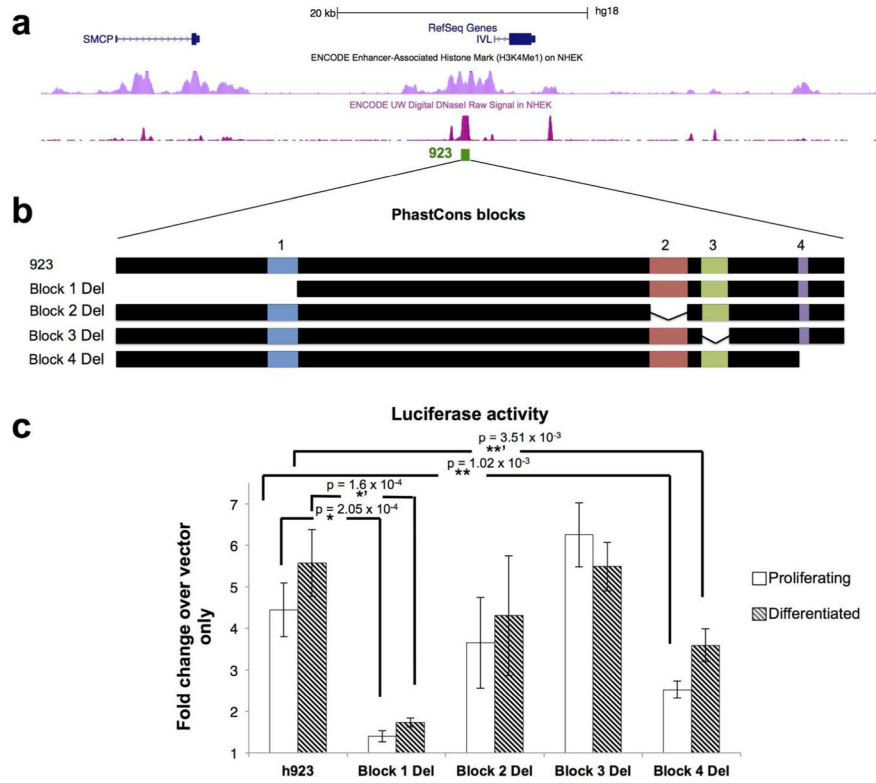


Figure 3. PhastCons (vertebrate conserved) blocks 1 and 4 are required for 923 enhancer activity
(a) 923 correlates with ENCODE-annotated strong enhancer (H3K4me1) and DNaseI hypersensitivity clusters in normal human epidermal keratinocytes (NHEK) and 4 PhastCons blocks (UCSC, hg18, block 1 (23 bp): chr1:151145182-151145204; block 2 (34 bp): chr1:151145525-151145558; block 3 (26 bp): chr1:151145572-151145597; block 4 (9 bp): chr1:151145661-151145669). **(b)** Individual deletion (del) of each PhastCons block reveals that **(c)** blocks 1 and 4 are required for enhancer activity based on transient dual-luciferase reporter assays in proliferating and differentiating keratinocytes (n=2). *, $P = 2.05 \times 10^{-4}$, *, 1.6×10^{-4} , **, $P = 31.02 \times 10^{-3}$, **/, 3.51×10^{-3} . P-values are based on a two-tailed t-test. Error bars represent mean \pm SE.

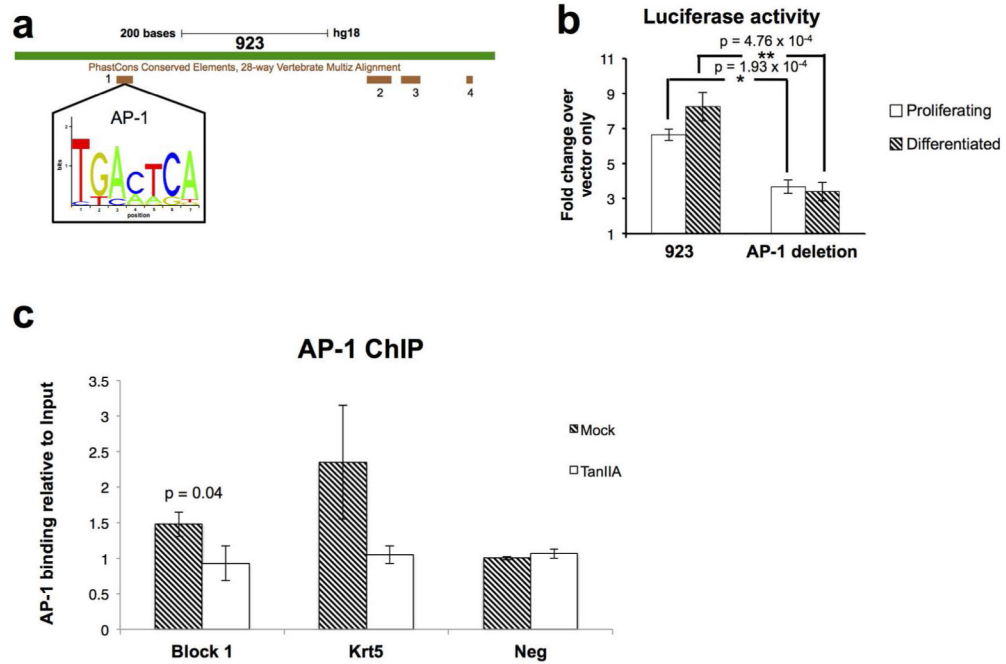


Figure 4. c-Jun/AP-1 transcription factor binding to PhastCons block 1 is required for 923 enhancer activity

(a) PhastCons block 1 contains a conserved AP-1 transcription factor binding sequence (UCSC, hg18, chr1:151145183-151145189). (b) Deletion of the AP-1 binding site significantly decreased luciferase and hence enhancer activity (n=3), * $P=1.93 \times 10^{-4}$, ** $P=4.76 \times 10^{-4}$ (two-tailed t-test). (c) Chromatin immunoprecipitation on differentiated cells demonstrates 1.6-fold decrease in AP-1 binding to PhastCons block 1 in TanIIA-treated versus mock-treated cells ($P=0.04$). The positive control, an ENCODE-annotated site within Keratin5 (*Krt5*), displayed 2.3-fold decrease in AP-1 binding in TanIIA-treated versus mock-treated cells ($P=0.06$). A negative control (no AP-1 site) showed no difference between mock-treated and TanIIA-treated cells (n=2). P-values based on a one-tailed t-test.

