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Chromatin-Bound I κ B α Regulates a Subset of Polycomb Target Genes in Differentiation and Cancer

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SUMMARY

I κ B proteins are the primary inhibitors of NF- κ B. Here, we demonstrate that sumoylated and phosphorylated I κ B α accumulates in the nucleus of keratinocytes and interacts with histones H2A and H4 at the regulatory region of *HOX* and *IRX* genes. Chromatin-bound I κ B α modulates Polycomb recruitment and imparts their competence to be activated by TNF α . Mutations in the *Drosophila* I κ B α gene *cactus* enhance the homeotic phenotype of *Polycomb* mutants, which is not counteracted by mutations in *dorsal/NF- κ B*. Oncogenic transformation of keratinocytes results in cytoplasmic I κ B α translocation associated with a massive activation of *Hox*. Accumulation of cytoplasmic I κ B α was found in squamous cell carcinoma (SCC) associated with IKK activation and *HOX* upregulation.

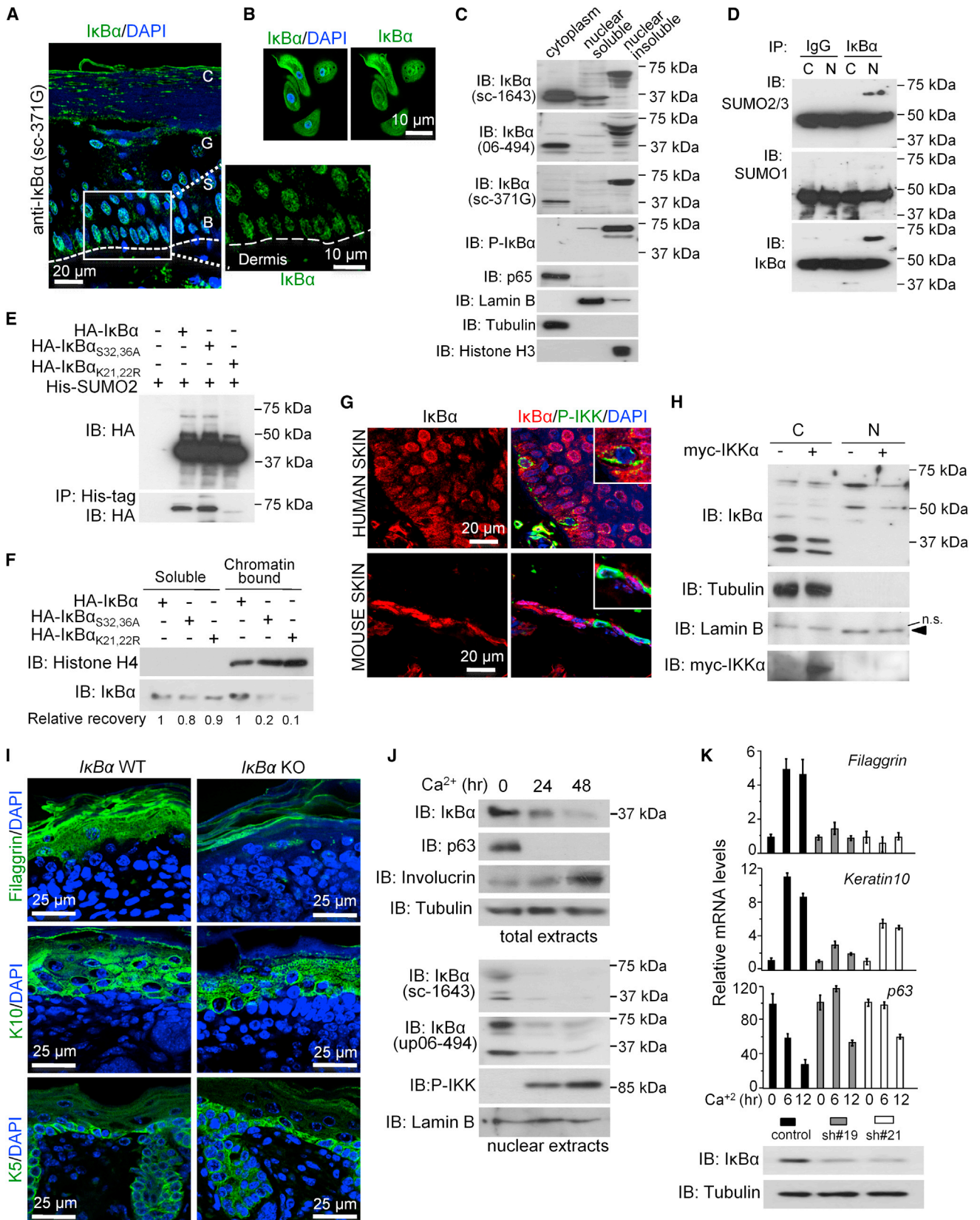
INTRODUCTION

NF- κ B plays a crucial role in biological processes, such as native and adaptive immune responses, organ development, cell proliferation, apoptosis, or cancer (Naugler and Karin, 2008; Vallabhapurapu and Karin, 2009). NF- κ B activation depends on the IKK-mediated degradation of the NF- κ B inhibitors,

I κ B proteins, that takes place in the cytoplasm and results in the translocation of the NF- κ B transcription factor to the nucleus, where it activates gene expression. Recent studies demonstrate the existence of alternative nuclear functions for regulatory elements of the pathway (reviewed in Espinosa et al., 2011), but their biological implications remain poorly understood. Recently, it has been demonstrated that nuclear I κ B β binds the promoter

Significance

Up to now, the only known function for I κ B α is as a repressor of NF- κ B. We now demonstrate that I κ B α plays an alternative role in the chromatin through binding to histones H2A and H4 and regulation of polycomb-dependent transcriptional repression. Consistent with the functional relevance of polycomb-associated I κ B α function, *Cactus/I κ B α* deficiency affects *Drosophila* development. In mammals, we found that I κ B α regulates keratinocyte differentiation and transformation associated with altered *Hox/IRX* expression.



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of NF- κ B target genes following lipopolysaccharide (LPS) stimulation to prevent I κ B α -mediated inactivation, thereby sustaining cytokine expression in immune cells (Rao et al., 2010). Numerous studies have reported nuclear translocation of I κ B α (Aguilera et al., 2004; Arenzana-Seisdedos et al., 1997; Huang and Miyamoto, 2001; Wuerzberger-Davis et al., 2011) and various partners for nuclear I κ B α , including histone deacetylases (HDACs) and nuclear corepressors, have been identified (Aguilera et al., 2004; Espinosa et al., 2003; Viatour et al., 2003). In fibroblasts, nuclear I κ B α associates with the promoter of Notch target genes correlating with their transcriptional repression, which is reverted by TNF α (Aguilera et al., 2004). Nevertheless, the mechanisms that regulate association of I κ B to the chromatin and its repressive function remain unknown.

I κ B α -deficient mice die around day 5 because of skin inflammation associated with high levels of IL1 β and IFN- γ in the dermis, CD8⁺ T cells, and Gr-1⁺ neutrophils infiltrating the epidermis, as well as altered keratinocyte differentiation (Beg et al., 1995; Klement et al., 1996; Rebholz et al., 2007), similar to keratinocyte-specific I κ B α -deficient mice (*I κ B α ^{K5 Δ /K5 Δ}*) (Rebholz et al., 2007). In all cases, disruption of TNF α signaling rescued the skin phenotype (Shih et al., 2009), suggesting that lethality was associated with an excessive inflammatory response, likely due to increased NF- κ B activity. However, mice expressing different I κ B α mutants that are equally able to repress NF- κ B in the skin showed divergent phenotypes. Specifically, mice expressing the nondegradable I κ B α mutant, I κ B α ^{S32-36A}, developed skin tumors resembling SCC (van Hogerlinden et al., 1999), whereas mice carrying a predominantly nuclear form of I κ B α show no overt skin defects (Wuerzberger-Davis et al., 2011).

Skin differentiation depends on the correct establishment and maintenance of specific gene expression patterns, including genes of the *HOX* family, which in the basal progenitor cells are repressed by EZH2, the catalytic subunit of the Polycomb repressive complex 2 (PRC2) (Ezhkova et al., 2009, 2011). PRC2 is composed by EZH2, the WD-repeat protein EED, RbAp48, and the zinc-finger protein SUZ12 (Zhang and Reinberg, 2001). Methylation of lysine 27 on histone H3 (H3K27me3) by EZH2 imposes gene silencing in part by triggering recruitment of PRC1 (Cao et al., 2002; Min et al., 2003) and histone deacetylases (HDACs). Here, we investigate an

alternative function for I κ B α in the regulation of skin homeostasis, development, and cancer.

RESULTS

Phosphorylated and Sumoylated I κ B α Localizes in the Nucleus of Keratinocytes

To investigate the physiological role for nuclear I κ B α , we performed an initial screen to determine its subcellular distribution in human tissues. We found that I κ B α localizes in the cytoplasm of most tissues and cell types as expected (Figure S1A available online); yet, a distinctive nuclear staining of I κ B α was found in human (Figure 1A) and mouse skin sections (Figures 1A, S1A, and S1C), more prominently in the keratin14⁺ basal layer keratinocytes. I κ B α distribution became more diffused in the suprabasal layer of the skin and gradually disappeared in the more differentiated cells. Specificity of nuclear I κ B α staining was confirmed using skin sections from newborn I κ B α -knockout (KO) mice (Figure S1B) and different anti-I κ B α antibodies and blocking peptides (Figure S1C). By immunofluorescence (IF) and immunoblot (IB), we detected I κ B α protein in both the cytoplasmic and the nuclear/chromatin fractions of human (Figures 1B and 1C) and mouse (Figure S1D) keratinocytes. Interestingly, nuclear I κ B α displayed a shift in its electrophoretic mobility (\approx 60 kDa) detected by different anti-I κ B α antibodies, including the anti-phospho-S32-36-I κ B α antibody. We next precipitated I κ B α from nuclear and cytoplasmic keratinocyte extracts and determined whether this low I κ B α mobility was a result of ubiquitin or SUMO modifications. We found that nuclear I κ B α was specifically recognized by anti-SUMO2/3, but not anti-SUMO1 or anti-ubiquitin antibodies (Figure 1D; data not shown). Hereafter, we will refer to this nuclear I κ B α species as phospho-SUMO-I κ B α (PS-I κ B α). By cotransfection of different SUMO plasmids in HEK293T cells, we demonstrated that SUMO2 was integrated to HA-I κ B α at K21,22 (Figure S1E), independently of S32,36 phosphorylation (Figure 1E). By subcellular fractionation, we found that most HA-PS-I κ B α was distributed in the nucleus of HEK293T cells (data not shown), and both K21,22R and S32,36A I κ B α mutants showed reduced association with the chromatin (Figure 1F). These results suggest that phosphorylation and sumoylation are both required for I κ B α nuclear functions *in vivo*. Of note, PS-I κ B α levels were always low in HEK293T cells when

Figure 1. Phosphorylated and Sumoylated I κ B α Is Found in the Nucleus of Normal Basal Keratinocytes

(A) Immunodetection of I κ B α (green) in normal human skin and detail of basal layer. B, basal; S, spinous; G, granular; and C, cornified layers of epidermis. Dashed line indicates the dermis interphase. DAPI was used for nuclear staining.

(B) IF of I κ B α in primary human keratinocytes.

(C) Subcellular fractionation of human keratinocytes followed by IB with the indicated antibodies.

(D) I κ B α was immunoprecipitated from primary murine keratinocyte extracts followed by IB with the indicated antibodies.

(E) IB analysis of His-tag precipitates from HEK293T cells transfected with the indicated plasmids. SUMO2 is incorporated in I κ B α when K21,22 are present.

(F) HEK293T cells were transfected with the indicated I κ B α plasmids and processed following the ChIP protocol to obtain the whole chromatin fraction that was analyzed by IB.

(G) IF of I κ B α and P-IKK in skin sections. Cells with P-IKK staining do not contain nuclear I κ B α .

(H) IB analysis of keratinocytes transduced with *myc-IKK α EE* or control.

(I) IF analysis of the indicated differentiation markers in skin sections of WT and I κ B α KO newborn mice.

(J) IB analysis of indicated proteins in control or Ca²⁺-treated murine keratinocytes. Total and nuclear/chromatin fractions are shown.

(K) Determination of *Filaggrin*, *K10*, and *p63* mRNA levels in control and I κ B α KD keratinocytes following Ca²⁺ treatment. Expression levels are relative to *Gapdh* and compared to control cells. Error bars indicate SD. I κ B α protein levels were analyzed by IB. Data correspond to one representative of three experiments. N, nuclear; C, cytoplasmic.

See also Figure S1.

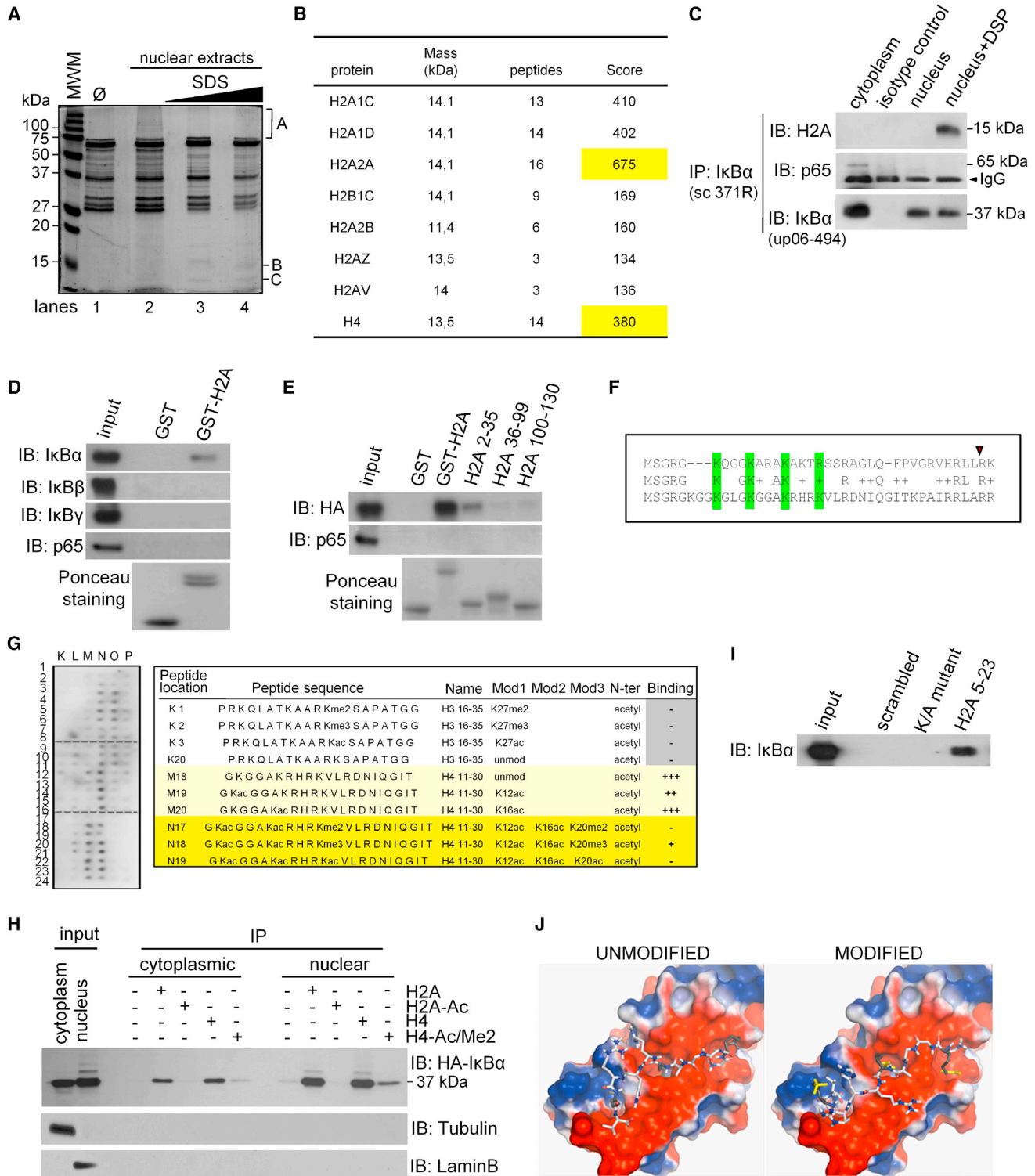


Figure 2. IκBα Binds Histones H2A and H4

(A) PD experiment using GST-IκBα and native (lane 2) or denatured-renatured (lanes 3–4) human keratinocyte nuclear extracts. One representative gel stained with Coomassie blue is shown (n = 3).

(B) Purification and analysis of B and C bands identified as histones H2A and H4 by mass spectrometry. Table indicates the number of peptides identified and their score factor. The highest score is highlighted.

(C) Coprecipitation from DSP-crosslinked nuclear extracts from human keratinocytes.

(D and E) PD using different GST-H2A proteins and total lysates from HEK293T cells expressing the indicated proteins.

(legend continued on next page)

compared with keratinocytes, even in overexpression conditions and cell lysates directly obtained under denaturing conditions (see inputs in Figures 1E and S1E).

It is well known that IKK activity regulates the cytoplasmic levels of I κ B α . By double staining of skin sections, we found that the few cells that were positive for active IKK contained I κ B α , but this I κ B α was excluded from the nucleus (Figure 1G). To directly investigate whether IKK regulates subcellular distribution of I κ B α , we transduced primary murine keratinocytes with lentiviral IKK α ^{EE}. We found that active IKK α induced a decrease in the nuclear levels of PS-I κ B α as determined by IB (Figure 1H) and IF (Figure S1G). Additional experiments comparing the effects of both IKK isoforms demonstrated that IKK α ^{EE} was more efficient than IKK β ^{EE} in decreasing nuclear PS-I κ B α levels (60% \pm 5% compared with 16% \pm 9% reduction; $p < 0.001$) (Figure S1F).

To directly address whether I κ B α was required for normal skin differentiation, we performed IF analysis using different markers comparing I κ B α wild-type (WT) and KO newborn skins. Consistent with previous reports, I κ B α -deficient mice do not show any obvious skin defect at birth with a normal K5-positive basal layer, although we observed a slight reduction in the thickness of the K10-positive suprabasal epidermal layer. Most importantly, I κ B α mutant skins showed a severe reduction of the more differentiated layer of cells identified by the accumulation of filaggrin granules (Figure 1I). This is a cause for impaired barrier function (Palmer et al., 2006). Next, we aimed to distinguish between cell-autonomous and non-cell-autonomous effects of I κ B α deficiency by using an in vitro system for keratinocyte differentiation induced by high Ca²⁺ exposure (Hennings et al., 1980). In this model, we found that keratinocyte differentiation was associated with a decrease in both I κ B α and PS-I κ B α levels and activation of nuclear IKK (Figures 1J and S1H). Notably, knockdown (KD) of I κ B α disturbs in vitro keratinocyte differentiation as indicated by the impaired K10 and filaggrin induction in response to Ca²⁺, which was accompanied by sustained expression of the progenitor marker p63 (Figure 1K). Together, these results strongly suggest that I κ B α plays a cell-autonomous function in skin differentiation.

I κ B α Directly Binds to the N-Terminal Tail of Histones H2A and H4

To further investigate the mechanisms underlying nuclear I κ B α functions, we searched for nuclear proteins that directly associate with I κ B α . Using GST-I κ B α and human keratinocyte nuclear extracts in pull-down (PD) experiments, we isolated proteins of estimated molecular weights of 15 and 14 kDa that were identified by mass spectrometry as histones H2A and H4 (Figures 2A and 2B). Interaction between histones H2A and H4

and I κ B α was further confirmed by coprecipitation of endogenous proteins from keratinocyte nuclear extracts. Of note, the NF- κ B subunit p65 was absent from nuclear I κ B α precipitates but coprecipitated in the cytoplasmic fraction (Figure 2C). By PD assays, we determined the specificity of I κ B α binding compared to other I κ B homologs (Figure 2D) and mapped the I κ B α -binding domain of histone H2A to be between amino acids 2 and 35 (Figure 2E). Preincubation of I κ B α with p65 prevented its association with histones (Figure S2A), suggestive of mutually exclusive complexes.

Comparative sequence analysis of the I κ B α -binding region of histone H2A (AA1–36) and the homologous region of H4 revealed the presence of a motif (3KXXXX/R) that was absent from other histone and nonhistone proteins (Figure 2F). To further study I κ B α binding specificity, we screened a histone peptide array using nuclear HA-I κ B α expressed in HEK293T cells as bait. We found that I κ B α bound to peptides containing AA11–30 of histone H4, but not the corresponding region of histone H3. Most importantly, binding of I κ B α to H4 was prevented by the combination K12/K16Ac and K20Ac or Me2 (Figure 2G). Because the equivalent peptides from histone H2A were not included in the array, we performed parallel precipitation experiments using biotin-tagged peptides (AA5–23) of histone H2A and H4 (Figures 2H and 2I). We found that I κ B α association was prevented by K12Ac, K16Ac, and K20me2 of histone H4 or the equivalent modifications of the H2A peptide (Figure 2H) and also when all K/R residues in the 3KXXXX motif were changed into A (Figure 2I). Of note that in these experiments histone-bound HA-I κ B α was mostly identified as a nonsumoylated band, which opens the possibility that posttranslational modifications are not essential to mediate this interaction in vitro. However, parallel binding experiments using keratinocyte extracts, PS-I κ B α , showed a preferential binding to the histone peptides compared with the cytoplasmic 37 kDa I κ B α form (Figure S2B). Together, these results strongly suggest that only PS-I κ B α can bind the chromatin, but in HEK293T cells this molecule is then desumoylated in vivo or as a consequence of the experimental processing.

To gain further insights into the molecular basis of I κ B α binding to histones, we completed the structure of I κ B α obtained from the Protein Data Bank (ID code 1IKN), which lacked part of the ankyrin repeat (AR) 1, using RAPPER (Depristo et al., 2005) and performed docking studies with AutoDock Vina (Trott and Olson, 2010) of the histone H4 peptide, GKGGAKRHRKV, that contains most of the KXXXX domain. Docking calculations showed two deep pockets for K interaction in I κ B α located between ARs 1-2 and 2-3 and an additional shallower patch between AR3 and 4. Overall, the peptide bound in a clearly negative region on the I κ B α surface (Figure 2J), with higher affinity than the modified peptide that was acetylated in the first and

(F) ClustalW alignment of the conserved KXXXX/R motifs in the N terminus of histones H2A and H4. Conserved K and R residues are in green. Red triangle indicates the last AA included in GST-H2A 2-35.

(G) Histone peptide array hybridized with nuclear HEK293T extracts expressing HA-I κ B α . One informative area of the blot image and the relative binding of selected peptides are shown.

(H) Coprecipitation of cytoplasmic and nuclear HA-I κ B α expressed in HEK293T cells with the indicated histone H2A and H4 peptides.

(I) HA-I κ B α was precipitated using the indicated histone H2A peptide, the K/A mutant, or scrambled peptide. In (G), (H), and (I), cell lysates were denatured-renatured previous to the precipitation to disrupt preformed complexes.

(J) Model for binding of the histone H4 peptide (unmodified or modified) to consecutive ankyrin repeats of I κ B α (3KXXXXK).

See also Figure S2.

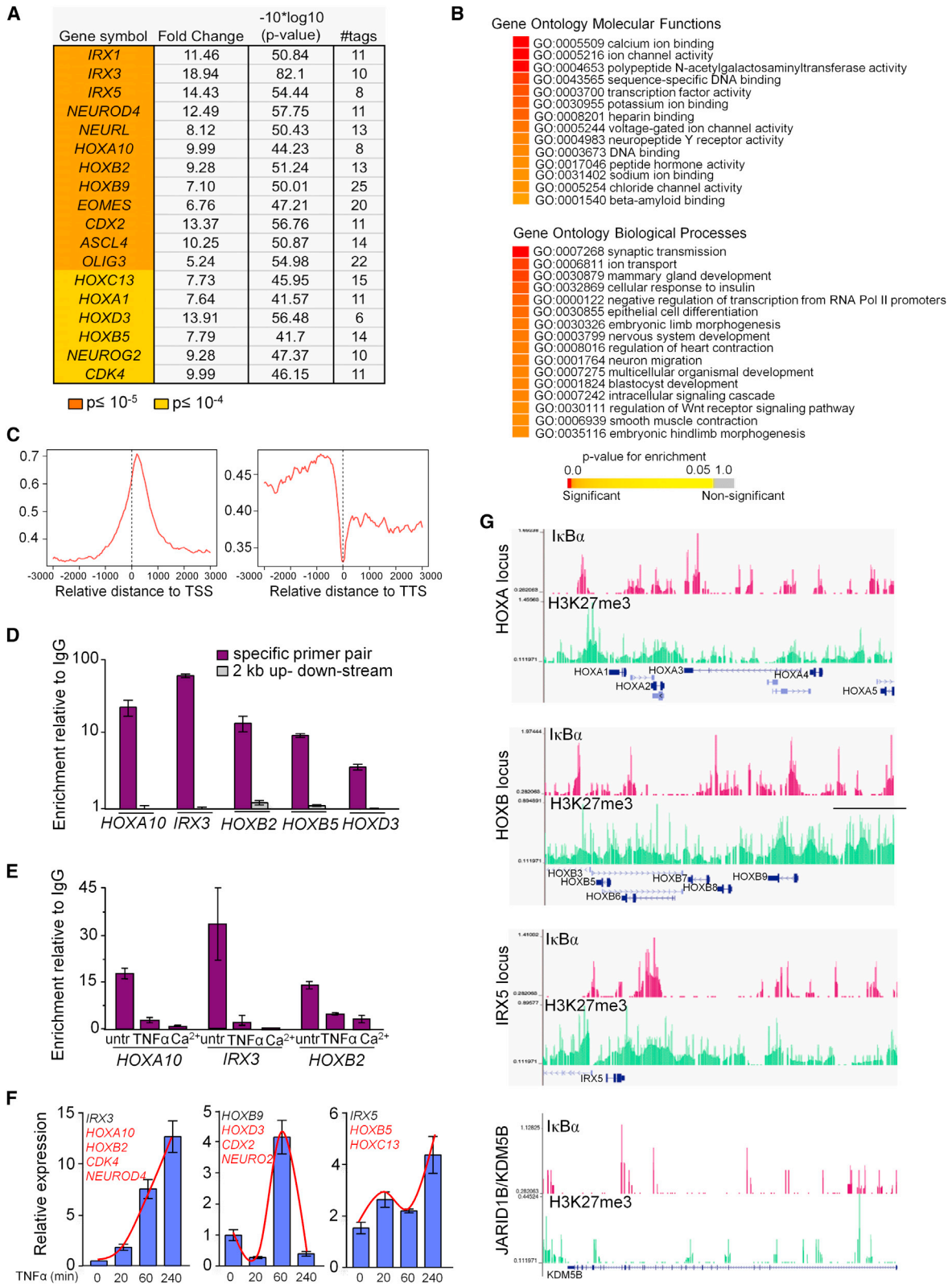


Figure 3. Analysis and Identification of I κ B α Target Genes

(A) Developmentally related genes selected from I κ B α targets identified in ChIP-seq analysis. Fold change over the random background is indicated.

(B) Functional enrichment of target genes with p value cutoff $\leq 10^{-5}$ based on gene ontology (GO) as extracted from Ensembl database using GiTools. Enriched categories are represented in heatmap with the indicated color-coded p value scale.

(legend continued on next page)

second K residues (K12 and K16). We experimentally validated that ARs of I κ B α participate in histone binding because the I κ B α Δ 55-106 mutant, lacking part of AR1, failed to bind GST-H2A (Figure S2C). Similarly, this association was prevented by 1% deoxycholate (Figure S2D), as described for interactions involving the ARs of I κ B α (Baeuerle and Baltimore, 1988; Savinova et al., 2009).

I κ B α Is Specifically Recruited to the Regulatory Regions of Developmental Genes

To identify putative PS-I κ B α target genes, we performed chromatin immunoprecipitation sequencing (ChIP-seq) using chromatin extracts from primary human keratinocytes and anti-I κ B α antibody. We identified 2,778 enriched peaks, corresponding to 2,433 Ensembl genes that were significantly enriched with p values $\leq 10^{-5}$. Gene ontology analysis showed that a significant proportion of genes participate in biological processes associated with embryonic development and cell differentiation. I κ B α targets included genes of the *HOX* and *IRX* families, *ASCL4*, *CDX2*, *NEUROD4*, *OLIG3*, and *NEURL*, among others (Figures 3A and 3B). Annotation of the peak genomic positions to the closest gene demonstrated that many peaks were positioned immediately after the transcription start site (TSS), with a sharp decrease near the transcription termination site (TTS) (Figure 3C), whereas others were located far from promoter regions. Some of the latter overlapped with regions enriched in H3K4me1, a histone mark associated with enhancer regions (data not shown). Randomly selected I κ B α targets were confirmed by conventional chromatin immunoprecipitation (ChIP) using primers flanking the regions identified in the ChIP-seq experiment (Figure 3D). Consistent with its overall effects on I κ B α levels, sustained Ca^{2+} treatment caused the loss of I κ B α from all tested gene promoters (Figure 3E). Similarly, short treatment with TNF α released chromatin-bound I κ B α in keratinocytes, as we previously found in fibroblasts (Aguilera et al., 2004). However, we did not detect a general effect of TNF α on PS-I κ B α levels, but we consistently found a partial redistribution of PS-I κ B α to the soluble nuclear fractions (Figure S3A). Next, we investigated whether TNF α and Ca^{2+} modulated *HOX* and *IRX* transcription in keratinocytes. All tested I κ B α targets ($n = 12$) were robustly induced by TNF α treatment (up to 12-fold) following different kinetics (Figures 3F) and to a lesser extent (up to 3-fold) by Ca^{2+} treatment (Figure S3B) or I κ B α KD (Figure S3C). Interestingly, 1 hr of TNF α treatment prevented Ca^{2+} -induced differentiation of murine keratinocytes (Figure S3D), supporting the notion that PS-I κ B α integrates inflammatory signals with skin homeostasis (see Discussion). We also tested whether p65 participated in *HOX* or *IRX* gene activation by TNF α . By ChIP analysis, we did not find any

recruitment of p65 to I κ B α target genes after TNF α treatment, in contrast to a canonical NF- κ B target gene promoter (Figure S3E). However, we detected low amounts of p65 at *HOX* genes under basal conditions that might contribute to gene repression (Dong et al., 2008), although the function of chromatin-bound p65 at regions distant from the TSS of both NF- κ B targets and nontargets is unresolved. Binding of p65 to *HOX* and *IRX* was reduced after TNF α treatment, suggesting that p65 was redistributed from noncanonical to canonical NF- κ B targets once activated.

Silencing of *HOX* genes in keratinocytes involves PRC2 and its core component the H3K27 methyltransferase EZH2 (Ezhkova et al., 2009; Mejetta et al., 2011). To explore a putative association between I κ B α and PRC2 function, we crossed our list of 2,433 I κ B α targets with available ChIP-seq data from keratinocytes. Approximately 50% of I κ B α targets corresponded to genes enriched for the H3K27me3 mark (Figures 3G and S3F), although I κ B α targeted only 13% of the H3K27 trimethylated genes. Most importantly, genomic sequences occupied by I κ B α essentially overlapped with those regions containing high H3K27me3 levels (Figure 3G). We also found a statistically significant overlap ($p < 10^{-16}$) between I κ B α target genes and PRC targets in ES cells (Birney et al., 2007; Ku et al., 2008) (Figure S3G).

I κ B α Interacts with and Regulates Association of PRC2 to Target Genes in Response to TNF α

In the mass spectrometry analysis of proteins that associate with GST-I κ B α , we identified a few peptides corresponding to chromatin modifiers, such as EZH2 and SUZ12, and SIN3A (Figure S4A). Specificity of I κ B α interactions with PRC2 elements, but also I κ B α association to the PRC1 protein BMI1, was confirmed by PD assays (Figure S4B). SUZ12 was able to interact with nuclear I κ B α , whereas p65 specifically associated with cytoplasmic I κ B α in the IP experiments (Figure 4A). Importantly, exogenous wild-type I κ B α , but not an I κ B α mutant that failed to bind histones, facilitated the association of SUZ12 to GST-H4 (Figure 4B). Moreover, ChIP experiments demonstrated that TNF α treatment induced the dissociation of SUZ12 from I κ B α target regions, but not non-I κ B α targets (Figure 4C). Sequential ChIP experiments demonstrated that I κ B α and SUZ12 simultaneously bound to I κ B α target genes (Figure 4D).

To test the functional relevance of I κ B α in PRC-mediated repression, we used WT murine embryonic fibroblast (MEFs), which expressed detectable levels of PS-I κ B α (Figure 4E) and I κ B α KO MEFs. By ChIP-on-chip experiments using three different I κ B α antibodies, we confirmed that several *Hox* genes were also targets of I κ B α in MEFs (Table S1). By ChIP, we found that SUZ12 and EZH2 bound I κ B α targets efficiently in WT MEFs

(C) Graphs show the relative distance to the nearest ChIPed region, 3 kb upstream and downstream of the RefSeq gene's TSS and TTS.

(D) Validation of the identified DNA regions (−222 to −200 for *HOXA10*, −9,380 to −9,360 for *HOXB2*, +6,166 to +6,186 for *HOXB5*, +4,451 to +4,471 for *HOXB3*, and −18,820 to −18,800 for *IRX3*) by conventional ChIP. Amplification of 2 kb distant regions was used as negative controls.

(E) ChIP analysis of I κ B α after 20 min of TNF α or 48 hr Ca^{2+} treatments. In (D) and (E), graphs represent mean enrichment relative to nonspecific immunoglobulin G (IgG) ($n = 2$).

(F) Expression levels of I κ B α target genes following TNF α treatment analyzed by qRT-PCR. Gene represented is in black, whereas genes following the same kinetics are indicated in red.

(G) ChIP-seq profiles of endogenous I κ B α occupancy in three enriched loci (*HOXA*, *HOXB*, and *IRX5*) and one negative locus (*JARID1B/KDM5B*) compared to H3K27me3 (from the UW ENCODE Project) in keratinocytes. (D–F) Bars represent mean, and error bars indicate SD.

See also Figure S3.

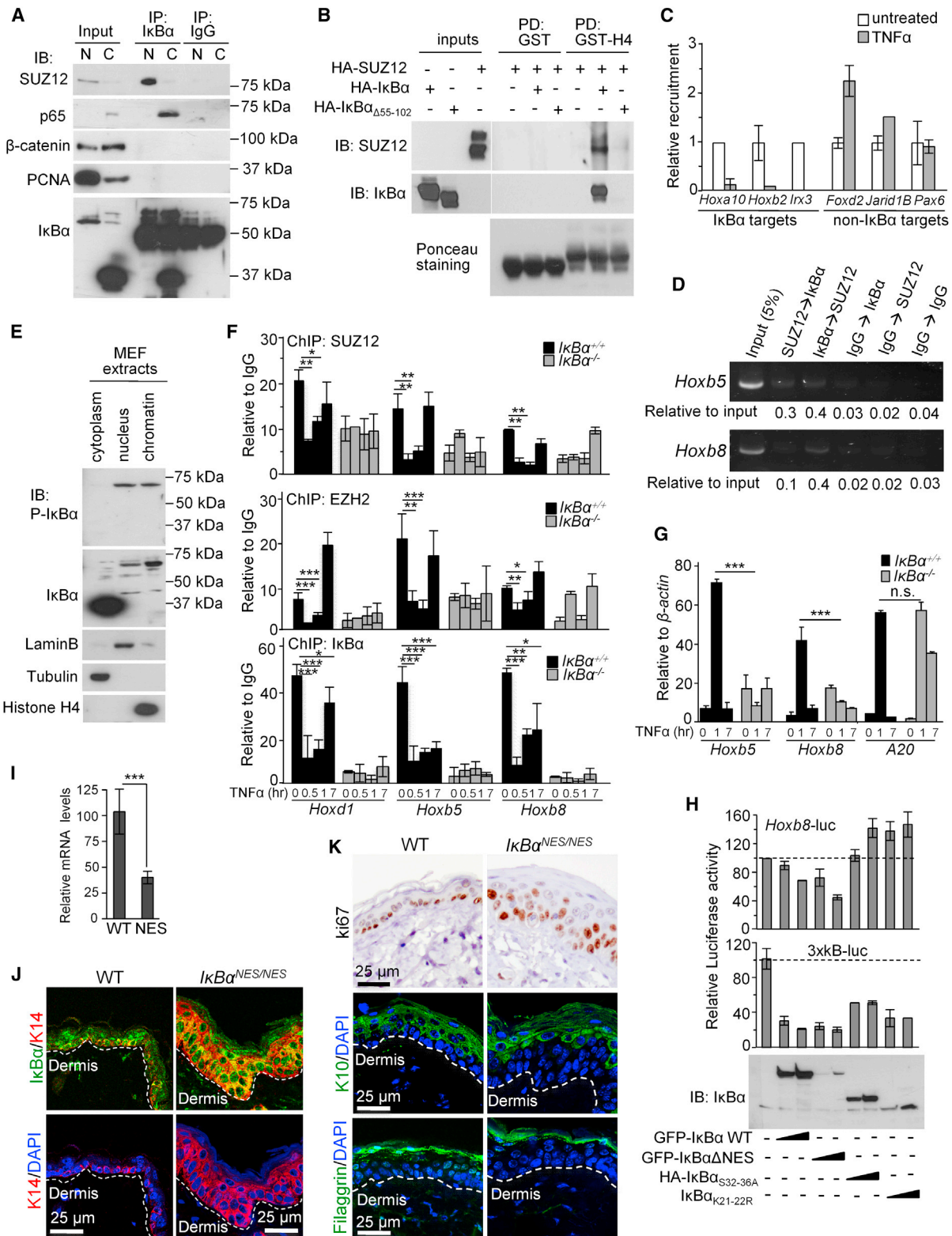


Figure 4. I κ B α Interacts with and Regulates Association of PRC2 in Response to TNF α

(A) IB analysis of I κ B α precipitates from nuclear and cytoplasmic primary murine keratinocyte extracts. Five percent of the input and 25% of the IP was loaded in all cases except for detection of I κ B α input that represents 0.5%.

(B) PD using GST-H4 and cell lysates from HEK293T expressing different combinations of HA-I κ B α and SUZ12.

(C) Relative recruitment assessed by ChIP of SUZ12 to different genes 40 min after TNF α in primary murine keratinocytes.

(legend continued on next page)

but only weakly in I κ B α KO MEFs (Figure 4F, time 0). In WT cells, TNF α treatment induced a significant but temporary release of SUZ12 and EZH2 from these loci, which peaked after 30–60 min of treatment (Figure 4F). The binding of PRC2 proteins at *Hox* genes inversely correlated with the expression of these *Hox* genes (Figure 4G). In contrast, I κ B α KO cells failed to activate *Hox* transcription in response to TNF α (Figure 4G), which is consistent with a defective release of PRC2 proteins (Figure 4F). Unexpectedly, we did not detect changes in H3K27me3 levels in these *Hox* genes upon TNF α treatment at any of the time points studied (20 min, 2 hr, and 7 hr) (data not shown), likely reflecting the high stability of this histone modification (De Santa et al., 2007). Supporting the possibility that activation of I κ B targets is independent of the enzymatic activity of EZH2, a 24 hr treatment with the EZH2 inhibitor DZNep does not affect *Hoxb8* or *Irx3* messenger RNA (mRNA) levels in keratinocytes (data not shown). Together, these results suggest that transcriptional repression-activation of these genes does not strictly depend on EZH2 enzymatic activity but rather PRC2 release might modulate the dissociation of PRC1 or HDACs (van der Vlag and Otte, 1999) that associate with more dynamic chromatin modifications. In agreement with this possibility, histone H3 is rapidly acetylated following TNF α treatment at different *Hox* gene promoters (Figure S4C).

To further study the involvement of NF- κ B in the regulation of I κ B α targets by TNF α , we attempted to use different mutant MEFs, including the *p65*, *Ikk α* , *Ikk β* , and the triple *p65;p50;c-Rel* KO. We found that TNF α induced *Hox* and *Irx* expression in both *p65* and *Ikk β* KO cells (Figure S4D) suggesting that it was NF- κ B independent. However, specific mutants contained variable levels of I κ B α and PS-I κ B α (Figures S4E and S4F), which make a more accurate quantitative analysis unproductive. Interestingly, phosphorylation of nuclear I κ B α was not reduced in the *Ikk α* or *Ikk β* KO cells (Figures S4F), indicating that other kinases are involved in generating PS-I κ B α . Only triple KO cells, which essentially lacked I κ B α (Figure S4G) and the *Ikk α* -deficient MEFs, showed a strong defect on *Hox* and *Irx* transcription (Figures S4D and S4H). To better understand the contribution of NF- κ B to *Hox* regulation, we next performed luciferase reporter assays measuring the ability of different I κ B α mutant proteins to repress a *Hoxb8*-promoter construct compared with a reporter containing three consensus sites for NF- κ B (3 \times κ B). We found that WT I κ B α and the nuclear I κ B α ^{NES} mutant (Huang et al., 2000) significantly repressed both promoters. However, mutations that affect chromatin-association (Figure 1F) prevented *Hoxb8* repression but still inhibited the expression of the 3 \times κ B reporter (Figure 4H). Consistently, *Hoxb8* mRNA levels were significantly reduced in the skin of mice expressing I κ B α ^{NES}

(Wuerzberger-Davis et al., 2011) (Figure 4I). Moreover, these animals showed an expansion of the K14-positive basal layer of keratinocytes containing nuclear I κ B α (Figure 4J), associated with increased proliferation measured by ki67 staining and impaired differentiation as indicated by the reduced thickness of the suprabasal K10-expressing layer (Figure 4K).

Genetic Interaction between I κ B α /cactus and polycomb in *Drosophila*

Polycomb group (PcG) I κ B and NF- κ B proteins are conserved from flies to humans. In addition, *Drosophila* contains one *Hox* cluster, compared with four clusters in vertebrates, which facilitates studying genetic interactions. We first confirmed that the single *Drosophila* I κ B homolog, cactus (*cact*) (Geisler et al., 1992), maintained the capacity to associate with histones (Figure 5A). By IF, we detected colocalization of cactus and Polycomb (Pc), a PRC1 protein that is essential for the repressive PRC2 function, in specific bands of polytene chromosomes (Figure 5B). Most of the cactus staining overlapped with Pc, but only a few Pc-positive bands contained cactus. Based on our mammalian data, our first attempt was to generate single PRC2 mutants and combine them with *cactus*-deficient mutants. All mutants were tested in heterozygosis because homozygous mutations in *cactus* or PcG genes are lethal.

We found that heterozygous mutations in PRC2 genes (e.g., the null mutations of *E(z)*) as well as the composed *E(z)* and *cact* exhibit no overt homeotic phenotypes. In contrast, heterozygous *Pc* mutants exhibit a variety of characteristic homeotic transformations, including the partial transformation of the second and third (mesothoracic and metathoracic) legs toward first (also known as prothoracic) legs that in males are characterized by the presence of sex combs. Modifications of this phenotype (also called “extra sex comb”) have been extensively used as a functional assay to validate new PcG proteins in vivo. Thus, we tested the effect of reducing *cact* on *Pc*-induced homeotic transformation using 12 recessive mutations of *cact* and two independently generated *Pc* alleles. All *cact* mutant alleles, but more prominently *cact*¹, enhanced the “extra sex comb” phenotype of *Pc* mutations (*Pc*³ and *Pc*^{X⁷¹⁰⁹}) (Figure 5C; Table S2).

Because cells lacking *cact* exhibit massive nuclear localization of the transcription factor dorsal (*dl*, *Drosophila* NF- κ B/Rel/p65 ortholog) during postembryonic stages (Lemaitre et al., 1995), we tested whether enhancement of homeotic defect of *Pc* by *cact* mutations is due to increased dorsal activity. Because stability of *cact* is under NF- κ B/Dorsal control (Kubota and Gay, 1995), we anticipated that for phenotypes due to increased dorsal, *dl* mutations would counteract *cact* mutations, whereas for phenotypes independent of dorsal, reducing *dl* should yield

(D) Sequential ChIP using the indicated combinations of antibodies. An analysis of two different *Hox* regulatory regions is shown.

(E) IB analysis of WT fibroblasts showing the presence of cytoplasmic and nuclear I κ B α .

(F) Relative chromatin binding of PRC2 and I κ B α in WT and I κ B α KO MEFs treated with TNF α . ChIP values were normalized by IgG precipitation.

(G) Relative levels of the indicated genes in WT and I κ B α KO MEFs.

(H) Luciferase assays to determine the effect of different I κ B α constructs on the activity of *HoxB8* compared to the 3 \times κ B reporter. Lower panels show expression levels of different constructs.

(I) Expression levels of *HoxB8* in the skin of WT and I κ B α ^{NES/NES} mice by qRT-PCR (n = 2).

(J and K) Analysis of skin sections from 7- to 8-week-old WT and I κ B α ^{NES/NES} mice by IF. K14 labels the basal layer keratinocytes (J). Immunostaining of ki67, the suprabasal marker K10, and filaggrin (K). Throughout the figure, bars represent mean, and error bars indicate SD.

See also Figure S4 and Table S1.

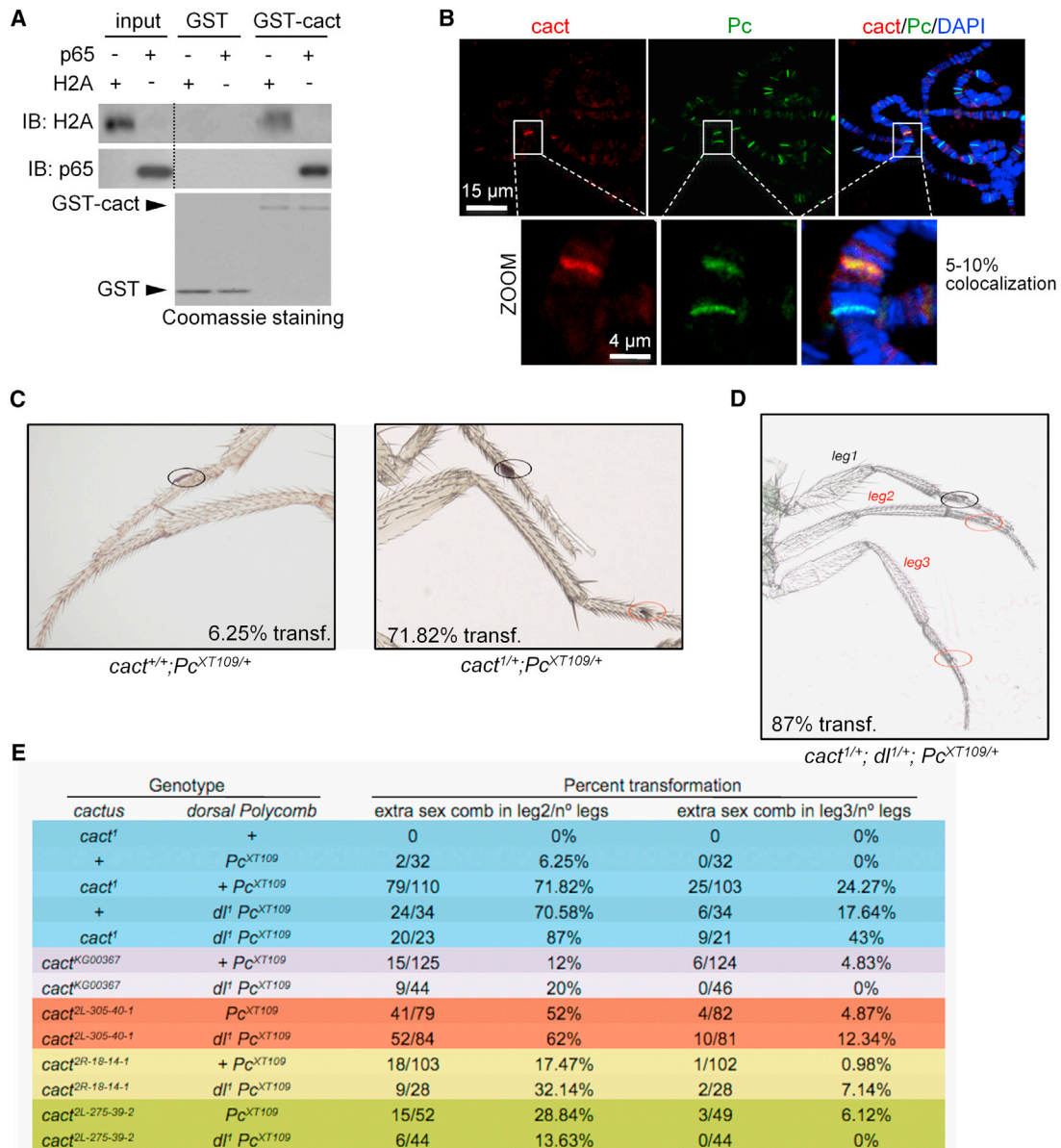


Figure 5. Mutations in *cactus*/IκBα Enhances the Homeotic Phenotype of Heterozygous *Pc* Mutants in *Drosophila melanogaster*

(A) PD using GST-cactus and detection of p65 expressed in HEK293T or H2A from a histone-enriched extract.

(B) Representative image of a double IF for *cact* and *Pc* in *Drosophila* salivary polytene chromosomes. Selected region is shown in the magnification. *cact*-positive bands always overlap *Pc* staining.

(C) The percent of mutant adults with “extra sex comb” in legs 2 and 3 (red circles) was measured in at least 100 legs of flies heterozygous for 12 *cact* mutations in two different *Pc*^{-/+} mutant backgrounds.

(D) Representative image of the homeotic phenotype induced by *cact* in *Pc* mutants, which is enhanced by heterozygous mutation of *dorsal*. In (C) and (D), the percentage of flies with “extra sex combs” in leg 2 is indicated.

(E) Frequency of the homeotic phenotypes corresponding to the number of “extra sex combs” in second and third leg in flies with the indicated *cactus*, *dorsal*, and *Polycomb* mutations.

See also Tables S2 and S3.

phenotypes similar to that of *cact* mutants. We found that heterozygous *dl* mutations significantly enhanced the extra sex comb transformation phenotype of *Pc* heterozygotes (Table S3), similar to the effect of *cact* mutations. Furthermore, by generating triple heterozygous strains carrying the null allele of *dorsal* (*dl*¹), the *Pc*^{XT109} allele and different *cact* alleles, we found that

most of the triple heterozygous *cact*;*dl*;*Pc* exhibited similar or stronger homeotic phenotypes than double heterozygotes for *dl*;*Pc* and *cact*;*Pc* of a particular allelic combination (Figures 5D and 5E). Note that the smaller number of animals scored in the triple heterozygotes also reflects the higher lethality of this genotype. These data support our finding that IκB/cactus

cooperates with Polycomb-mediated repression, independently of NF- κ B/dorsal, which is relevant for the regulation of epidermal development in the fly.

Requirement of I κ B α for Keratinocyte Transformation and HOX Upregulation

Deregulated HOX expression is a common trait in cancer (De Souza Setubal Destro et al., 2010; Hassan et al., 2006; Yamatoji et al., 2010; Zhai et al., 2007), including squamous cell carcinoma (SCC). To study whether I κ B α participates in Hox deregulation of SCC, we induced transformation of primary murine keratinocytes by expressing mutant H-RasV12 plus Δ Np63 α using retroviral infection (Keyes et al., 2011). We found that PS-I κ B α , which was consistently detected in the nuclear fraction of control-transduced keratinocytes, was mainly lost in transduced cells associated with P-IKK induction (Figure 6A). By ChIP assay, we found that binding of SUZ12 and EZH2 at I κ B α targets was significantly decreased in these cells (Figure 6B), concomitant with a massive increase in I κ B α target gene expression (Figure 6C). Furthermore, transformed keratinocytes growing in three-dimensional (3D) matrigel cultures showed a complete cytoplasmic distribution of I κ B α (Figure 6D), whereas I κ B α KD abrogated the capacity of transformed keratinocytes to grow in matrigel (Figure 6E), similar to the nontransformed cells. These results suggest that accumulation of cytoplasmic I κ B α favors oncogenic transformation.

We further explored this possibility by analyzing the subcellular distribution of I κ B α protein in a tissue microarray (TMA) that included different human nonmalignant or premalignant skin lesions (n = 21), skin cancer (n = 32), and normal control samples (n = 3). We detected nuclear I κ B α in different nonmalignant/noninflammatory lesions and normal skin samples. In noninvasive tumors, such as actinic keratosis and Bowen's disease, a variable proportion of cells displayed localization of I κ B α in the cytoplasm, but most of them retained nuclear I κ B α , including chromosomal staining of the mitotic keratinocytes (Figure 6H). In more aggressive cutaneous SCC, around 50% of the tumor cells showed a complete loss of nuclear I κ B α and accumulation of cytoplasmic I κ B α (Figures 6F and 6G). By qRT-PCR, we measured the mRNA levels of several I κ B α targets in 21 SCC and 13 normal skin samples. We found that HOXD3 was significantly increased in human SCC compared with normal skin (p = 0.04). We also detected a consistent upregulation of HOXB9 and HOXB5 that did not reach statistical significance due to the dispersion between samples (Figure 6I; data not shown).

We extended our study to a cohort of 112 patients with urogenital SCC at different stages of tumor progression. We found that cytoplasmic I κ B α accumulation started before the stage of tumor invasion, but total loss of nuclear I κ B α was most predominant in the invasive areas of the tumors and in the metastases. The significance of the correlations was p < 0.0001 for normal adjacent versus in situ tumor, p < 0.0001 for in situ compared with infiltrating tumors, and p = 0.3 when comparing the tumor with the metastasis, using the Fisher's exact test (Figures 6J and 6L). Cytoplasmic accumulation of I κ B α significantly associated with IKK α activation in these samples (Figures 6K and 6L).

DISCUSSION

Here, we show that I κ B α plays a function in the chromatin associated with repression of developmental genes, including the HOX and IRX families. We focused our study on the skin keratinocytes, where we found a significant accumulation of nuclear I κ B α ; however, this mechanism is not exclusive of this cell type and operates at least also in fibroblasts. In addition, we deciphered part of the mechanism of I κ B α -mediated repression that involves posttranslational I κ B α modifications and association with histones H2A/H4 and Polycomb proteins. The evolutionary conservation of this mechanism is illustrated by the strong homeotic phenotype of double *Polycomb* and *cactus* mutants in *Drosophila*.

Compiled data on NF- κ B signaling demonstrate that I κ B α plays a major role as inhibitor of the p65/p50 dimer and termination of NF- κ B signal after stimulation. In the latter, establishment of a nuclear I κ B α -NF- κ B ternary complex actively facilitates dissociation of NF- κ B from specific binding sites in the DNA (Zabel and Baeuerle, 1990), leading to the notion that I κ B α and chromatin binding are irreconcilable. Our experiments demonstrate that a specific fraction of I κ B α that is phosphorylated at S32,36 and posttranslationally modified by SUMO2 at K21,22 is able to bind the histones. Sumoylation of I κ B α had been previously described, but the functional relevance of this modification was mainly unknown (Desterro et al., 1998). Although phosphorylation of S32,36 is the instructive mark that targets I κ B α for K21,22 ubiquitination and its subsequent degradation by the proteasome, we now propose that K21,22 sumoylation protects phosphorylated I κ B α from degradation, thus generating an unexpected functional I κ B α species. Whether calcium-induced degradation of PS-I κ B α involves its desumoylation and subsequent ubiquitylation, and which modifications in both I κ B α and histones are imposed by TNF α and induce PS-I κ B α release from the chromatin, is currently under investigation.

Our data suggest that other I κ B homologs cannot compensate I κ B α chromatin function because *Hoxb5* and *Hoxb8* failed to be induced by TNF α in I κ B α KO cells. However, expression of I κ B β from the I κ B α locus compensates most of the defects of I κ B α deficiency (Cheng et al., 1998), which suggest that a specific fraction of I κ B β (i.e., hypophosphorylated nuclear I κ B β) might overlap nuclear I κ B α functions in the skin or that ectopic I κ B β affects regulation of cytokines that mediate lethality in I κ B α KO models (Rebholz et al., 2007). It is the strong association between inflammation and the skin phenotype that has impaired distinguishing between cell-autonomous and non-cell-autonomous effects of I κ B α deletion on keratinocyte differentiation. Our data indicate that I κ B α plays an essential role in this process, as shown by the strong decrease in the number of Filaggrin-expressing cells in the skin of I κ B α KO newborn mice, which results in a defective skin barrier that might explain the massive inflammatory response observed in these animals after cytokine exposure. Moreover, this function is cell autonomous as indicated by our in vitro data. However, in transformed keratinocytes and human SCC, I κ B α is accumulated in the cytoplasm associated with tumor progression and increased HOX expression, which is consistent with the tumorigenic phenotype of mice expressing the chromatin binding-defective I κ B α super-repressor mutant in the skin (Dajee et al., 2003; van Hogerlinden et al.,

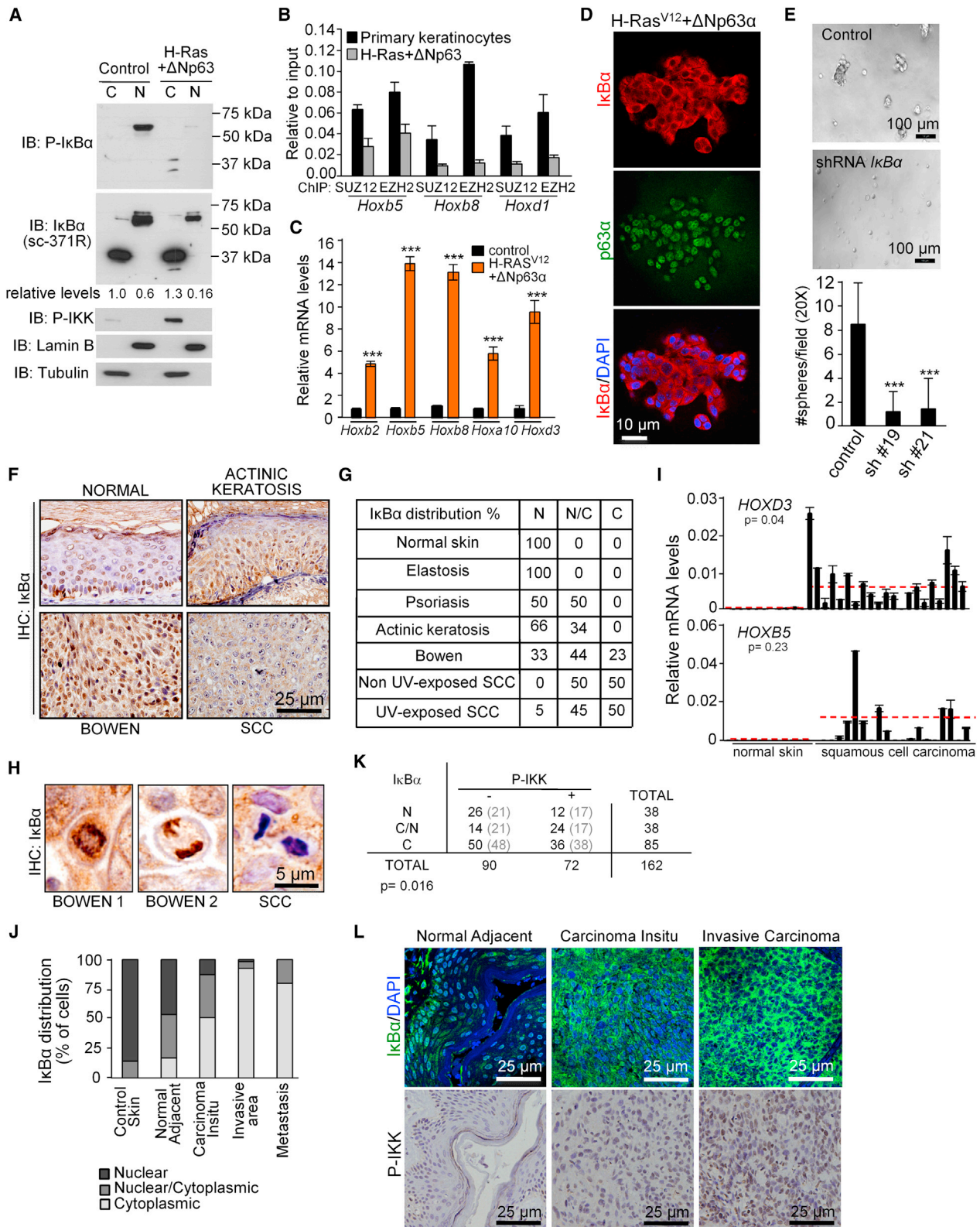


Figure 6. IκBα Accumulates in the Cytoplasm after Keratinocyte Transformation Associated with Hox Induction

(A) IB analysis of IκBα in the subcellular fractions of primary and H-Ras/ΔNp63 transformed keratinocytes.

(B) ChIP analysis of PRC2 recruitment on the indicated genes in control and transformed keratinocytes.

(C) Expression levels of the indicated genes relative to *Gapdh*.

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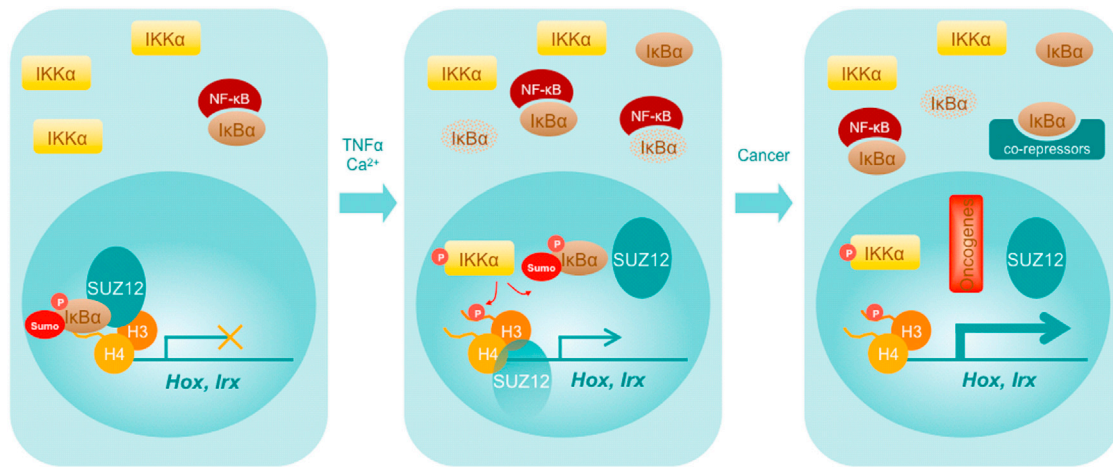


Figure 7. Model for Nuclear I κ B α Function in Normal and Transformed Cells

In brief, sumoylated and phosphorylated I κ B α binds the chromatin of noninduced and nontransformed cells to maintain gene repression, which is relieved after cytokine induction or keratinocyte transformation. Release of I κ B α from the chromatin is, at least in part, mediated by IKK α and results in PRC dissociation and specific gene activation. These effects are maximized by stimuli that lead to tumorigenesis.

1999, 2004). We propose that accumulation of I κ B α in the cytoplasm, or even in the nucleoplasm as it is observed after TNF α exposure, contributes to gene activation by sequestering nuclear repressors. In contrast, knockin mice carrying a nuclear I κ B α mutant do not show defective skin function or signs of SCC, even in aged mice (data not shown). Although we cannot exclude the involvement of NF- κ B in the I κ B α mutant phenotypes, these reports and our data support the concept that nuclear I κ B α through *Hox/Irx* regulation might provide a platform to integrate control of barrier integrity with cytokine response, while preventing cell transformation.

On the other hand, extensive *in silico* analysis of the I κ B α target promoters failed to reveal any enrichment of specific sequences that could mediate binding specificity, which is coherent with the absence of known DNA elements that recognize PRC2 in mammalian genes and is different from *Drosophila* genes that contain PRE elements (Simon et al., 1993). Instead, our data show that I κ B α binds the chromatin in regions enriched for H3K27me3, which is antagonized by specific combinations of histone H2A/H4 modifications. Such H2A/H4 modifications are involved in skin differentiation (Frye et al., 2007) and are known to regulate chromatin structure (Kimura et al., 2002; Shogren-Knaak et al., 2006; Suka et al., 2002), which could be connected with the I κ B α function described here.

Based on our results, we propose a model (Figure 7) in which nuclear PS-I κ B α negatively regulates transcription of developmentally related genes in collaboration with Polycomb proteins. Keratinocyte differentiation or TNF α treatment result in the loss of chromatin-associated I κ B α and Polycomb release, associated with a moderate or temporary activation of *Hox* and *Irx* transcription, respectively. Upon oncogenic transformation, nuclear I κ B α is lost, but it accumulates in the cytoplasm, leading to a massive activation of *Hox* and *Irx* genes, likely related with cytoplasmic retention of negative regulators of gene transcription (Aguilera et al., 2004; Viatour et al., 2003). Future studies will focus on establishing the exact histone code that is recognized by I κ B α and the elements involved in its regulation, both in physiological and pathological situations.

EXPERIMENTAL PROCEDURES

Tissue Samples

Human tissue samples were obtained from the archive of the Pathology Department of the Hospital del Mar with the consent of Bank Tumor Committee, following Spanish Ethical regulations and guidelines of the Declaration of Helsinki. Patient identity remained anonymous. Mouse samples were obtained from pathogen-free mice colonies that included C57Bl6/J, CD1, I κ B α -deficient (Beg et al., 1995), and I κ B α ^{NES/NES} mice (Wuerzberger-Davis et al., 2011). The

(D) IF of I κ B α in the transformed keratinocytes growing in 3D cultures.

(E) Transformation capacity of *H-Ras*/ Δ Np63 α transduced keratinocytes in control or I κ B α KD cells. Image of 3D cultures (upper panels) and graph represents the number of spheres from 5×10^3 cells after 5 days in culture (lower panel). Sixteen fields (20x) from two independent experiments were counted.

(F and G) Analysis by immunohistochemistry of I κ B α distribution in different human skin lesions. (G) Table indicates the percent of nuclear (N), diffuse (N/C), or cytoplasmic (C) distribution in the analyzed samples.

(H) Chromosomal localization of I κ B α in mitotic cells from Bowen's disease samples, but not in SCC cells.

(I) Expression analysis of the indicated genes by qRT-PCR. Red line indicates the mean expression in normal and SCC samples. Significance of the differences was determined using Student's *t* test analysis.

(J) I κ B α distribution in control and different grades of tumor progression in a collection of 112 human urogenital SCC samples.

(K) Correlation between subcellular distribution of I κ B α and P-IKK levels in this group of patients. Statistical significance of the association was determined by a chi-square test.

(L) Representative images showing I κ B α and P-IKK staining in different lesions from the same patient. In (C) and (E) ****p* < 0.001, as determined by Student's *t* test analysis. In (B), (C), (E), and (I), bars represent mean, and error bars indicate SD.

Animal Care Committee of Generalitat de Catalunya, USCD and University of Wisconsin approved all procedures.

Cell Fractionation

Cells were lysed in 10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 0.05% NP40 at pH 7.9 for 10 min on ice and spun at 3,000 rpm. Supernatants were the cytoplasmic fraction. Pellets were lysed in 5 mM HEPES, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and 26% glycerol at pH 7.9, sonicated 5 s three times, left on ice 20 min, and then centrifuged 15 min at 13,000 rpm to recover the soluble nuclear fractions. Pellets included the non-soluble chromatin-enriched fraction.

Coimmunoprecipitation Assay and Peptide Array

For precipitation of nuclear extracts, nuclei were either crosslinked with dithio-bis succinimidyl propionate (DSP, Pierce) 15 min at room temperature and then sonicated in a bioruptor (Diagenode) in 1% SDS-containing buffer and then neutralized by 1% Triton X-100, or directly sonicated in RIPA buffer plus protease inhibitor cocktail (Roche) and 10 mM N-ethyl maleimide. In both cases, nuclear lysates were centrifuged at 13,000 rpm for 15 min and then incubated with 5 μ g of the indicated antibodies. Precipitates were captured with 35 μ l of protein A-sepharose, extensively washed, and analyzed by IB. In most of the experiments, we used the Clean-Blot IP Detection Kit instead of standard secondary antibodies, which is optimized for postimmunoprecipitation IB.

Histone peptides were synthesized as biotinylated N-terminal and C-terminal amides (Peptides and Elephants). Peptides were incubated overnight at 4°C with the indicated cell extracts and precipitated with streptavidin-sepharose beads (Amersham) for 45 min. Denatured HEK293T nuclear extracts were used to hybridize a histone peptide array from Active Motif (N.13001). Peptide composition can be found at <http://www.activemotif.com/catalog/667/histone-peptide-array> and includes different combinations of acetylated, methylated, or unmodified peptides.

Chromatin Immunoprecipitation, ChIP-on-Chip, and ChIP-Seq

Cells were subjected to chromatin immunoprecipitation (ChIP) as previously described (Aguilera et al., 2004). Briefly, formaldehyde crosslinked cell extracts were sonicated, and chromatin fractions were incubated with sc-371, sc-371G, sc-1643 (Santa Cruz Biotechnology), or 06-494 (Upstate) anti-I κ B α antibodies in RIPA buffer and precipitated with protein A/G-sepharose (Amersham). Crosslinkage was reversed, and DNA was analyzed by PCR, sequenced, or labeled to hybridize the Mouse BCBC PromoterChip 5 from the University of Pennsylvania School of Medicine (<http://www.cbil.upenn.edu/EPConDB/>). Data obtained were analyzed with AFM 4.0 software (Breitkreutz et al., 2001). Anti-I κ B α antibody 06-494 (Upstate) was used for ChIP-seq, and 6 ng of precipitated chromatin from human keratinocytes was directly sequenced in these experiments. Data are deposited at the GEO database with accession numbers GSM744581 and GSE24011. For mapping, peak calling, and functional enrichment analysis, see the [Supplemental Experimental Procedures](#).

ACCESSION NUMBERS

The GEO accession number for the ChIP-on-chip and ChIP-seq reported in this paper are GSM744581 and GSE24011, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.ccr.2013.06.003>.

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