



Published in final edited form as:

*J Invest Dermatol.* 2015 April ; 135(4): 1016–1024. doi:10.1038/jid.2014.519.

## RNA-Seq and ChIP-Seq reveals SQSTM1/p62 as a key mediator of JunB suppression of NF- $\kappa$ B-dependent inflammation

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### Abstract

Mice with epidermal deletion of *JunB* transcription factor displayed a psoriasis-like inflammation. The relevance of these findings to humans and the mechanisms mediating JunB function are not fully understood. Here, we demonstrate that impaired JunB function via gene silencing or overexpression of a dominant negative mutant increased human keratinocyte cell proliferation but decreased cell barrier function. RNA-seq revealed over 500 genes affected by JunB loss-of-function which included an upregulation of an array of proinflammatory molecules relevant to psoriasis. Among these were TNF $\alpha$ , CCL2, CXCL10, IL6R and SQSTM1, an adaptor protein involved in NF- $\kappa$ B activation. ChIP-Seq and gene reporter analyses showed that JunB directly suppressed SQSTM1 through binding to a consensus AP-1 cis-element located around 2 Kb upstream of *SQSTM1*-transcription start site. Similar to JunB loss-of-function, SQSTM1-overexpression induced TNF $\alpha$ , CCL2 and CXCL10. Conversely, NF- $\kappa$ B-inhibition genetically with a mutant I $\kappa$ B $\alpha$  or pharmacologically with PDTC prevented cytokine, but not IL6R, induction by JunB-deficiency. Taken together, our findings indicate that JunB controls epidermal growth, barrier formation and proinflammatory responses through direct and indirect mechanisms, pinpointing SQSTM1 as a key mediator of JunB-suppression of NF- $\kappa$ B-dependent inflammation.

### Keywords

JunB; SQSTM1/p62; NF- $\kappa$ B; keratinocyte; proinflammatory cytokine

### Introduction

Psoriasis and psoriatic arthritis affect nearly 3% of the US population(Helmick *et al.*, 2014; Rachakonda *et al.*, 2014). While the etiology of the process is known to be multifold

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### Conflict of Interest

Authors declare no conflict of interest

involving multiple cell types and various genetic and environmental factors, it is clear that a disarrayed T-cell driven immune response is critical for the pathogenesis of psoriasis (Clark, 2010). Current therapies are mostly designed to target immune cells, and treatment results are often mixed and unpredictable (Belge *et al.*, 2014). Recently, it has become apparent that defects in epidermal cells, the predominant barrier cells of the skin, can initiate a wide spectrum of immune responses. This was best demonstrated using conditional genetic animal models. Of particular interest, mice with epidermal deletion of *JunB*, an AP-1 family transcription factor, alone or together with c-Jun, developed joint and skin lesions that bared a remarkable resemblance to human arthritis and psoriasis, respectively (Zenz *et al.*, 2005). Additionally, targeted *JunB* deletion in epidermal cells led to a multi-organ inflammation involving blood, kidney and lymph nodes (Meixner *et al.*, 2008). These studies establish that JunB plays an important role in epidermal cell-mediated innate and adaptive immune responses in mice.

In humans, *JunB* is located in the previously identified psoriasis susceptibility locus *PSOR6* (19p13) (Huffmeier *et al.*, 2009), though the significance of which has not been validated by later GWAS studies. Interestingly, JunB mRNA levels were found increased in psoriatic lesions as compared to nonlesional skin (Haider *et al.*, 2006; Johansen *et al.*, 2004; Kulski *et al.*, 2005), whereas JunB protein levels were reduced in the basal and spinous layers of the lesional epidermis (Park *et al.*, 2009; Zenz *et al.*, 2005). The discrepancy between the mRNA and protein levels may be due to the presence of lymphocytes in the lesional skin or a posttranslational effect. Regardless, the reduced protein expression suggests that JunB-hypofunction in epidermal cells may have a causal effect on epidermal cell proliferation and inflammation. In agreement with this possibility, our recent studies have shown that JunB inhibits, whereas its dominant negative mutant promotes, epidermal growth (Jin *et al.*, 2011).

In this study, we demonstrated that gene silencing of JunB in normal epidermal cells resulted in increased cell proliferation and compromised epidermal barrier function. By using global RNA-seq and CHIP-seq, we found that JunB regulated expression of a wide-range of molecules involved in cell growth, interaction, differentiation and inflammation through both direct and indirect mechanisms. In particular, JunB directly interacted with the consensus AP-1 response element to suppress gene transcription of SQSTM1/p62, an adaptor protein linked to Paget's disease of bone and neurodegenerative disorders, and also upregulated in psoriasis (Rea *et al.*, 2014; Sundaram *et al.*, 2011). SQSTM1/p62 has been characterized as a key regulator involved in NF- $\kappa$ B activation and autophagy (Wooten *et al.*, 2005; Zotti *et al.*, 2014). We found that NF- $\kappa$ B, but not autophagy, is essential for the expression of various psoriasis-relevant chemokines induced by decreased JunB-function. These findings demonstrate that JunB-hypofunction impairs epidermal barrier function, and triggers a proinflammatory response through direct and indirect target gene regulation, and identified SQSTM1 and NF- $\kappa$ B signaling axis as a potential therapeutic target for skin diseases such as psoriasis.

## Results

### JunB loss-of-function leads to an increased keratinocyte cell proliferation but reduced barrier function

To determine the role of JunB in human skin, we first examined JunB loss-of-function effects on epidermal growth and barrier function via siRNA-mediated gene silencing in primary human keratinocytes. The efficiency of gene silencing was confirmed by RT-PCR and Western blotting (Fig. 1a). Cell growth analyses were performed in the absence or presence of TNF $\alpha$  which has been shown to inhibit normal keratinocyte growth in culture (Detmar and Orfanos, 1990). JunB gene silencing induced a significantly higher rate of cell proliferation, and diminished the growth inhibitory effects of TNF $\alpha$  (Fig. 1b). To assess effects on barrier function, we plated cells on permeable membrane inserts for induction of differentiation with 1.2 mM Ca<sup>++</sup>, and then measured the transepithelial electrical resistance (TER) as described (Sumigra *et al.*, 2012). A higher TER value corresponds to a healthier barrier function. Gene silencing of JunB significantly reduced the TER at both 24h and 48h time-points (Fig. 1c), indicating that JunB is important for the epidermal cell barrier formation.

To confirm the growth effects observed with gene silencing, we transduced keratinocytes with retrovirus for stable overexpression of JunB or the dominant negative deletion mutant of JunB (DNJunB) (Fig. 1d). Monolayer cell growth analysis revealed that overexpression of JunB significantly reduced cell proliferation, while DNJunB induced a modest increase of cell growth (Fig. 1e). Consistently, skin grafts regenerated on immunodeficient SCID mice with the transduced keratinocytes showed that JunB reduced epidermal proliferation, whereas DNJunB induced hyperproliferation as indicated by the over 2-fold changes of the number of Ki-67-positive cells (Fig. 1f). These findings indicate that JunB is involved in controlling epidermal cell proliferation and barrier function.

### Global RNA-seq analysis reveals an important role for JunB in regulation of epidermal inflammation and cell adhesion

To examine how JunB regulates epidermal homeostasis, we performed RNA-seq with mRNA isolated from primary human keratinocytes that had been transfected with siRNA oligonucleotides for JunB gene silencing. Around 8-20 million sequence reads passed quality control for each sample, accounting for over 15,000 genes that were expressed at a detectable level in normal human keratinocytes. In response to JunB gene silencing, about 500 genes displayed significant changes with 138 decreased and 84 increased by over 2-fold (Table S1-S2). The JunB responsive genes include many of those involved in inflammation and cell adhesion as shown by the heatmap analysis (Fig. 2a-b). Of the 29 inflammation related genes, over 70% of them were found increased in patient psoriatic lesions in a recent large scale RNA-seq data set (GSE54456) (Li *et al.*, 2014). Additionally, a majority of these molecules have been previously linked to psoriasis and psoriatic arthritis. These include CCL2, CXCL10, CXCL11 and TNF $\alpha$ , as well as receptors such as IL6R and the leukocyte interaction molecule ICAM1 (Antonelli *et al.*, 2009; Danik *et al.*, 2009; Fraticelli *et al.*, 2001; Paukkonen *et al.*, 1995; Plant *et al.*, 2006). Similarly, 70% of the 16 downregulated cell-adhesion molecules were found decreased in patient psoriatic lesions (GSE54456).

To validate the RNA-seq data, we performed RT-PCR for a number of proinflammatory molecules, including TNF $\alpha$ , CXCL10, IL6R and SQSTM1, which were increased in response to reduced JunB expression (Fig. 2c). To further examine whether the altered mRNA levels were reflected at protein levels, we collected conditioned keratinocyte culture media for cytokine analysis. We found that CCL2 and CXCL10 were significantly upregulated in cells with reduced JunB expression (Fig. 2d). In agreement with results observed with gene silencing, overexpression of the dominant negative JunB (DNJunB) also increased the expression levels of multiple cytokines, such as TNF $\alpha$ , CXCL10 and CCL2, as demonstrated by RT-PCR or cytokine analysis (Fig. S1a-b). Additionally, we confirmed by RT-PCR that the cell adhesion molecule CNTN1 was reduced, whereas MMP7, MMP9 and ICAM1 was upregulated, whereas the terminal differentiation marker filaggrin was not changed in response to reduced JunB expression (Fig. S2a). Western blotting further validated the increased expression of ICAM1 in response to JunB gene silencing (Fig. S2b). These results indicate that JunB loss-of-function induces cell adhesion defects and proinflammatory responses reminiscent of psoriasis.

### ChIP-Seq analysis identifies SQSTM1 as a direct JunB target

Next, we performed ChIP-seq analysis to identify JunB direct target genes. To do this, genomic DNA was isolated from primary human keratinocytes for chromosomal immunoprecipitation (ChIP) with an antibody against JunB and then subjected to ChIP-seq analyses. Peak calling using Chipseeqer identified a total of 2377 sequences. Alignment with the RefSeq database revealed that 43% of these sequences were located in gene promoter regions with 17% in the proximal region (2kb+/-) and 26% in the distal region (2-50kb) (Fig. 3a). The rest were located in the introns (32.7%), intergenic regions (20.4%) and exons (0.2%). Motif analysis with ChipSeeqerFIRE demonstrated that AP-1 consensus elements were present with a high confidence score of 10 out of 10 (Giannopoulou and Elemento, 2011) (Fig. 3b), confirming that JunB ChIP achieved a satisfactory enrichment of the putative AP-1 target genes.

Focused peak analysis on UCSC genome browser (hg19) identified a peak on chromosome 5 at about 1.8 kb upstream of the transcription start site of *SQSTM1* (Fig. 3c), which encodes an adaptor protein important for NF- $\kappa$ B activation and autophagy (Sanz *et al.*, 2000), and was recently found upregulated in psoriatic lesions (Lee *et al.*, 2011). In agreement with *SQSTM1* being an actively transcribed gene, its promoter region was enriched with H3K27Ac modification, a marker for active enhancers, and DNase-sensitive, an indicator for open chromatin state. In addition, within the peak harbored a putative AP-1-response element (TGACTCA) (Fig. 3e). To validate the ChIP-seq result, we performed quantitative ChIP-PCR with primers designed to cover the 5' and 3' ends of the AP-1 cis-element. As expected, JunB ChIP achieved an over 10-fold enrichment of *SQSTM1*-sequence (Fig. 3e). To further examine the importance of the AP-1 cis-element, we performed luciferase gene reporter analysis, and found that JunB inhibited gene expression driven by the WT *SQSTM1*-promoter but not the mutant containing a defective AP-1 response-element (Fig. 3f). Thus, JunB suppresses *SQSTM1* expression via the AP-1 cis-element.

### SQSTM1 mediates chemokine expression

Interestingly, among the JunB-responsive genes, only 27 of them were enriched by JunB ChIP-seq (Table S3), which suggests that some of those responsive genes were caused by secondary effects. Specifically, we predicted that SQSTM1 is involved in mediating the upregulation of the inflammatory cytokines. To test this idea, we first confirmed that SQSTM1 was increased at the protein level by JunB gene silencing (Fig. 4a). We then performed gene silencing or retrovirally mediated overexpression of exogenous SQSTM1 (Fig. 4b-c). Quantitative RT-PCR revealed that exogenous expression of SQSTM1 increased CXCL10 and CCL2 mRNA levels as compared to LacZ control (Fig. 4d). Conversely, gene silencing of SQSTM1 prevented the induction of both chemokines by reduced JunB expression (Fig. 4e). These results indicate that SQSTM1 positively regulates chemokine expression.

### NF- $\kappa$ B is required for the induction of CCL2 and CXCL10

Since SQSTM1 is a key mediator of NF- $\kappa$ B activation by TRAF6/IKK $\gamma$  signaling axis (Wooten *et al.*, 2005; Zotti *et al.*, 2014), we predicted that NF- $\kappa$ B is responsible for the increased cytokine expression. In agreement with this possibility, NF- $\kappa$ B activation was induced by gene silencing of JunB as indicated by the increased levels of pIKK $\alpha/\beta$  and pI $\kappa$ B $\alpha$  and NF- $\kappa$ B-driven luciferase expression (Fig. 5a-b). Inhibition of NF- $\kappa$ B via expression of the I $\kappa$ B $\alpha$  mutant (I $\kappa$ B $\alpha$ M), a well-known super-repressor of NF- $\kappa$ B abolished the induction of CXCL10 by SQSTM1 (Fig. 5c). To further test whether NF- $\kappa$ B is required for the cytokine expression, we treated cells with the pharmacological NF- $\kappa$ B inhibitor pyrrolidine dithiocarbamate (PDTC) or autophagy inhibitor chloroquine (CQ) at 24h post-gene silencing. The conditioned culture media were collected for cytokine analyses at the 72h time-point. We found that PDTC but not CQ markedly reduced the protein levels of CCL2 and CXCL10 (Fig. 5d). In line with the results obtained with the pharmacological agents, expression of I $\kappa$ B $\alpha$ M significantly reduced the mRNA levels of TNF $\alpha$ , CCL2 and CXCL10, as shown by quantitative RT-PCR (Fig. 5e). In contrast, IL6R, a direct target of JunB which was previously validated in mouse keratinocytes (Zenz *et al.*, 2005), was not affected by I $\kappa$ B $\alpha$ M. In agreement with the responsiveness to NF- $\kappa$ B, TNF $\alpha$ , CCL2 and CXCL10, but not IL6R, all contained one or more putative NF- $\kappa$ B cis-elements on the promoter region (Fig. S2). These findings indicate that NF- $\kappa$ B is required for the induction of the proinflammatory cytokines.

Taken together, our findings support a working model in which JunB-hypofunction leads to increased expression of SQSTM1 and consequent NF- $\kappa$ B-dependent induction of proinflammatory cytokines (Fig. 6). Additionally, JunB loss-of-function increases cell proliferation, and compromises barrier functions through direct and indirect target gene regulations. Future studies are needed to determine whether the proinflammatory cytokines indirectly affect cell growth and barrier function.

## Discussion

Our studies demonstrate that reduced JunB expression affects multiple cellular processes through direct and indirect regulation of an array of target genes. The most pronounced

consequences of JunB reduction is the increased expression of various proinflammatory chemokines and reduced expression of cell adhesion molecules. SQSTM1, as a direct downstream target of JunB, serves as a critical mediator to the uncontrolled NF- $\kappa$ B activation and subsequent proinflammatory responses.

Our findings suggest that the reduced JunB protein expression in psoriatic lesions, as demonstrated in earlier studies (Park *et al.*, 2009; Zenz *et al.*, 2005), may contribute to the development of the lesions due to the uncontrolled expression of the inflammatory cytokines. In this respect, JunB may be important for the inhibition of the disease onset or progression. Given that JunB mRNA level is increased in psoriatic lesions (Haider *et al.*, 2006; Johansen *et al.*, 2004; Kulski *et al.*, 2005), it is conceivable that the reduced JunB protein level in the lesional skin is due to a posttranscriptional deregulation. Future studies will be necessary to determine whether and how JunB protein translation or stability is altered in psoriatic lesions.

Current therapies for psoriasis are mainly focused on targeting T-cell mediated disease processes (Belge *et al.*, 2014). A chosen treatment option may produce good outcomes for some patients but be less efficient for others, which is likely due to the complex nature of the etiology involving genetic and environmental heterogeneities. Our findings suggest that it might be possible to block the disease at an early stage by interfering keratinocyte-mediated proinflammatory responses. Strategies that modulate the SQSTM1/NF- $\kappa$ B signaling axis in keratinocytes might be applied for disease prevention.

SQSTM1 is implicated in cancers of various organs, including kidney (Li *et al.*, 2013), tuberous sclerosis complex (Parkhitko *et al.*, 2011), pancreatic (Ling *et al.*, 2012), prostate (Chang *et al.*, 2014), lung (Inoue *et al.*, 2012), and oral (Inui *et al.*, 2013; Kuo *et al.*, 2014). However, in cutaneous squamous cell carcinoma, SQSTM1 displays an inverse correlation with malignant progression (Yoshihara *et al.*, 2014), suggesting that SQSTM1 may have epidermis-specific functions. Similar to SQSTM1, NF- $\kappa$ B is known to stimulate inflammation and tumorigenesis in a wide range of tissues. On the other hand, persistent NF- $\kappa$ B activation promotes epidermal aging and inhibits cutaneous neoplasia (Adler *et al.*, 2007; Dajee *et al.*, 2003; Zhang *et al.*, 2004; Zhang *et al.*, 2005b). Thus, NF- $\kappa$ B-inhibition predicts both beneficial and harmful results (Wullaert *et al.*, 2011). It will be important to determine whether SQSTM1 is involved in the regulation of the functional dichotomy of NF- $\kappa$ B, and may be exploited to maximize the beneficial effects of NF- $\kappa$ B-targeted therapy.

## Materials and Methods

### Cell culture, gene transfection and barrier function analysis

Primary human keratinocytes were isolated from surgically discarded foreskin samples obtained from Duke Children's Hospital in accordance to in institutionally approved IRB protocol. Patient consent for experiments was not required because French laws consider human tissue left over from surgery as discarded material. Cells were grown and passaged in keratinocyte serum free media (Invitrogen, Grand Island, NY) at 37 °C with 5% CO<sub>2</sub>, and used at passage 2-3. 293T cells were obtained from ATCC and cultured in 10% FBS/DMEM. Gene transfection was performed with Genjet transfection reagent (SignaGen Laboratories,

Rockville, MD). siRNA oligonucleotides targeting JunB (S7776 and S7662), SQSTM1 (HSS113116) and non-silencing control were purchased from Invitrogen and used at 100nM concentrations. Unless noted, siJunB were performed with S7662. The SQSTM1 cDNA expression construct was obtained from Addgene (Fan *et al.*, 2010), and subcloned into the LZRS retroviral construct. For barrier function analysis, cells were seeded onto to Costar Transwell Permeable membrane inserts at 24h post-transfection, and induced with 1.2 mM calcium for 24h and 48h. TER was measured by using a Millipore Millicell ERS-2 ohm meter. Readings were normalized against wells with no cells (set as zero resistance) and to insert area. A higher resistance indicates better barrier function. The retroviral LZRS expression constructs for JunB and DNJunB were described previously (Jin *et al.*, 2011).

### RNA-Seq and RT-PCR

For RNA-seq, total RNA was isolated from triplicate sets of human keratinocytes at 48h after transfection using RNeasy column purification (Qiagen, Germantown, MD). cDNA library was prepared via oligo-dT-directed reverse transcription (Ambion, Grand Island, NY), and subject to deep sequencing on Illumina HiSeq2000 (50bp single-read sequencing) and analyzed at Duke Genome Sequencing & Analysis Resource. The analysis pipeline includes the initial QC to remove sequencing adaptors and low quality bases to facilitate mapping using fastx toolkit ([http://hannonlab.cshl.edu/fastx\\_toolkit/index.html](http://hannonlab.cshl.edu/fastx_toolkit/index.html)). High quality reads were mapped to the human reference genome (hg19) using tophat (Trapnell *et al.*, 2009). The gene differential expression analysis was performed between control and treatment using cuffdiff with upper quartile normalization, multi-hit correction and 0.5% FDR (Trapnell *et al.*, 2013). Three biological replicates were used for statistical analysis of differential expression for each comparison. Pathway analyses were performed using WEB-based GENE SeT AnaLysis Toolkit (Wang *et al.*, 2013; Zhang *et al.*, 2005a). SYBR green-based real-time RT-PCR was performed in Bio-Rad iCycler with primers listed in (Table S4). Ribosomal 18S RNA or GAPDH was used as an internal control.

### ChIP-Seq and ChIP-PCR

ChIP was performed as described (Guo *et al.*, 2012). Briefly, primary keratinocytes cultured in eight 30-cm plates were scraped off the dish with PBS at about 60-80% confluence, and cross-linked with 1% formaldehyde for 8 minutes, and then treated with 1.25 M glycine for 5 minutes to stop crosslink. Cells were pelleted by centrifuging 5 minutes at 1500 rpm, and lysed with ChIP lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) for cell nuclei isolation. DNA fragmentation was performed by sonication with Branson Sonifier. The fragmented DNA underwent IP with the antibody against JunB (Cell Signaling Technology, Beverly, MA) followed by purification with MinElute DNA kit (Qiagen) and library construction with the NEBNext® DNA library preparation kit (New England BioLabs). The library was sequenced using Illumina HiSeq 2000 platform and analyzed at Duke Genome Sequencing & Analysis Resource. The analysis pipeline includes the initial QC to remove sequencing adaptors and low quality bases to facilitate mapping using fastx toolkit (Martin, 2011). High quality reads were mapped to the human reference genome (hg19) using bwa (Li and Durbin, 2009), and genes lists were from the Ensemble Gene database (<http://www.ensembl.org>) and the RefSeq ([www.ncbi.nlm.nih.gov/RefSeq](http://www.ncbi.nlm.nih.gov/RefSeq)). Distal promoter and terminator regions of the genes were defined as those sequences that mapped +/-2 kb

upstream or  $\pm$  50 kb from transcription “start” and “end” sites, respectively. Peak detection, annotation, de novo motif discovery and pathway analysis were performed using ChIPseeq tool (Giannopoulou and Elemento, 2011), and visualized by UCSC genome browser (Kent *et al.*, 2002). Motif analysis was performed with FIRESeq as described (Mathelier and Wasserman, 2013). ChIP-PCR was performed with primers listed in (Table S4). The raw data and additional information of ChIP-seq and RNA-seq are available from NCBI Gene Expression Omnibus (Accession number GSE63081).

### Multiplex cytokine analysis

For cytokine analysis, human keratinocytes cultured in 12-well plates were transfected in duplicates with 100nM siRNA oligonucleotides targeting non-silencing control and JunB (Invitrogen). At 24h post-transfection, cells were treated with DMSO control, Pyrrolidine Dithiocarbamate (PDTC) or Chloroquine (Sigma, St Louis, MO) for 48h. Cell culture conditioned media were analyzed in tetrads with custom ordered Luminex Screening Assay System (R&D Systems, Pittsburgh, PA). The readings were obtained on a Bioplex reader-200 (Bio-Rad, Hercules, CA) at a flow rate of 60ul/min and 50ul/sample.

### Skin regeneration

Human skin regeneration was performed as described in our previous studies in accordance with protocols approved by the Duke Animal Care and Use Committee (Jin *et al.*, 2011). Briefly, primary human keratinocytes were transduced with retrovirus encoding genes of interest, and seeded onto devitalized human cadaver dermis (National Disease Research Institute) for grafting onto the back of CB17.SCID mice (n=3-5 per group, purchased from National Cancer Institute). Animals were euthanized at 6 weeks post-grafting for tissue collection.

### Western blotting and immunostaining

For western blotting, 20ug protein lysates of primary human keratinocytes collected at 48-72h post transfection were separated on SDS-PAGE, and immunoblotted with antibodies against JunB, SQSTM1, I $\kappa$ B $\alpha$  and actin, followed by IR-dye-conjugated secondary antibodies (Invitrogen). The blots were scanned on the Odyssey imaging system (Li-COR, Lincoln, NE). For immunostaining, 5um frozen tissue sections were fixed with cold methanol, incubated with primary antibodies against Ki-67, and detected with a secondary antibody conjugated to Alexa 5 (Invitrogen) and counterstaining with Hoechst 33342. The images were obtained with the Olympus imaging system.

### Gene reporter analysis

NF- $\kappa$ B-luciferase (Promega) and *SQSTM1*-luciferase reporter constructs were transfected together with the CMV-renilla luciferase and genes of interest into 293T cells using Genjet transfection reagent. Dual luciferase assay was performed at 24h post-transfection. The relative luciferase reading unit (RLU) was obtained by normalizing the readings of firefly luciferase to that of renilla luciferase, and then to that of the control cells. The p-values were obtained in Excel with two-tiered student T-test.



## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgement

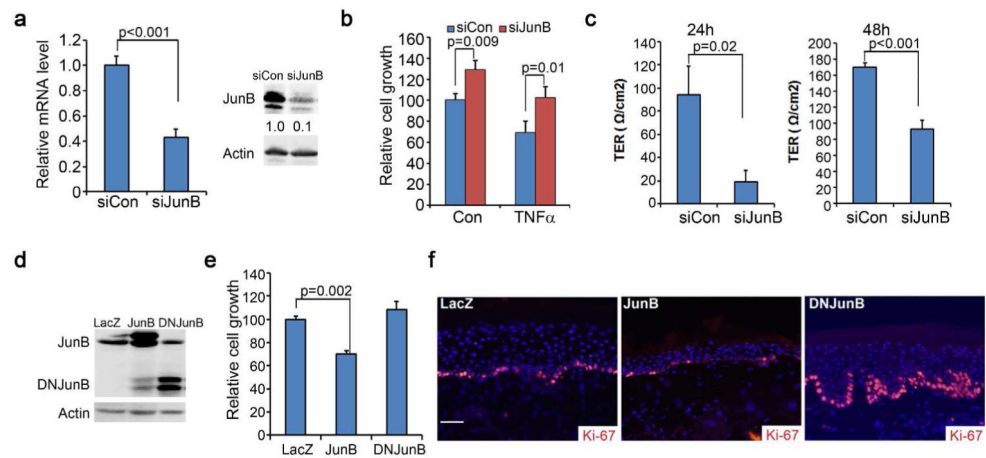
This work was supported by NIH/NIAMS R01 to Jennifer Zhang. We thank Olivier Fedrigo, Yiping He and Changcun Guo of Duke University, Howard Change and Paul Khavari of Stanford University for their advice on ChIP-seq experiments, and Terry Lechler of Duke University for assistance in barrier function analysis. We also thank Mitsuyasu Kato of University of Tsukuba for the cDNA expression constructs of JunB and DNJunB, and Paul Chiao of Texas University for the *SQSTM1*-promoter driven luciferase expression construct.

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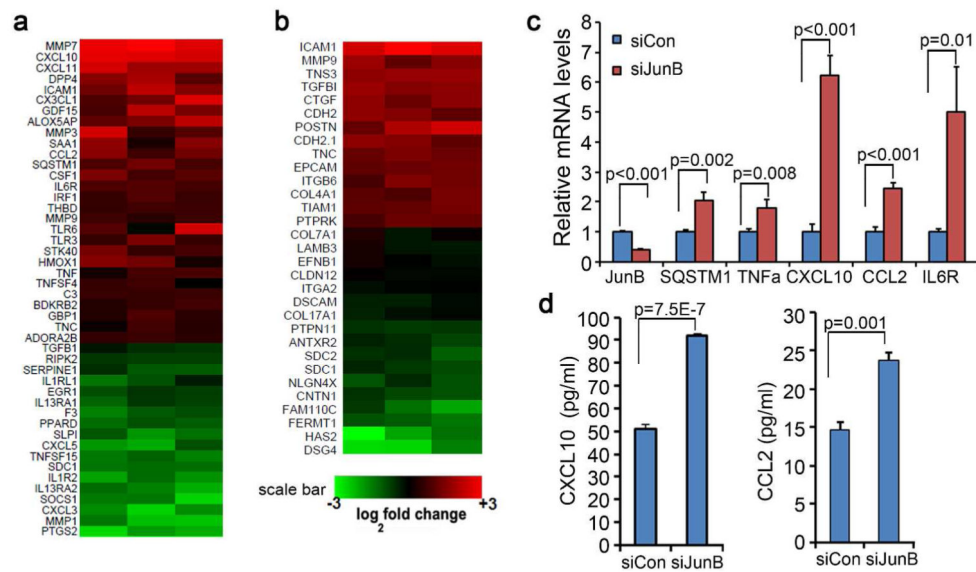
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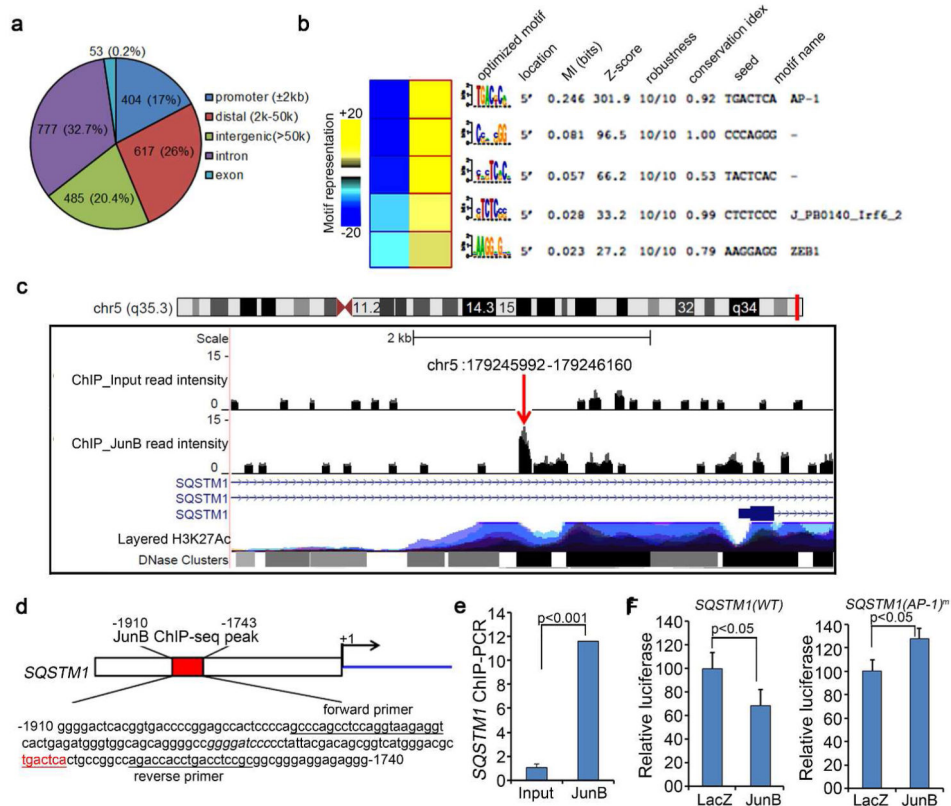


**Figure 1. JunB loss-of-function leads to increased epidermal cell proliferation and reduced barrier function**

(a) Confirmation of gene silencing via real-time RT-PCR and Western blotting. Relative expression levels of JunB were shown below each band via densitometry. (b) Cell growth of human keratinocytes treated with or without 50ng/ml TNF $\alpha$  for 72h. (c) Barrier functions as measured by the Transepithelial Electrical Resistance (TER). Keratinocytes were induced to differentiate with 1.2 mM Ca $^{++}$  on permeable membranes for 24h and 48h prior to TER measurement. (d) Confirmation of gene expression via immunoblotting. (e) Cell growth analysis. (f) Immunostaining of 6-week old skin grafts generated on SCID mice with human keratinocytes transduced to express genes as indicated. Ki-67 [orange] and nuclei [blue]. Graphs represent the average values of triplicate samples + SD. P-values were obtained via two-tiered student T-test. Scale bar=100 $\mu$ m.

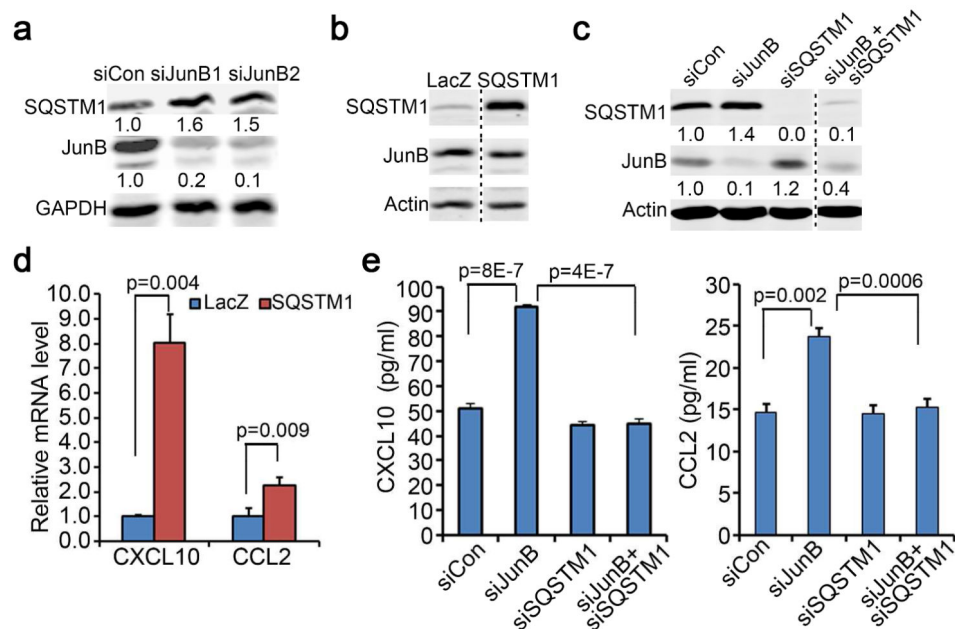


**Figure 2. RNA-seq reveals JunB-targets involved in inflammation and cell adhesion**  
**(a-b)** Heat map analysis of gene expression of 3 independent sets of human keratinocytes underwent gene-transfection with siRNA oligonucleotides targeting JunB (siJunB) or nonsilencing control (siCon). **(c)** Real-time RT-PCR for JunB, TNF $\alpha$ , IL6R, CCL2, CXCL10 and SQSTM-1. Graph represents average fold changes of triplicate samples + SD. **(d)** Cytokine analysis of cell culture conditioned media. Graph represents average of tetrad samples + SD.



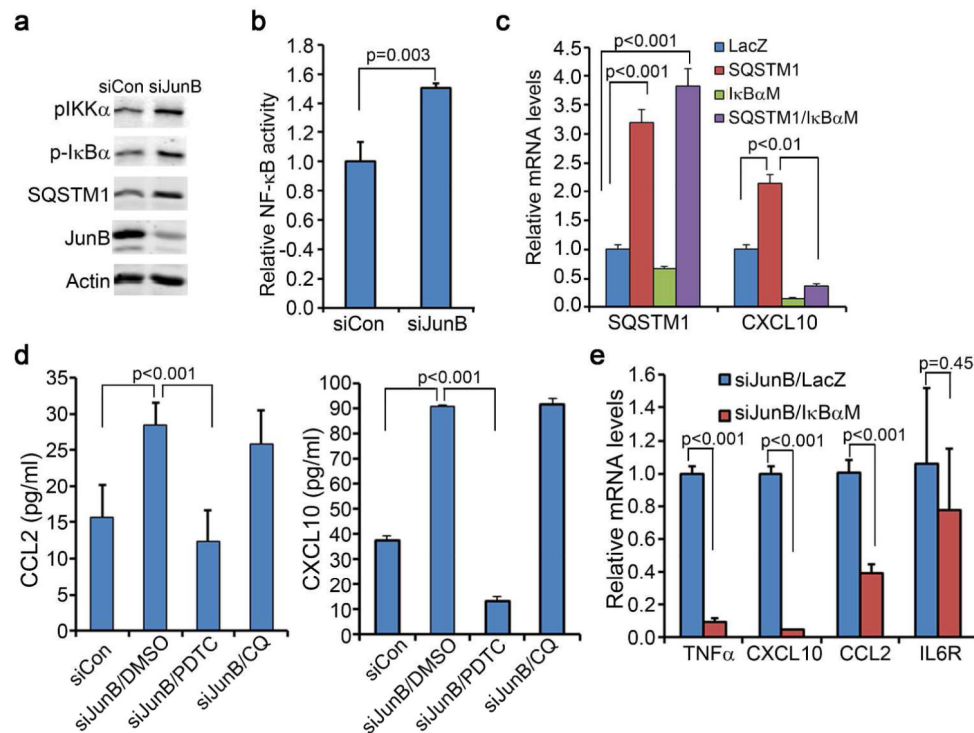
**Figure 3. ChIP-seq identifies p62/SQSTM1 as a JunB-target gene**

(a) Percent of ChIP-seq distribution. (b) Motif analysis with ChIPseekerFire. (c) A representative ChIP-seq peak located on *SQSTM1*. (d) DNA sequence of the *SQSTM1*-promoter (Promoter ID: 33839; <http://rulai.cshl.edu/cgi-bin/TRED>). The consensus AP-1 element  $-5'TGACTCA3'$  was underlined in red. (e) ChIP-PCR with primers marked in (d). Graph represents the average fold enrichment of triplicate samples by JunB-ChIP over the input DNA + SD. (f) Dual luciferase gene reporter analysis of 293T cells transfected with the firefly luciferase reporter constructs containing the WT *SQSTM1*-promoter or the mutant with a defective AP-1 element along with the CMV-renilla luciferase and JunB expression constructs. Graph represents the average of relative luciferase reading units + SD.



#### Figure 4. SQSTM1 positively regulates chemokine expression

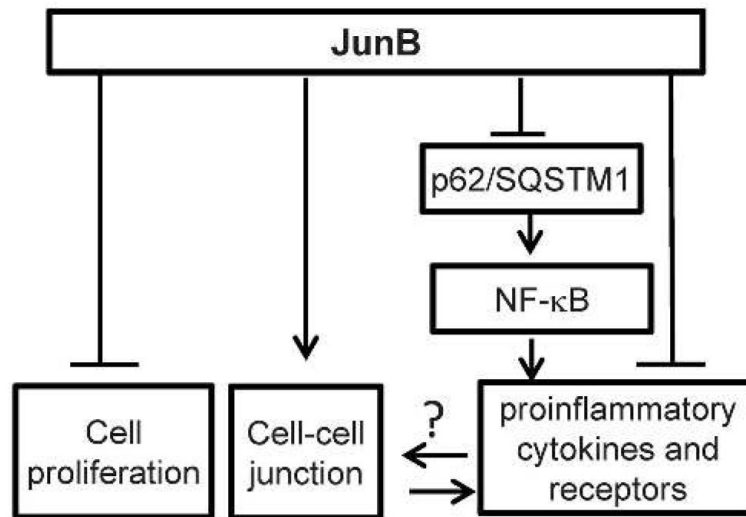
(a-c) Immunoblotting of SQSTM1, JunB, Actin or GAPDH with protein lysates collected from human keratinocytes at 48h post gene transduction. Relative expression levels of SQSTM1 and JunB were shown below each band. Note: Results obtained with two different siRNA oligonucleotides (Invitrogen S7776 and S7662) targeting JunB were comparable. Most studies were performed with the second oligonucleotide. (d) Relative mRNA levels of CXCL10 by real-time RT-PCR. Graph represents relative mRNA levels of triplicates + SD with GAPDH used as the internal control. (e) Cytokine analysis of cell culture conditioned media. Graph represents average of tetrad samples + SD.



### Figure 5. NF- $\kappa$ B is required for cytokine induction

(a) Immunoblotting for pIKK $\alpha$ , pI $\kappa$ B $\alpha$ , p62 and Actin of protein lysates isolated from keratinocytes at 48h post-transfection. (b) Dual luciferase NF- $\kappa$ B-gene reporter assay. (c) Relative mRNA levels of SQSTM1 and CXCL10 in human keratinocytes at 48h post-gene transduction as determined by real-time RT-PCR with 18S RNA used as the internal control. (d) Cytokine analysis of keratinocyte conditioned culture media collected at 48h after treatment with 20uM PDTC or 10uM chloroquine (CQ). (e) Relative mRNA levels of TNF $\alpha$ , CXCL10, CCL2 and IL6R analyzed by RT-PCR as in described above. Graphs represent the average values of triplicate or tetrad samples + SD. P-values were obtained via two-tiered student T-test.





**Figure 6. Working model**

JunB-suppression of inflammation. JunB regulates epidermal cell proliferation, cell adhesion and inflammation through direct and indirect target genes. In particular, JunB-hypofunction leads to increased expression of SQSTM1 and consequently NF- $\kappa$ B-dependent expression of multiple inflammatory cytokines.