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p63-Specific Activation of the *BPAG-1e* Promoter

Motonobu Osada,* Yuichi Nagakawa,† Hannah Lui Park,* Keishi Yamashita,* Guojun Wu,* Myoung Sook Kim,* Alexey Fomenkov,‡ Barry Trink,* and David Sidransky*
*Department of Otolaryngology, Division of Head and Neck Cancer Research, †Department of Surgery, and ‡Department of Dermatology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

***p63*, a member of the *p53* superfamily, is an essential cell fate determinant for stratified epithelium. Deficiency of *p63* leads to lack of differentiated epithelium from the skin and the presence of trace undifferentiated cells left in the dermis. We found that transcriptionally active isoforms of p63, TAp63β and TAp63γ, activated the skin-specific promoter of bullous pemphigoid antigen 1 (BPAG-1). The p63-response element was localized between bases −177 and −153 upstream of exon 1 in the *BPAG-1e* promoter, whereas regions surrounding the response element suppressed transcriptional responses to p53 and TAp73β, resulting in p63-specific activation of the promoter. This represents a novel molecular mechanism by which target gene induction by p63 is distinguished from induction by other p53 family members.**

Key words: BPAG-1/p53/p53-response element/p63
J Invest Dermatol 125:52–60, 2005

p63 is a member of the p53 tumor suppressor gene family. Similar to p53, p63 is a transcription factor that activates target genes through sequence-specific DNA binding (Schmale and Bamberger, 1997; Osada *et al*, 1998; Senoo *et al*, 1998; Trink *et al*, 1998; Yang *et al*, 1998). The *p63* gene plays a pivotal role in skin development and the *p63*-deficient mouse displays defects in its skin epithelium and appendages. The surface epithelium of *p63* knockout mice consists of a single cell layer that fails to differentiate into mature stratified epithelium. This altered morphology is due to crucial roles for p63 in the maintenance of stem cells in the skin cell lineage or through failure of commitment to the stratified epithelial lineage (Mills *et al*, 1999; Yang *et al*, 1999). It has been reported that mutations in *p63* cause several ectodermal dysplasia and limb abnormalities, such as EEC (ectodactyly, ectodermal dysplasia), AEC (ankyloblepharon, ectodermal dysplasia, clefting), LMS (limb-mammary syndrome), SHFM (split hand/foot malformation), and ADULT (acro-dermato-ungual-lacrimal-tooth) syndromes. The ectodermal symptoms of these syndromes are dependent on the specific types of p63 mutations (Celli *et al*, 1999; Ianakiev *et al*, 2000; Amiel *et al*, 2001; McGrath *et al*, 2001; van Bokhoven and Brunner, 2002).

Recently, in an effort to identify candidate p63 target genes by microarray analysis, we identified several plakin family genes, including *bullous pemphigoid antigen 1* (*BPAG-1*), *envoplakin*, and *periplakin*, induced by TAp63β and/or TAp63γ.[1] Plakins are cytolinker proteins that associate with cytoskeletal elements and junctional complexes (Leung *et al*, 2001; Leung *et al*, 2002). Mutations of plakin genes or autoimmune diseases targeting plakins cause skin blistering disorders (Borradori and Sonnenberg, 1999; Leung *et al*, 2002). In the *EVPL* promoter, a p63-specific response element was found in the 5′ promoter region. Moreover, we found that *BPAG-1* was uniquely activated by p63, not by p53 or p73.

The human *BPAG-1* gene has various alternatively spliced isoforms (Okumura *et al*, 2002; Roper *et al*, 2002). These isoforms are expressed from 4 different promoters, P1, P2, P3, and Pe, and exhibit tissue-specific expression (Roper *et al*, 2002). One of the major transcripts, *BPAG1-e*, is induced from promoter Pe and is expressed mainly in the epidermis. Several skin-specific regulatory elements on the *BPAG1-e* promoter have been described (Tamai *et al*, 1993, 1994a, b, 1995; Matsuzaki *et al*, 2003). The effect of p63 on the regulation of *BPAG-1e* promoter activity, however, has not been reported.

To clarify the p63-specific transactivation mechanism of the *BPAG-1e* promoter, we examined the ~1.1 kb region upstream of exon 1 and identified a p53-type response element at −177 to −153. The response element consisted of two half-sites of a p53-type binding motif (RRRCWWGYYY) (el-Deiry *et al*, 1992; Vogelstein and Kinzler, 1992) with several mismatches and a 5-bp gap between the two half-sites. Although the response element itself was activated by all p53 family members, the surrounding sequences suppressed activation by p53 and p73. Our studies thus demonstrate a novel mechanism of expression regulation, which confers p63-specific gene activation.

Results

***p63*-deficient mice lack skin BPAG-1 expression** Recently we demonstrated that BPAG-1 was specifically in-

Abbreviations: BPAG, bullous pemphigoid antigen 1; EEC, ectodactyly, ectodermal dysplasia; SHFM, split hand/foot malformation
[1]Manuscript, in preparation.

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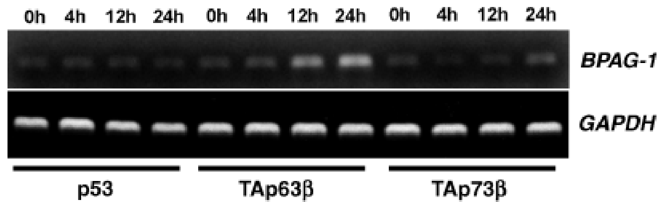

Figure 1
Bullous pemphigoid antigen 1 (BPAG)-1 mRNA induction in TAp63-β-inducible Saos2 cells. Cells were treated with tetracycline to induce p53, TAp63β, and TAp73β and harvested for BPAG-1 expression analysis by RT-PCR. BPAG-1 was clearly induced by TAp63β.

duced by TAp63γ in a Saos2-inducible cell line by oligonucleotide microarray analysis.[2] As shown in Fig 1, *BPAG-1* was endogenously expressed at low levels and was clearly upregulated by TAp63β induction. To test whether BPAG-1 expression was also affected by p63 *in vivo*, we stained p63-deficient mouse skin with BPAG-1 antibody (Fig 2). Consistent with previous reports, *p63−/−* mouse skin did not have stratified epithelium, displaying only remnants of undifferentiated cells on the surface of the dermis. BPAG-1 was detected throughout the entire epithelium in wild-type mouse skin, especially in the basal layer of the epithelium and also relatively weakly in the dermis, which is similar to the staining pattern of TAp63β and TAp63γ (Nylander *et al*, 2002). BPAG-1 was, however, not detected in the single layer epithelium on the surface of the *p63−/−* mouse. The 4A4-p63 antibody stained the basal layer of the stratified epithelium in wild-type *p63* mice (Yang *et al*, 1998). Although the 4A4 antibody can recognize all p63 isoforms, the transcriptionally active isoforms, TAp63β and TAp63γ, which are expressed suprabasally in stratified epithelium, are very labile and may not be detected by the 4A4 antibody (Osada *et al*, 2001; Ghioni *et al*, 2002; Nylander *et al*, 2002; Serber *et al*, 2002). ΔNp63α has also been shown to act as a transactivator in keratinocytes and is the most predominant isoform in skin (Dohn *et al*, 2001 and King *et al*, 2003). These results show the coordinate expression between p63 and BPAG-1 in the skin. p53 and p73 protein expression were also examined in p63−/− mouse skin; however, no protein expression was detected in the single layer epithelium.

The *BPAG-1* promoter is activated by some p63 family members To examine the ability of p63 to activate skin-specific *BPAG-1* expression, we cloned the 1.1 kb *BPAG-1e* promoter, which induces *BPAG-1* expression specifically in the skin, and examined its response to p63 and also to other p53 family members. Figure 3*a* shows the transactivation activity of various p53 family members on the *BPAG-1e* promoter in Saos2 cells. Our rationale for using Saos2 cells is that the Saos2 cell line has a homozygous deletion of p53 and thus has been extensively used in p53 research (Chen *et al*, 1990; Diller *et al*, 1990). Whereas various levels of p63 expression in Saos2 cells have been reported by other investigators, the Saos2 cells used in our laboratory express undetectable levels of p53, p63, and p73 proteins (Wu *et al*, 2005) (Fig 3*c*). Thus, transactivation is presumably not inhibited by either mutant p53 or ΔN isoforms of p63 and/or p73. To study *BPAG-1* promoter regulation in a relevant cellular environment, we also used HaCaT keratinocytes, which express ΔNp63α and mutant p53 (Lehman *et al*, 1993; Papoutsaki *et al*, 2004). ΔNp63α has also been shown to exhibit transactivation ability in keratinocytes (Dohn *et al*, 2001; King *et al*, 2003).

In both cell lines, TAp63β and TAp63γ strongly activated the reporter, and ΔNp63β, which has a second activation domain in its carboxy-terminus (Ghioni *et al*, 2002; Serber *et al*, 2002), activated the reporter with less efficiency than TAp63β (Fig 3*a* and *b*). Other p63 isoforms and p53 family members activated the promoter less than 10-fold when compared to mock transfection. Transactivation levels were relatively lower in HaCaT cells than in Saos2 cells except for transactivation by TAp63β, perhaps due to a dominant negative effect of mutant p53 and/or ΔNp63α. To test for potential synergistic activation or dominant negative inhibition of promoter activity, p53 or TAp73β were co-transfected with TAp63γ or TAp63β. No activation or repression, however, was observed in these co-transfection experiments (data not shown). Figure 3*c* shows the protein expression of exogenously expressed p53, p63, and p73 in Saos2 and HaCaT cells. Consistent with previous reports, TAp63β and TAp63γ isoforms exhibited lower steady state levels of protein expression than TAp63α and ΔNp63 isoforms (Osada *et al*, 2001; Ghioni *et al*, 2002; Serber *et al*, 2002). Thus, the higher levels of *BPAG-1e* promoter activation by TAp63β and TAp63γ were not due to higher levels of protein expression.

TAp63β and TAp63γ respond to the p53-type response element in the −177 to −153 region of the *BPAG-1e* promoter In order to clarify the p63-response element in the *BPAG-1e* promoter, we made a series of 5′ ∼ 100 bp deletion plasmids. The deletion plasmids were co-transfected with TAp63β, and luciferase activity was measured in Saos2 cells (Fig 4*a*). From −1072 to −192, luciferase activity gradually decreased. Between −192 and −152, the transactivation by TAp63β was completely lost. The same sets of transfections were performed in HaCaT cells in order to examine the effect of other tissue-specific transcription factors on BPAG-1e promoter activation by p63. Similar to the results seen in Saos2 cells, the region between −192 and −152 was critical for transactivation by TAp63β. In the case of HaCaT cells, the *BPAG-1e* promoter was also activated by endogenous transcription factors. Between −272 and −192, there was a strong *cis*-element responsive to tissue-specific transcription factors and the region corresponds to KRE3 which was previously reported (Tamai *et al*, 1995). This tissue-specific responsive region (−272 to −192) was, however, distinguished from the p63 responsive region (−192 to −152).

In the p63 responsive region's proximity, four potential p53-type response element half-sites, each with two to three mismatches, were observed, each within 13 bp of the next half-site (−190 to −136, Fig 5). In order to determine which element responds to p63, six different combinations of putative elements were cloned into the luciferase plasmid and examined for activation by p53 family members in Saos2 and HaCaT cells. As shown in Fig 5, the combination of elements 2 and 3 was activated by p53, TAp63γ, TAp73β,

[2]Manuscript, submitted.

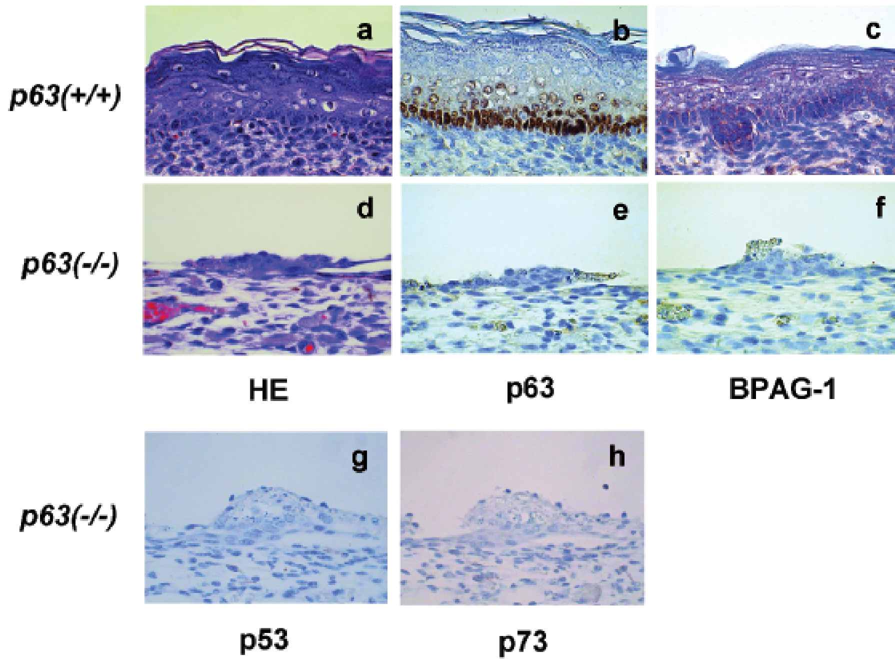

Figure 2
Immunohistochemical staining of bullous pemphigoid antigen 1 (BPAG)-1 in p63+/+ and p63−/− mice skin. (*a–c*) e *p63*+/+ and (*d–h*) *p63*−/−. *a* and *d* were stained with hematoxylin–eosin, *b* and *e* were stained for p63, *c* and *f* were stained for BPAG-1, *g* was stained for p53 and h was stained for p73. Absence of p63 and BPAG-1 staining are evident in the skin of *p63* knockout mice.

and especially TAp63β. Reporters that contained elements 1 or 4 were less activated by p53 and TAp73β than reporters containing elements 2 and 3. These data suggest that elements 2 and 3 constitute the two half-sites of the p63-response element and that the specific activation of the *BPAG-1e* promoter by p63 was due to the presence of suppressive sequences for p53 and TAp73β surrounding the response element.

To test whether p63 proteins bind to the endogenous *BPAG-1e* promoter, we performed a chromatin immunoprecipitation assay with ectopic expression of p53 family members in Saos2 cells. As shown in Fig 6, the *BPAG-1e* promoter was PCR-amplified from the TAp63β and TAp63γ precipitates but not from p53 or TAp73β precipitates. These data demonstrate the unique binding affinity of p63 to the *BPAG-1e* promoter *in vivo*.

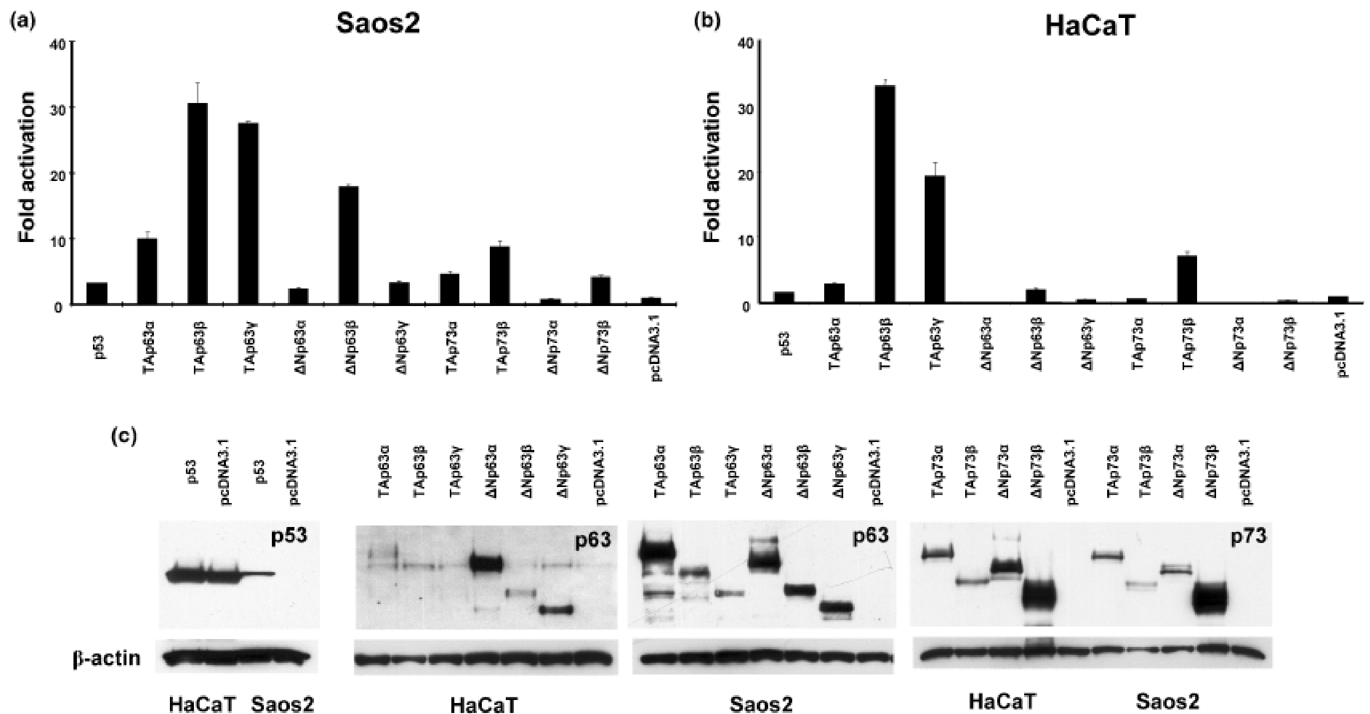

Figure 3
Activation of the bullous pemphigoid antigen 1 (*BPAG-1*)e promoter by p63. (*a*) A luciferase plasmid containing 1.1 kb of the *BPAG-1e* promoter upstream of the luciferase gene was co-transfected into Saos2 cells with p53 family gene expression plasmids. Y-axis represents fold activation of luciferase activity exhibited by the *BPAG-1e* (−1072/+27) promoter in the presence of p53 family members. Luciferase activity of the *BPAG-1e* promoter with empty pcDNA3.1 was arbitrarily set to 1. (*b*) The same set of reporter assays was performed in HaCaT keratinocyte cells. (*c*) Immunoblotting of exogenously expressed p53, p63, and p73 protein in HaCaT and Saos2 cells.

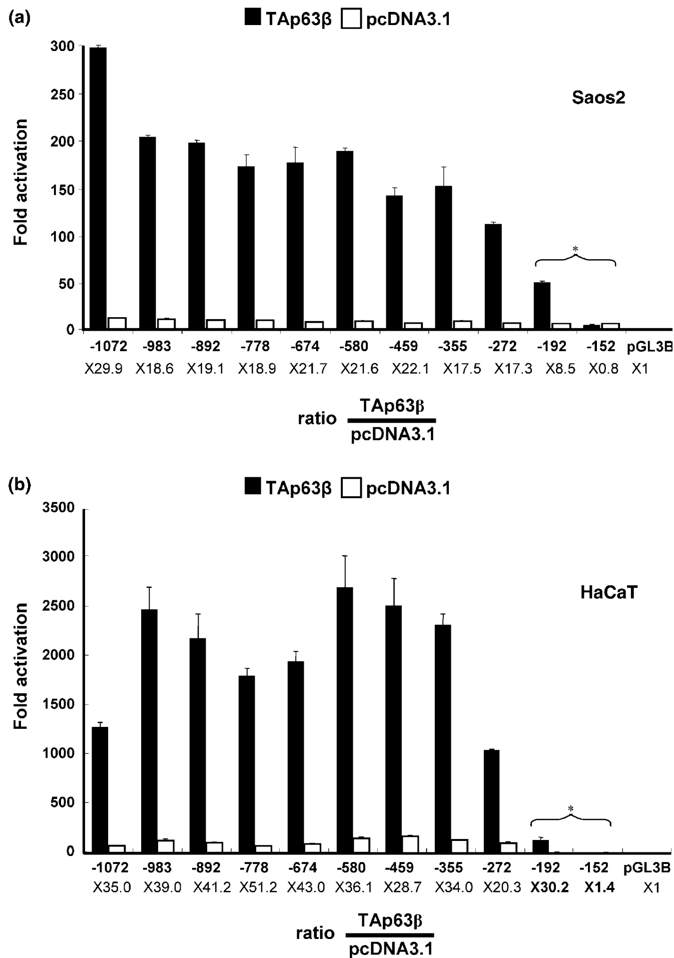

Figure 4
p63-response elements are located between −192 and −152 in the bullous pemphigoid antigen 1 (BPAG-1)e promoter. (a) Serial deletion mutants of *BPAG-1e* promoter plasmids were co-transfected with TAp63β and luciferase activity was measured in Saos2 (*a*) and HaCaT (*b*) cells. Luciferase activity exhibited by cells transfected with pGL-3Basic was arbitrarily set to 1. The transcriptionally active isoforms of p53 family members have a general repressor effect on transcription; thus, the values were normalized by using an internal control vector for Renilla luciferase (Johnson *et al*, 2003). p63-specific activation is represented by TAp63β/pcDNA3.1 ratio values. (*) indicates location of p63-response elements.

SHFM and EEC syndrome-derived mutants differentially activate the *BPAG-1e* promoter SHFM and EEC syndromes are hereditary syndromes characterized by mutations in the DNA binding domain of p63 (Brunner *et al*, 2002; van Bokhoven and McKeon, 2002). Individuals with EEC syndrome exhibit various skin and appendage symptoms, whereas individuals with SHFM do not exhibit such symptoms. The p63 mutations present in EEC syndrome, such as R204, R279, R280, and R304, correspond to R173, R248, R249, and R275 in p53. These p53 mutations have typically been found in human cancers and are located in the residues that have direct contact with DNA. On the other hand, the SHFM mutations, K193 and K194, presumably do not involve residues that have direct contact with DNA and are not assumed to have a severe inhibitory effect on DNA binding. In order to clarify the functional differences between EEC syndrome-derived and SHFM-derived p63 mutations, we examined the effect of these mutations on the transcriptional activity of three p63 target gene promoters: *BPAG-1e* as a p63-specific target, *WNT-4* as a target for p63 and p73, and *CLCA-2* as a common target for all p53 family members.

Figure 7*a* shows the transactivation activity of SHFM and EEC syndrome-derived mutant p63 proteins on the *BPAG-1e* promoter. K193E and K194E, which were derived from SHFM, retained 30% to 100% transactivation activity compared to wild-type p63. On the other hand, the mutants derived from EEC syndrome almost completely lost all transactivation function on the *BPAG-1e* promoter. On the *WNT-4* promoter, the K193E and K194E mutations of TAp63γ exhibited 20% to 25% transactivation ability compared with wild-type TAp63γ (Fig 7*b*). On the other hand, TAp63β and TAp63γ with K193E or K194E mutations activated the *CLCA-2* promoter to virtually to same extent as wild-type proteins (Fig 7*c*). The mutations of EEC syndrome-derived mutants resulted in loss of transactivation activity on all three promoters (Fig 5*a–c*). Based on these observations, we speculate that the mutations of EEC syndromes are loss of function mutations, whereas the mutants derived from SHFM mutations possess differential transactivation activity depending on the promoter structure. These differences could underlie the differential skin symptoms observed between these two syndromes.

Discussion

p63 plays an essential role in stratified epithelium development. The *p63*-deficient mouse lacks stratified epithelium due to the absence of stem cells in the skin and/or the inhibition of commitment to the stratified epithelium lineage (Mills *et al*, 1999; Yang *et al*, 1999). ΔNp63α is highly expressed in holoclone-generating keratinocytes, which are thought to constitute a peripheral stem cell fraction in the skin (Pellegrini *et al*, 2001). TAp63α expression under the control of the Keratin 5 promoter induces the early stages of differentiation in skin but inhibits terminal differentiation (Koster *et al*, 2004). By using a TA-specific antibody, it has been shown that TAp63 proteins are expressed in the supra-basal layer of stratified epithelium (Nylander *et al*, 2002). ΔNp63α, a major isoform expressed in skin, produces dominant negative effects against TAp63 isoforms (Yang *et al*, 1998); however, ΔNp63α also functions as a sequence-specific transcription factor in keratinocytes (Dohn *et al*, 2001; King *et al*, 2003). Thus, clarifying the target genes of p63 via sequence-specific transcriptional activation is important for understanding skin development.

We analyzed the *BPAG-1e* promoter in two cell lines, Saos2 and HaCaT. In both cell systems, we found that p63 activated the *BPAG-1e* promoter to a higher extent than other p53 family members. Interestingly, the surrounding sequences suppressed transcriptional activation by p53 and TAp73β. Previously, we found two criteria for p63-specific transactivation: (1) the response element (RRRCW WGYYY, RE1) itself has higher affinity for p63 than for p53 and (2) the p63-response element (RE2) together with the RRRCWWGYYY (RE1)-type response element results in p63-specific transactivation (see footnote 2). In this study, we demonstrated that the structure exhibited by the *BPAG-*

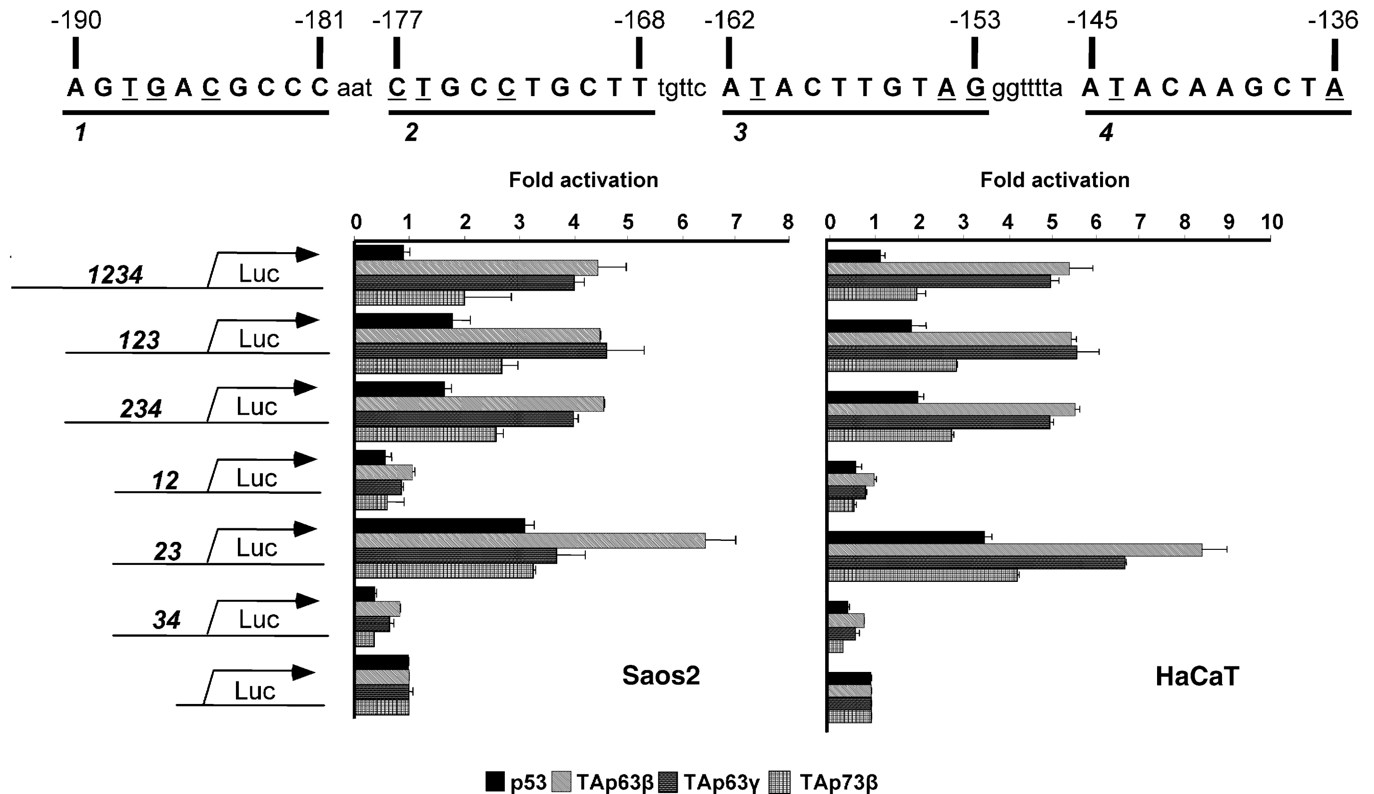

Figure 5
Identification of the p63-response element in the bullous pemphigoid antigen 1 (*BPAG-1*)*e* promoter. The putative p63-response elements were cloned into the pGL3-Basic plasmid and luciferase activity was examined upon co-transfection with p53, TAp63β, TAp63γ and TAp73β in Saos2 (a) and HaCaT (b) cells. −191/−181 (element 1), −177/−168 (element 2), −162/−153 (element 3), and −145/−136 (element 4) are putative p63-response elements. Underlined bases are mismatched bases from the p53 consensus sequence.

1e promoter uncovers another pathway for specific p63 activation. Several p63 or p73 unique target genes, such as *JAG1*, *JAG2*, and *IL4R*, have been reported (Sasaki *et al*, 2002, 2003). In the case of *JAG1* and *IL4R*, the p63- and p73-response elements were reported to reside within their introns. We also examined the 5 tandem repeats of p53-type binding elements in intron 2 of the *JAG2* gene (unpublished data). However, these response elements themselves are activated not only by p63 or p73, but also by p53. Thus, it is likely that suppressive elements specific for p53, and not for p63 or p73, are located elsewhere in these gene promoters as in the case of the *BPAG-1e* promoter.

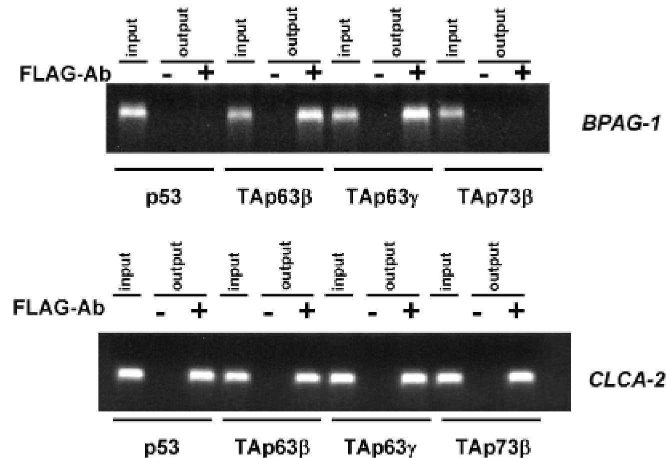

Figure 6
Chromatin immunoprecipitation of the bullous pemphigoid antigen 1 (*BPAG-1*)*e* promoter by p63 protein. Chromatin immunoprecipitation analysis using ectopically expressed FLAG-tagged p53 family proteins. After induction of p53, TAp63β, TAp63γ, and TAp73β with 1 μg per mL of tetracycline, cells were fixed with formaldehyde and immunoprecipitated with anti-FLAG antibody. Only p63 precipitated the *BPAG-1e* promoter fragment. *CLCA-2* (*calcium-dependent chloride channel-2*) was used as a positive control for all p53 family members. The *CLCA-2* promoter has a p53-type response element in the −73 to −54 region of its promoter and is activated by p53, p63, and p73.

The mechanisms responsible for suppression of transactivation by p53 and p73 based on the sequences surrounding the p63-RE in the *BPAG-1e* promoter are unknown. *In vitro* protein–DNA interaction analysis by electromobility shift analysis using −177/−153 and −190/−136 oligonucleotides did not show p63-specific binding. These oligonucleotides gave very little to no binding signals for p53 and p63, presumably due to the 5-base gap between each half-site, a result which is consistent with our previous EMSA observations (see footnote 2). The sequences flanking the p63-RE have two potential half-sites within a 13-bp gap. However, both sequences functioned as suppressive elements rather than activating elements. The obvious differences between these half-sites and the canonical p53-type response element (RRRCWWGYYY) are: (1) the 5′ element, −191/−181, has a mismatch in the fourth position (C to G) and (2) the 3′ element, −145/−136, has a 7-bp gap between it and the next response element, which is relatively rare in previously reported p53-RE (Tokino *et al*, 1994;

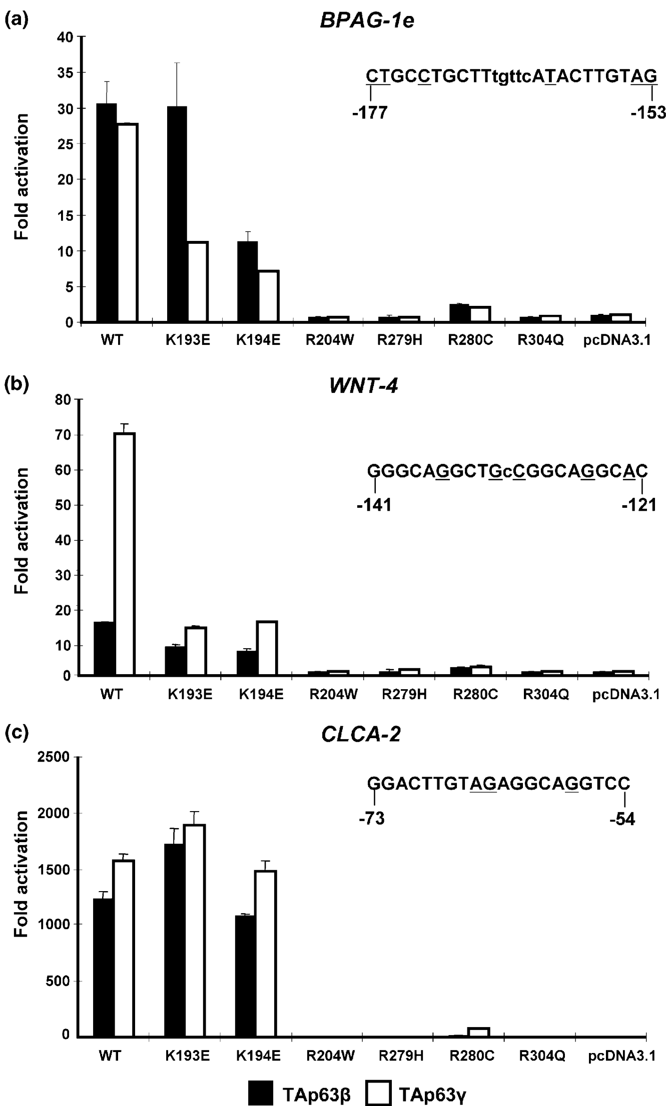

Figure 7
Differential regulation of p63 target gene promoters by split hand/ foot malformation (SHFM) and ectodactyly, ectodermal dysplasia (EEC) syndrome-derived mutants. The transactivation activity of TAp63β and TAp63γ mutants derived from SHFM and EEC syndromes were examined on the promoters of p63 target genes. K193E and K194E were derived from SHFM, and R204W, R279H, R280C, and R304Q were derived from EEC syndrome mutants. (*a*) *BPAG-1e* promoter (−1072/+27), (*b*) *WNT-4* promoter (−1200/−37), and (*c*) *CLCA-2* (*calcium-dependent chloride channel-2*) promoter (−1241/+101). Upper case bases represent p53-type response element half-sites and underlined bases are mismatched bases from the p53 consensus sequence.

Inga *et al*, 2002). These differences may contribute to the suppressive effect of the flanking elements.

It has been shown that several hereditary epidermal and/ or limb anomalies are caused by p63 mutations (Celli *et al*, 1999; Ianakiev *et al*, 2000; Amiel *et al*, 2001; McGrath *et al*, 2001; van Bokhoven and Brunner, 2002). Although both SHFM and EEC syndromes have mutations in the DNA binding domain of the p63 protein, EEC syndrome manifests epidermal abnormalities, whereas SHFM does not (Brunner *et al*, 2002; van Bokhoven and McKeon, 2002). The transactivation activity of the EEC syndrome-derived mutants was almost completely lost, whereas that of the mutants derived from SHFM varied depending on the promoter. The SHFM-derived p63 mutants, K193E and K194E, activated the *CLCA-2* promoter to the same extent or higher than their wild-type counterpart; on the other hand, they were less active on the *BPAG-1e* and *WNT-4* promoters when compared to wild-type. It is possible that the lysine residues are modified by acetylation or binding to ubiquitin-protein family members and thus modulate the function of the wild-type p63 protein. The p63-response element in the *CLCA-2* promoter has three mismatches in two half-sites and no gap, whereas the response elements in the *BPAG-1e* and *WNT-4* promoters have six and five mismatches in two half-sites and a 5- and 1-bp gap between the half-sites, respectively. We have previously shown that an increased number of mismatches and presence of a gap in the response element greatly reduce transactivation ability and protein-DNA binding (see footnote 2). The p63 lysine mutants derived from SHFM might not be correctly modified and it is possible that lack of proper modification more severely affects DNA binding of less conserved consensus sequences.

Recently, through microarray analysis, we obtained a list of more than 100 candidate target genes, which were induced by p63 and demonstrated a consensus sequence for p63-specific transcriptional activation (see footnote 2). Included on this list were several plakin family genes, such as *EVPL*, *periplakin*, and *BPAG-1*. These plakin family members are components of hemidesmosomes in the epidermis. Hemidesmosomes are junctional complexes that contribute to the attachment of epithelial cells to the underlying basement membrane predominantly in stratified epithelium (Borradori and Sonnenberg, 1999). Genetic mutations of hemidesmosome components or autoimmunity to their components cause diseases manifested by dermo-epidermal separation, including bullous pemphigoid and epidermolysis bullosa (Borradori and Sonnenberg, 1999). Koster *et al* (2004) showed that TAp63s induced *Keratin 5* and *Keratin 14*, which are intermediate filaments that bind to the BPAG-1e protein at the hemidesmosome. We also demonstrated that *BPAG-2*, a transmembrane component of the hemidesmosome that serves as a transmembrane receptor connecting the cell interior to the extracellular matrix, was induced by TAp63γ (Stappenbeck *et al*, 1993; Fontao *et al*, 2003). Thus, the deficiency of mature epidermis in the *p63−/−* mouse could be due, at least in part, to the dysregulation of hemidesmosome components in the skin.

Materials and Methods

RT-PCR The First Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, California) was used for reverse transcription. PCR was performed with recombinant Taq polymerase (Invitrogen) as follows: 30 cycles consisting of 94°C for 1 min, 58°C for 1 min and 72°C for 30 s. Primer sequences are BPAG7210F, TCAGGGCAGCAATATCAGTG and BPAG7418R, ACTAACCGGCTCAGCAAAGA.

Luciferase assay For luciferase analysis, one μg of expression vector and 200 ng of pGL3-Basic reporter plasmid (Promega, Madison, Wisconsin) were co-transfected by calcium-phosphate into Saos2 cells in 24-well plates. Data reflect fold-change in luciferase activity in experimental cells over cells co-transfected with empty pGL3-Basic and pcDNA3.1-Hygro vectors unless otherwise

stated in the figure legend. Means and standard deviations were calculated after three independent transfections.

Immunostaining *p63 +/−* mice (Jackson Laboratory, Bar Harbor, Maine) were mated and homozygous mutants were produced (Mills *et al*, 1999). 18.5 dpc embryos were formalin fixed and embedded in paraffin. Each representative section was stained by hematoxylin and eosin (HE), and immunohistochemical detection was performed with the avidin–biotin–peroxidase complex method using Vectastain ABC Kits (Vector Laboratories, Burlingame, California). Antigen retrieval was achieved by five cycles of boiling and cooling in 0.1 M citric acid and 0.1 M trisodium citrate. Monoclonal anti-p63 4A4 antibody (Santa Cruz Biotechnology, Santa Cruz, California) (1:100), polyclonal anti-BPAG1 C-17 (Santa Cruz Biotechnology) (1:200) antibody, anti-p73 Ab-4 antibody (Neomarker, Fremont, California) (1:100) and anti-p53 rabbit polyclonal antibody (Neomarker) (1:100) were used. The antibodies were incubated at 4°C overnight. The sections were developed with diaminobenzidine and counterstained with hematoxylin. The ACU (animal care and use) committee of Johns Hopkins University approved all described studies.

Western blotting Total protein was extracted from cells with sample lysis buffer (50 mM Tris-HCl (pH 6.8), 2% SDS, 6% (v/v) β-mercaptoethanol and 10% glycerol) and run on a 9% SDS-polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell, Limerick, Ireland) and blocked with 3% skim milk/PBS-Tween20. Ab-8 (DO-7 + BP53-12) antibody (Neomarker) was used to detect p53, 4A4 antibody (Santa Cruz Biotechnology) was used to detect p63 and Ab-4 (ER-13 + ER-15 + GC-15) (Neomarker) was used to detect p73. Anti-mouse IgG conjugated with horseradish peroxidase was used as secondary antibody (Amersham-Pharmacia, Buckinghamshire, England) and chemiluminescent signals were detected by Supersignal WestPico Chemiluminescent Substrate Kit (Pierce, Rockford, Illinois).

Inducible cell line Flp-in, T-Rex Saos2 cells have been described elsewhere. p53, TAp63β, and TAp73β with and without a 2XHA tag at the amino and 3XFLAG tag at the carboxyl terminus were subcloned into the *Bam*HI and *Xho*I sites of pcDNA5/FRT/TO (Invitrogen). The pcDNA5/FRT/TO expression constructs were introduced along with the pOG44 (Invitrogen) plasmid into Flp-in, T-Rex Saos2 cells and the resulting cells were selected by resistance to hygromycin. Final stable clones were induced with 1 μg per mL tetracycline.

Plasmids The *BPAG-1e* promoter region (−1072 to +27) was PCR-amplified with BPAG1F-1072*Mlu*I, TGACACGCGTTGCCTAC TACATTGGGTGGTTATG, and BPAG1R + 72*Xho*I, GATCCTCGAGC TACTTCTAACGGTGAAAAGTGGC.

The amplified promoter fragment was cloned into the *Mlu*I and *Xho*I sites of pGL3-Basic. Each truncation mutant was constructed by using the following primers and cloning into the same site of pGL3-Basic:

BPAG1F-983*Mlu*I, TGACACGCGTCCAGGTGCCACTGT-TATTGTTT;
BPAG1F-892*Mlu*I, TGACACGCGTAAGCAATTGGTGTGG-CACTCTC;
BPAG1F-778*Mlu*I, TGACACGCGTGGCTGGTTGGA-TAAATGCCTTC;
BPAG1F-674*Mlu*I, TGACACGCGTTGGGAAAGAGC-CAGTGTTCTTA;
BPAG1F-580*Mlu*I, TGACACGCGTCTACTTGGCACTTTATAG-TAGG;
BPAG1F-459*Mlu*I, TGACACGCGTATGGAAGCG-TACTTTGCTTCTC;
BPAG1F-355*Mlu*I, TGACACGCGTGCAGAAGTCAGACTAT-GATTGG;
BPAG1F-272*Mlu*I, TGACACGCGTGACATCTGTGAGGCATCT-GAGT;
BPAG1F-232*Mlu*I, TGACACGCGTAATTGGATGACTCTTAGTGC;
BPAG1F-192*Mlu*I, TGACACGCGTCTGCTTTGTTCATACTTG-TAGG;
BPAG1F-152MluI, TGACACGCGTGGTTTTAATACAAGCTACAA; and
BPAG1F-110*Mlu*I, TGACACGCGTCGAATGGCTTTAGGCTA-GATTC.

The *CLCA-2* promoter was PCR-amplified using the primers CLCA2F-*Mlu*I, TGACACGCGTCCGTTTCCTAAATGCTACACTCCC, and CLCA2R-*Xho*I, GATCCTCGAGGTTGTAGAGAAGCCTCCAGT TCAC.

The fragment (−1241/+101) was cloned into the *Mlu*I and *Xho*I sites of pGL3-Basic. *WNT-4* promoter plasmid was provided by Dr Melissa H. Little at the University of Queensland, Australia (Sim *et al*, 2002).

Response element oligonucleotides were cloned into the *Mlu*I and *Xho*I sites of pGL3-Basic. The oligonuclelotide sequences were as follows:

BPAG1-RE1234S, CGCGTCAGTGACGCCCAA TCTGCCTGCTTTGTTCATACTTGTAGGGTTTT AATACAAGCTACC;
BPAG1-RE1234A, TCGAGGTAGCTTGTATTAAAACCCTACAAG TATGAACAAAGCAGGCAGATTGGGCGTCAC TGA;
BPAG1-RE123S, CGCGTCAGTGACGCCCAATCTGCCTGCTTT GTTCAT ACTTGTAGGC;
BPAG1-RE123A, TCGAGCCTACAAGTATGAACAAAGCAGGCA G ATTGGGCGTCACTGA;
BPAG1-RE234S, CGCGTTCTGCCTGCTTTGTTCATACTTGTAG GGTTTTAATACAAGCTACC;
BPAG1-RE234A, TCGAGGTAGCTTGTATTAAAACCCTACAAGTA TGAACAAAGCAGGCAGAA;
BPAG1-RE12S, CGCGTCAGTGACGCCCAATCTGCCTGCTTTC;
BPAG1-RE12A, TCGAGAAAGCAGGCAGATTGGGCGTCACTGA;
BPAG1-RE23S, CGCGTTCTGCCTGCTTTGTTCATACTTGTAGGC;
BPAG1-RE23A, TCGAGCCTACAAGTATGAACAAAGCAGGCAGAA;
BPAG1-RE34S, CGCGTCATACTTGTAGGGTTTTAATACAAGCTAC C; and
BPAG1-RE34A, TCGAGGTAGCTTGTATTAAAACCCTACAAGTATGA.

PCR-amplified 2XHA and 3XFLAG tagged p53, TAp63β, TAp63-γ, and TAp73β were cloned into the *Bam*HI and *Xho*I sites of pcDNA5/FRT/TO. The PCR primers are as follows:

hp53-2HAF*Bam*HI, TGCATGGATCCCCACCATGTATCCGTAC-GATGTACCTGACTATGCATATCCGTAC-GATGTTCCAGACTATGCTATGGAG-GAGCCGCAGTCAGATCCT;
hp53-3FLAGR*Xho*I, GAATCTCGAGTCACTTGTCATCGT-CATCCTTGTAGTCGATGTCATGATCTTTA-TAATCACCGTCATGGTCTTTGTAGTCGTCT-GAGTCAGGCCCTTCTGT;
hp63TA-2HAF*Bam*HI, TGCATGGATCCCCACCATGTATCCGTAC-GATGTACCTGACTATGCATATCCGTAC-GATGTTCCAGACTATGCTATGTCCCA-GAGCACACAGACAAAT;
hp63b-3FLAGR*Xho*I, GAATCTCGAGTCACTTGTCATCGT-CATCCTTGTAGTCGATGTCATGATCTTTA-TAATCACCGTCATGGTCTTTGTAGTC-GACTTGCCAGATCCTGACAAT;
hp63g-3FLAGR*Xho*I, GAATCTCGAGTCACTTGTCATCGT-CATCCTTGTAGTCGATGTCATGATCTTTA-TAATCACCGTCATGGTCTTTG-TAGTCTGGGTACACTGATCGGTTTGG;
hp73TA-2HAF*Bam*HI, TGCATGGATCCCCACCATGTATCCGTAC-GATGTACCTGACTATGCATATCCGTAC-GATGTTCCAGACTATGCTATGGCC-CAGTCCACCGCCACCT; and
hp73b-3FLAGR*Xho*I, GAATCTCGAGTCACTTGTCATCGT-CATCCTTGTAGTCGATGTCATGATCTTTA-TAATCACCGTCATGGTCTTTG-TAGTCGGGCCCCCAGGTCCTGACGAG.

Mutant TAp63β and TAp63γ were made using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California). The following primers were used for the mutagenesis:

K193Esense, ATGCCTGTCTACGAAAAAGCTGAGCACGTC;
K193Eanti, GACGTGCTCAGCTTTTTCGTAGACAGGCAT;
K194Esense, ATGCCTGTCTACAAAGAAGCTGAGCACGTC;
K194Eanti, GACGTGCTCAGCTTCTTTGTAGACAGGCAT;

R204Wsense, ACGGAGGTGGTGAAGTGGTGCCCCAACCATGAGC;
R204Wanti, GCTCATGGTTGGGGCACCACTTCACCACCTCCGT;
R279Hsense, GTTGGAGGGATGAACCACCGTCCAATTTTAATCA;
R279Hanti, TGATTAAAATTGGACGGTGGTTCATCCCTCCAAC;
R280Csense, GGGATGAACCGCTGTCCAATTTTAATCATT;
R280Canti, AATGATTAAAATTGGACAGCGGTTCATCCC;
R304Qsense, CGCTGCTTTGAGGCCCAGATCTGTGCTTGCCCAG; and
R304Qanti, CTGGGCAAGCACAGATCTGGGCCTCAAAGCAGC G.

Chromatin immunoprecipitation (ChIP) A Chromatin Immunoprecipitation Kit (Upstate Cell Signaling Solutions, Waltham, Massachusetts) was used for ChIP analysis according to the manufacturer's protocol. FLAG M-2 antibody (SIGMA, St Louis, Missouri) was used for immunoprecipitation. PCR consisted of 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s using Taq polymerase (Invitrogen). The PCR primers used for ChIP were as follows: BPAG1F-355*Mlu*I; BPAG1R + 72*Xho*I; CLCA2F, TGACACGCGTGCTCCTTTGGTCTTATTTCCCT; and CLCA2R.

DOI: 10.1111/j.0022-202X.2005.23801.x

Manuscript received September 24, 2004; revised March 3, 2005; accepted for publication March 18, 2005

Address correspondence to: Motonobu Osada, Department of Otolaryngology, Division of Head and Neck Cancer Research, Johns Hopkins University School of Medicine, 818 Ross Research Building, 720 Rutland Avenue, Baltimore, MD 21205, USA. Email: osadamotonobu@yahoo.com

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