RESEARCH ARTICLE

Dimeric combinations of MafB, cFos and cJun control the apoptosis-survival balance in limb morphogenesis

Natsuno Suda1, Takehiko Itoh2, Ryuichiro Nakato3, Daisuke Shirakawa1, Masashige Bando3, Yuki Katou3, Kohsuke Kataoka4, Katsuhiko Shirahige3, Cheryll Tickle5 and Mikiko Tanaka1,∗

ABSTRACT

Apoptosis is an important mechanism for sculpting morphology. However, the molecular cascades that control apoptosis in developing limb buds remain largely unclear. Here, we show that MafB was specifically expressed in apoptotic regions of chick limb buds, and MafB/cFos heterodimers repressed apoptosis, whereas MafB/cJun heterodimers promoted apoptosis for sculpting the shape of the limbs. Chromatin immunoprecipitation sequencing in chick limb buds identified potential target genes and regulatory elements controlled by Maf and Jun. Functional analyses revealed that expression of p63 and p73, key components known to arrest the cell cycle, was directly activated by MafB and cJun. Our data suggest that dimeric combinations of MafB, cFos and cJun in developing chick limb buds control the number of apoptotic cells, and that MafB/cJun heterodimers lead to apoptosis via activation of p63 and p73.

KEY WORDS: AP-1 transcription factor, Limb, Chick

INTRODUCTION

During vertebrate development, apoptosis is a crucial process that sculpts the shape of embryos. In this regard, vertebrate limbs provide one of the best models in which to study apoptosis, as it has major morphogenetic functions. In avian limbs, the elimination of cells is observed in four distinct mesenchymal areas: the anterior necrotic zone (ANZ), posterior necrotic zone (PNZ), opaque patch (OP) and interdigital necrotic zones (INZs). In the ectoderm, cells are eliminated in the apical ectodermal ridge (Todt and Fallon, 1986; Zuzarte-Luis and Hurle, 2002). Apoptosis in these regions is particularly important because disruptions in apoptosis affect the final morphology of the limb (Ros et al., 1997; Yokouchi et al., 1996; Zuzarte-Luis and Hurle, 2002). Numerous studies have shown that bone morphological proteins (BMPs) trigger apoptosis both in the mesodermal cells (Ganan et al., 1996; Macias et al., 1997; Tang et al., 2000; Yokouchi et al., 1996; Zou and Niswander, 1996) and in the ectoderm of the apical ectodermal ridge (Wang et al., 2004). Overexpression of a dominant-negative form of BMP receptor Iib or Ia in chick limb represses apoptosis and induces the formation of interdigital webs (Yokouchi et al., 1996; Zou and Niswander, 1996), whereas transplantation of a BMP protein-soaked bead in limb mesenchymal induces apoptosis in chick embryos (Ganan et al., 1996; Macias et al., 1997; Tang et al., 2000). However, the molecular cascades that connect BMP signalling with the expression of genes controlling apoptosis in limbs remain largely unclear.

The AP-1 transcription factor superfamily is well conserved, and its members regulate the transcription of genes involved in diverse cellular processes, including proliferation, transformation and death (Shaulian and Karin, 2002). During vertebrate embryogenesis, some AP-1 transcription factors are crucial for the proper development of bones and the lens, liver and heart (Ogino and Yasuda, 1998; Shaulian and Karin, 2002). AP-1 transcription factors are also known to modulate gene expression triggered by free radicals (Sen and Packer, 1996). Interestingly, high levels of reactive oxygen species (ROS) are present in the INZ, and antioxidants can reduce apoptosis in limb buds (Salas-Vidal et al., 1998; Schnabel et al., 2006), suggesting that ROS stress may trigger apoptosis in the INZ. Indeed, the AP-1 family members cFos and cJun are regulated by oxidants at both transcriptional and translational levels (Sen and Packer, 1996).

Here, we examine the molecular cascades that control apoptosis in developing limb buds and tested the involvement of AP-1 transcription factor members in this process. Global expression profiles of all 41 reported AP-1 superfamily members showed that expression of MafB was restricted to the area of apoptosis. Thus, we examined whether MafB, as well as its candidate heterodimer partners cJun and cFos (Deppmann et al., 2006), are involved in controlling apoptosis during limb morphogenesis. Gain- and loss-of-function experiments revealed that MafB/cFos heterodimers inhibited apoptosis, whereas MafB/cJun heterodimers promoted apoptosis for sculpting the shape of chick limb buds. Integrating chromatin immunoprecipitation sequencing (ChIP-Seq) and functional analyses showed that expression of the transcription factors p63 and p73 was directly activated by MafB and cJun. Our results suggest that the balance of MafB, cFos and cJun heterodimer combinations controls the number of apoptotic cells and that the MafB/cJun heterodimer triggers pathways that lead to apoptosis via activation of p63 and p73 during chick limb morphogenesis.

RESULTS

Global gene expression profiles of AP-1 superfamily members in chick limb buds

In vertebrates, AP-1 superfamily transcription factors are involved in many processes that are crucial to the function of an organism. Some are necessary for the proper development of several organs and tissues (Shaulian and Karin, 2002). We explored whether any genes that encode AP-1 transcription factors are involved in regulating apoptosis in chick limb buds. Expression patterns of genes encoding all 41 AP-1 members that are present in chickens were examined in chick wing buds at stage 26 and in leg buds at
stage 31 (supplementary material Fig. S1). Of these, MafB transcripts were detected in the areas of apoptosis, including the ANZ, the PNZ and the apical ectodermal ridge at stage 26 (Fig. 1A) and the INZ at stage 31 (Fig. 1B), as previously reported (Lecoin et al., 2004). Based on their structural similarities, MafB was predicted to form heterodimers with either Fos or Jun family members (Deppmann et al., 2006). Among all Fos and Jun family members, only cFos and cJun transcripts were detected throughout the wing and leg buds of chick embryos, including apoptotic regions (Fig. 1C-F; supplementary material Fig. S1). Thus, we next examined whether MafB and its candidate heterodimer partners cFos and cJun were involved in regulating apoptosis during limb morphogenesis.

**MafB/cFos inhibits apoptosis, whereas MafB/cJun promotes apoptosis**

To investigate whether MafB has important roles in regulating apoptosis in chick limb buds, we introduced a retrovirus expressing the dominant-negative form of chicken MafB (DN-MafB), which lacks a transcriptional activation domain. Nile Blue staining of wing buds electroporated with the RCAS-DN-MafB construct showed that the number of apoptotic cells was reduced in the PNZ (5/6; dashed circle in Fig. 1G) and increased in the apical ridge (4/6; arrowheads in Fig. 1G), although control wing buds electroporated with a RCAS-AP vector did not display any changes in apoptosis (4/4; supplementary material Fig. S2A,B) or in the apical ridge formation (4/4; supplementary material Fig. S2C). These results suggest that AP-1 factor(s) that were inhibited by the RCAS-DN-MafB construct were involved in regulating the number of apoptotic cells in the apoptotic regions both in the mesenchyme and in the apical ectodermal ridge. By contrast, wing buds electroporated with RCAS-MafB showed almost no changes in apoptosis (6/7; supplementary material Fig. S2D,E), although they did form a considerably disturbed ridge (2/5; supplementary material Fig. S2F). Therefore, the phenotypes of wing buds electroporated with the RCAS-DN-MafB construct may be caused by suppression of multiple AP-1 factors, probably MafB and its heterodimer partner(s). We thus evaluated dimeric formation between endogenous MafB and cFos or cJun proteins using co-immunoprecipitation from lysates of stage 30 interdigital regions of distal leg buds. Maf did indeed interact with Fos and Jun in chick leg buds (Fig. 1H). We also examined whether MafB and cFos or cJun colocalized in chick limb buds by using antibodies against MafB, Fos, and Jun (supplementary material Fig. S3). Fos colocalized with MafB in interdigital regions of stage 30 interdigital regions of distal leg buds (white arrowheads in supplementary material Fig. S3B). Likewise, Jun and MafB co-localized in interdigital regions at stage 30 (white arrowheads in supplementary material Fig. S3C). These results suggest that MafB proteins colocalize and interact with Fos and Jun proteins in interdigital regions of chick leg buds.

To investigate our hypothesis that MafB/cFos and MafB/cJun heterodimers are involved in regulating apoptosis in limb buds, we overexpressed MafB and either cFos or cJun constructs in limb buds. Electroporation of retrovirus constructs expressing MafB together with a human cFos-expressing pEF vector (Kataoka et al., 1994a) in the presumptive wing field led to the reduction of apoptotic cells in transfected areas, including the apical ridge (5/6; dashed circle in Fig. 1I), which caused the disruption of the apical ridge formation (5/6; Fig. 1J). By contrast, electroporation of RCAS-MafB, together with a human cJun-expressing pEF vector (Kataoka et al., 1994a) in the wing field caused ectopic apoptosis in the transfected regions (4/5; Fig. 1K-L’). These results suggest that, in chick limb buds, MafB/cFos heterodimers repress apoptosis and MafB/cJun heterodimers promote it.

To confirm our hypothesis, we electroporated a Tol2-flanked construct expressing either MafB and cFos or MafB and cJun under
the control of a bipolar CMV promoter (pT2AL-MafB-BI-CMV-cFos in Fig. 2A; pT2AL-MafB-BI-CMV-cJun in Fig. 2B) along with a Tol2 transposase-expressing vector (pCAGGS-T2TP) (Kawakami and Noda, 2004) and pCAGGS-EGFP in the presumptive wing field at stage 14. Apoptotic cells in wing buds were examined with LysoTracker 2 days after electroporation. In mesenchymal cells of the PNZ (dashed circle in Fig. 2C) and in ectodermal cells of the apical ridge (dashed circle in Fig. 2E) that were electroporated with pT2AL-MafB-BI-CMV-cFos, apoptosis was clearly repressed (10/13; Fig. 2C-F). Apoptosis was, however, ectopically activated both in mesenchymal and ectodermal cells (arrowheads in Fig. 2G) of wing buds that were electroporated with pT2AL-MafB-BI-CMV-cJun (11/17; Fig. 2G,H). Thus, the co-existence of MafB and cFos repressed apoptosis, whereas co-existence of MafB and cJun promoted apoptosis both in mesenchymal and ectodermal cells of chick limb buds.

**Overexpression and downregulation of cFos and cJun in chick wing buds**

We electroporated pEF-cFos or -cJun alone into the PNZ of presumptive wing fields in stage 14-16 chick embryos and observed the effect on apoptosis after 48 h. Interestingly, overexpression of pEF-cFos alone in the PNZ caused both mild increases and decreases in the number of apoptotic cells (n=5; supplementary material Fig. S2G,H) and did not lead to any alterations in the apical ridge formation (5/7; supplementary material Fig. S2J,K) and did not lead to any alterations in the apical ridge formation (6/6; supplementary material Fig. S2L). Unlike MafB, both cFos and cJun have more than 10 other possible dimeric partners (Deppmann et al., 2006). Thus, electroporated cFos or cJun might have dimerized with other AP-1 transcription factors and may have activated both pro- and anti-apoptotic genes.

To investigate the function of endogenous cFos or cJun, we used siRNAs targeted to chick cFos or cJun. We first evaluated the effect of cFos-siRNA and cJun-siRNA in vitro by transfecting COS7 cells with cFos-siRNA and vectors expressing cFos-GFP fusion protein (pAc-cFos-GFP; supplementary material Fig. S4A) or with cJun-siRNA and vectors expressing cJun-GFP fusion protein (pAc-cjun-GFP; supplementary material Fig. S4B). At 24 h after transfection, the number of GFP-positive cells transfected with cFos-siRNA or cJun-siRNA was significantly reduced when compared with control cultures (P<0.00001, Student’s t-test; supplementary material Fig. S4A,B). We then investigated the effect of depleting cFos or cJun in limb development 48 h after co-electroporation of cFos- or cJun-siRNA together with pCAGGS-EGFP into the PNZ of the presumptive wing region at stage 14-16 (supplementary material Fig. S4C-H). Interestingly, electroporation of cFos-siRNA led to both mild increases and decreases in the number of apoptotic cells (n=5; supplementary material Fig. S4E,F). By contrast, electroporation of cJun-siRNA in the wing field led to minimal changes in apoptosis (n=5; supplementary material Fig. S4G,H). It is possible that the freed partners of cFos or cJun dimerized with other AP-1 transcription factors after introduction of the cFos- or cJun-siRNA, and may have upregulated both pro- and anti-apoptotic genes. Control embryos in which control siRNA
and pCAGGS-EGFP were electroporated did not show any changes in apoptosis (n=3; supplementary material Fig. S4C,D).

Function of other AP-1 transcription factors
As seen in supplementary material Fig. S1, genes encoding other AP-1 transcription factors were also expressed in the apoptotic regions (e.g. c-maf, Nfe2l1, Nfe2l2, Xbp1). We examined whether c-maf, Nfe2l1, Nfe2l2 and Xbp1 could affect the number of apoptotic cells by introducing vectors that express those genes into the PNZ of stage 14-16 chick wing buds (supplementary material Fig. S5). Misexpression of c-maf, Nfe2l1 and Xbp1 in wing buds showed hardly any changes in apoptosis (3/5; 5/6 and 4/5, respectively), whereas that of Nfe2l2 showed only a mild reduction in apoptosis in some cases (4/6; dashed circle in supplementary material Fig. S5C). Thus, it is possible that AP-1 transcription factors other than MafB, cFos and cJun, including Nfe2l1 and its unknown dimeric partners, are also involved in regulating the number of apoptotic cells in chick limb buds.

Expression of MafB, but not cFos or cJun, is regulated by BMP signalling in limb buds
Apoptosis in limbs is controlled by BMP signalling (Ganan et al., 1996; Macias et al., 1997; Tang et al., 2000; Yokouchi et al., 1996; Zou and Niswander, 1996). We thus investigated whether expression of MafB, cFos and cJun was regulated by BMP signalling. For this purpose, we inserted a bead soaked with BMP2, Noggin or PBS into stage 28 leg buds. Twenty-four hours after implantation, expression of MafB was activated by BMP2 (5/8; arrowheads in Fig. 3A) and repressed by Noggin (6/7; dashed circle in Fig. 3B), suggesting that MafB expression in the INZ is controlled by BMP signalling. By contrast, expression of cFos and cJun showed no changes after implantation of a bead soaked with BMP2 (5/5; Fig. 3C; 5/5, Fig. 3E) or Noggin (5/5, Fig. 3D; 4/4, Fig. 3F). No changes were observed in the expression of MafB, cFos or cJun after implantation of a PBS-soaked bead (4/4, 5/5 and 5/5, respectively; supplementary material Fig. S6A-C). These results suggest that expression of MafB, but not cFos or cJun, is controlled by BMP signalling. Similarly, ectopic MafB expression was induced in the PNZ of stage 24 wing buds 24 h after implantation of a BMP2-soaked bead (7/7; Fig. 3G). By contrast, no changes were seen in MafB expression after application of a PBS-soaked bead (4/4; supplementary material Fig. S6D). We then investigated expression of MafB 3 h after implantation of a BMP2-soaked bead in the INZ at stage 28. As seen in Fig. 3H, ectopic MafB expression was induced around a BMP2-soaked bead (6/6; Fig. 3I), but not around a PBS-soaked bead (5/5; supplementary material Fig. S6E). Therefore, MafB is most likely directly regulated by Smads, phosphorylated by serine/threonine kinases BMP receptors, or through only a few intermediate transcription factor(s), in this signalling pathway.

Retinoic acid signalling is specifically active in the interdigital region (Lussier et al., 1993; Rodriguez-Leon et al., 1999) and is sufficient to trigger interdigital apoptosis (Alles and Sulik, 1989; Dupe et al., 1999; Hernandez-Martinez et al., 2009). To see whether MafB expression is controlled by retinoic acid signalling in chick limb buds, we implanted a retinoic acid-soaked bead into the PNZ of stage 21-22 wing buds and into the INZ of stage 28 leg buds and examined expression of MafB (Fig. 3J). Twenty-four hours after application of a retinoic acid-soaked bead, expression of MafB was weakly activated around the bead in the PNZ (3/4; arrowheads in Fig. 3I) and slightly activated in a single case in the INZ (1/9; arrowheads in Fig. 3J). By contrast, no obvious changes were observed after implantation of a DMSO-soaked bead in the PNZ and in the INZ (5/5 and 6/6, respectively; supplementary material Fig. S6F,G). Thus, expression of MafB seems to be only weakly activated by retinoic acid in early limb buds.

Maf proteins are detected both in macrophage-positive and -negative regions in limb buds
In the hematopoietic system, MafB is expressed at high levels in macrophages and promotes macrophage differentiation (Bakri et al., 2005; Kelly et al., 2000). To investigate whether MafB expression in the limb buds is due to the presence of macrophages, we examined the distribution of macrophages and MafB proteins in interdigital regions of stage 30 leg buds (supplementary material Fig. S3D,E). Double staining of leg buds with macrophage and MafB antibodies showed that MafB proteins were distributed in macrophages (white arrowheads in supplementary material Fig. S3D). MafB proteins were also distributed in nuclei of several non-macrophage cells in
interdigital regions (open arrowheads in supplementary material Fig. S3E).

**Identification of Maf and Jun binding sites**

We then decided to look for downstream targets of MafB/cJun heterodimers that promote apoptosis in chick limb buds. To identify target genes and regulatory elements controlled by Maf and Jun, we dissected the distal region of 60 leg buds from stage 30 chick embryos (Fig. 4A) and performed ChIP using these tissue samples with Maf, Jun or p300 antibodies (Fig. 4C-E). Epigenomic profiling of p300, a transactivational co-activator, identifies active enhancers (Visel et al., 2009). Immunoprecipitated DNA fragments were analysed using massively parallel sequencing, and the resulting 50 base pair (bp) sequence reads were aligned with the reference chick genome (galGal3).

In leg buds, we identified 26,009, 29,815 and 18,303 regions of the genome with substantial enrichment in sequences associated with Maf, Jun and p300, respectively (in all cases, the presence of binding sites was indicated by fold enrichment >2.5 and peak width >400 bp; supplementary material Table S2). To identify direct targets of MafB/cJun heterodimers in chick limb buds, we looked for Maf and Jun binding sites that were coincident with p300 binding sites and identified 1347 peaks (supplementary material Table S3). Of these, the transcriptional start sites (TSSs) of 1717 genes resided within ±50 kb of these identified peaks, and these genes were highly expressed in limb buds based on published transcriptome data (normalized counts ≥100; supplementary material Table S4) (Wang et al., 2011). We considered genes that were within ±50 kb of these identified peaks as potential target genes of MafB/cJun heterodimers in chick limb buds. To select candidates among potential target genes of AP-1 (supplementary material Table S4), we looked for genes encoding core components that regulate the cell cycle and/or apoptosis (supplementary material Table S5); we identified p73, Chk1 and DAPK1 (Fig. 4D; supplementary material Fig. S7). An AP-1 binding site was also found near the TSS of caspase 6, but we did not choose caspase 6 as a candidate because Caxp6 knockout mice exhibit normal limb phenotype (Zheng et al., 1999). In addition, we selected p63 as a potential AP-1 target, as p63 belongs to the same family as p73 (Fig. 4C). Furthermore, a possible binding site for AP-1 proteins that was coincident with a p300 binding site was identified within ±50 kb of TSSs of p63, and transcription of p63 is controlled by AP-1 transcription factors (Leventaki et al., 2007; Vartanian et al., 2011; Yao et al., 2010). To see whether these candidates are expressed in the apoptotic regions, we examined their expression patterns in stage 26 chick wing buds and stage 31 chick leg buds (Fig. 4F-K; supplementary material Fig. S7). Of these, p63, p73 and Chk1, but not DAPK1, were highly expressed in chick limb buds, including apoptotic regions (Fig. 4F-K) such as the apical ridge (p63 in Fig. 4F) and the mesenchyme (p73 and Chk1 in Fig. 4H-K). Chk1 initiates cell cycle arrest when ATM and/or ATR recognizes DNA damage, and they induce transcription of p73, which mediates apoptosis (Fig. 4B; Urist et al., 2004). p63 can also induce apoptosis (Fig. 4B; Pyati et al., 2011).

To investigate whether MafB/cJun control the transcription of candidate target genes during limb development, we misexpressed RCAS-MafB together with pEF-cJun in the presumptive wing field at stage 14 and examined the expression patterns of potential target genes (Fig. 5A,B; supplementary material Fig. S8A). Thirty-six hours after misexpression, p63 and p73, but not Chk1, were ectopically expressed in the region electroproated with RCAS-MafB, pEF-cJun and pCAGGS-RFP (p63, 6/6; arrowheads in Fig. 5A; p73, 5/7; arrowheads in Fig. 5B; Chk1, 0/6, see supplementary material Fig. S8A). Expression of p63 and p73 was not altered after misexpression of control constructs (5/8 and 2/3, respectively; supplementary material Fig. S8B,C). These results suggest that expression of p63 and p73 is controlled by MafB/cJun.

Binding profiles suggest that AP-1 binds within the first intron of p63 (BS-p63; Fig. 4C) and 8.5 kb upstream of p73 (BS-p73; Fig. 4D). To confirm binding of p300 in these regions, we carried out ChIP-qPCR analysis with stage 30 chick leg buds using anti-p300 (supplementary material Fig. S9). ChIP-qPCR analyses showed distinct degrees of enrichment of p300 binding to BS-p63 and BS-p73 (supplementary material Fig. S9). These results suggest that p300 binds to these AP-1 binding sites.
were tested in reporter assays. Data are mean±s.d., * indicates activated expression of 

The electroporated sites were visualized with RFP. Arrowheads indicate activated expression of p63 (A) and p73 (B). (C,D) The binding sites were tested in reporter assays. Data are mean±s.d., *P<0.05 (Student’s t-test).

To test the functions of these AP-1-binding sites, each identified site was cloned directly in front of a minimal promoter that is followed by a luciferase reporter to test whether it is responsive to MafB/cJun transactivation (Fig. 5C,D). Transient reporter assays showed that the enhancer activities of BS-p63 and BS-p73 were highly activated by co-expression of cJun and MafB (Fig. 5C,D), suggesting that BS-p63 and BS-p73 can function as enhancer elements to control gene expression in limb buds, and that their activities are dependent on MafB and cJun. These results suggest that MafB/cJun heterodimers directly activate p63 and p73 and promote apoptosis. Taken together, our data demonstrate that dimeric combinations of MafB, cFos and cJun control the apoptosis-survival balance and that MafB/cJun heterodimers trigger apoptosis by activating expression of p63 and p73 during chick limb morphogenesis (Fig. 6).

**DISCUSSION**

We showed that MafB/cJun promotes apoptosis both in mesenchymal cells and in the apical ectodermal ridge, although it is likely that this heterodimer acts through different targets in the two regions. For example, one target, p63, is expressed only in the ectoderm. A p63/p73-responsive element is present in the second intron of Jagged1 (Sasaki et al., 2002), which is one of a few components known to control apoptosis in the apical ridge (Francis et al., 2005). Furthermore, p63 and p73 are required for triggering p53-dependent apoptosis in response to DNA damage (Flores et al., 2002). By contrast, p63 also directly upregulates the expression of Dlx5 (Kouwenhoven et al., 2010), which promotes proliferation in the median apical ridge (Robledo et al., 2002), and p63 knockout mice fail to form the apical ectodermal ridge (Mills et al., 1999; Yang et al., 1999). p63 in the apical ectodermal ridge likely activates multiple genes, as it is known to do in other systems (Melino et al., 2003). Such genes may be involved in pro-apoptotic cascades (including Jagged1) and proliferation (including Dlx5), and the sum of all signals is likely to determine the fate of cells. Further studies are required to determine the detailed functions of p63 targets that regulate the formation of the apical ridge. p73 contributes to p53-independent apoptosis by directly activating expression of Noxa, mutation of which causes mitochondrial dysfunction (Flintner et al., 2005). In agreement with these p73 functions, Bax is specifically expressed in the INZ (Dupe et al., 1999), and mice deficient in both Bax and Bak show soft-tissue syndactyly (Lindsten and Thompson, 2006). Thus, in apoptotic regions of chick limb buds, p73 very likely triggers a p53-independent apoptosis cascade probably by activating Bax. However, in mice, the absence of p73 does not affect limb development (Yang et al., 2000). This may be because levels of p73 transcripts are very low in mouse limb buds (EMBRYS; http://embrys.jp/embrys/html/MainMenu.html; Yokoyama et al., 2009) when compared with those in chick limb buds (Fig. 4H,I). In this study, we looked for only direct targets of MafB/cJun, the functions of which could explain the phenotypes noted here; it is also reasonable to expect that MafB/cFos directly activate pro-apoptotic genes. Importantly, several other AP-1 family members activate both pro-apoptotic and anti-apoptotic genes (Shaulian and Karin, 2002). Indeed, Shaulian and Karin (2002) proposed that there is a "balance between the pro-apoptotic and anti-apoptotic target-genes that determines whether the final outcome will be cell survival or cell death". Hence, we must consider the possibility that MafB/cFos and Maf/cJun activate both pro- and anti-apoptotic genes, and that the balance between these activated genes determines the outcome. Furthermore, MafB/cFos and/or MafB/cJun are likely also to inhibit the expression of pro-apoptotic or survival genes, and cFos/cJun combinations may also control the expression of genes throughout limb buds. For example, MafB/cJun binds to the regulatory region of Hoxb9 to repress its transcription (Mehta-Grigoriou et al., 2003), and cFos/cJun stably binds to regulatory elements in many other systems (Shaulian and Karin, 2002). Although MafB seems to dimerize with either cFos or cJun in chick limb buds (Fig. 1H; supplementary material Fig. S3B,C), both cFos and cJun have more than 10 other possible dimer partners (Deppmann et al., 2006). As we have shown in supplementary material Figs S2 and S4, overexpression or knockdown of cJun alone produced only a slight increase or decrease in the number of apoptotic cells in some cases. Moreover, overexpression or knockdown of cFos alone both increased and decreased the number of apoptotic cells (supplementary material Fig. S2G,H and Fig. S4E, F). These results suggest that cFos and cJun can dimerize with other AP-1 transcription factors and activate both pro- and anti-apoptotic genes. It is also plausible that other combinations of AP-1 transcription factors have roles that are similar to those of MafB, cFos and cJun in regulating apoptosis during limb morphogenesis.

---

**Fig. 5.** MafB/cJun activate expression of p63 and p73 in chick limb buds. (A,B) Expression of p63 (A) and p73 (B) in chick wing buds 36 h after electroporation with a cocktail of RCAS-MafB, pEF-cJun and pCAGGS-RFP. Right (experimental) and left (control) wing buds of the same embryo are shown. (A,B’) The electroporated sites were visualized with RFPт

**Fig. 6.** Mechanisms that control the apoptosis-survival balance during chick limb development. Dimeric combinations of MafB, cFos and cJun control the apoptosis-survival balance in limb patterning.
In fact, misexpression of Nfe2L1 caused mild repression of apoptosis in chick limb buds (supplementary material Fig. S5). Although overexpression of c-maf, Nfe2L1 or Xbp1 resulted in minimal changes in the number of apoptotic cells (supplementary material Fig. S5), it is still possible that they are also involved in this process by forming heterodimers or are involved in the development of different structures in the embryo. Future studies should clarify the complex regulation of AP-1 factors in various morphological processes.

In this study, we show that MafB is involved in controlling apoptosis in chick limb buds. Importantly, a recent study in mice revealed a novel heterodimers or are involved in the development of different structures still possible that they are also involved in this process by forming in the number of apoptotic cells (supplementary material Fig. S5), it is overexpression of in chick limb buds (supplementary material Fig. S5). Although in fact, misexpression of in chick limb buds as shown in other systems (Sen and Packer, 1996). We conclude that the balance of MafB and cFos or cJun heterodimer combinations, specifically in apoptotic regions, controls the apoptosis-survival balance, and MafB/cJun heterodimers trigger apoptosis by activating expression of p63 and p73 during limb patterning in chick embryos.

**MATERIALS AND METHODS**

**Whole-mount in situ hybridization**

The A9300001N09Rik, Atf1, Atf2, Atf3, Atf4, Atf6, Bach1, Bach2, Batf3, Cebp, CEBPβ, Cebpβ, Creb, Creb3, Creb31, Creb32, Creb33, Creb5, Crc, Crem, cFos, Hlf, MafB, nfe2l1, nfe2l1, NfI3 and Xbp1 probes were transcribed from Biotechnology and Biological Sciences Research Council (BBSRC; http://www.chick.manchester.ac.uk/) chick EST cDNA clones (ChEST595m10, ChEST547e5, ChEST913d7, ChEST927011, ChEST90a19, ChEST222013, ChEST92610, ChEST1024h14, ChEST543k2, ChEST72f15, ChEST70p4, ChEST86h12, ChEST50d14, ChEST256g16, ChEST3351, ChEST553f15, ChEST98d7, ChEST9648, ChEST860a20, ChEST272b15, ChEST262m21, ChEST837k20, ChEST52m15, ChEST179p8, ChEST813j4, ChEST679h3 and ChEST151f18, respectively) (Boardman et al., 2002; Hubbard et al., 2005). Atf7, Cebpa, Cebpb, Fog2 and Jun probes were transcribed from the University of Delaware Chick EST cDNA clones (pT1n1.pkn008b16, pT1n1.pkn007.g3, pT1n1.pkn007.118, pT1n1.pkn014.g7 and pT1n1.pkn012.d17, respectively; http://www.chicest.udel.edu/). Crebf, Jdp, MafB, MafG, MafK, Tef, p63, p73, Chk1 and Dapk1 cDNAs for in situ probes were amplified from cDNA pools prepared from stage 20-28 chick embryos using primers that hybridize to the published sequences described in supplementary material Table S1. The L-Maf, MafB, c-maf and Fog9 probes were transcribed as described previously (Crossley et al., 1996; Kataoka et al., 1994a). Whole-mount in situ hybridization was performed as described previously (Wilkinson, 1992).

**Plasmid construction**

For RCAS-MafB, the open reading frame (ORF) of chick MafB was amplified using primers that include the EcoRI, Kozak and FLAG epitope sequences at the N terminus and the HindIII sequence at the C terminus (5'-CCCGGAATTCCTCCACCTGAGTACATAAGGACGATGAGATAAGATCCGCTCCACAACTGTC-3' and 5'-CCCAAGTCTGACTCATGAG-AGGAAGGTGGTATGGCCGGAGAGCTGAGCATC-3'; all PCR primers given as forward and then reverse). The resulting PCR products were inserted into the pGEM-T Easy vector (Promega; pGEMTeasy-MafB) and then cloned into the EcoRI and HindIII sites of SLAX12 NCO (Morgan and Fekete, 1996). A Clal fragment was cloned into the Chl site of the RCAS-AP retroviral vector (Homburger and Fekete, 1996; Hughes et al., 1987). For DN-MafB, the cDNA sequence encoding amino acid residues 111 to 311 of chick MafB was amplified with oligonucleotide primers including the EcoRI, Kozak and FLAG epitope sequences at the N terminus and the HindIII sequence at the C terminus (5'-CCCGGAATTCCTCCACCTGAGTACATAAGGACGATGAGATAAGATCCGCTCCACAACTGTC-3' and 5'-CCCAAGTCTGACTCATGAG-AGGAAGGTGGTATGGCCGGAGAGCTGAGCATC-3'; all PCR primers given as forward and then reverse). The resulting PCR products were inserted into the pGEM-T Easy vector and then cloned into SLAX12 NCO. A Clal fragment was cloned into the Chl site of the RCAS-AP retroviral vector. For pEF-MafB, the ORF of chick MafB was amplified using specific primers (5'-ATCTCTTCTAGGTGCAACCCAATCATGAGTACATAAGGACGATGAGATAAGATCCGCTCCACAACTGTC-3' and 5'-CCCAAGTCTGACTCATGAG-AGGAAGGTGGTATGGCCGGAGAGCTGAGCATC-3') and inserted into the NotI and SalI sites of the plhHyg-EF2 vector (Nishizawa et al., 2003) via the In-Fusion reaction (Clontech). For pT2AL-MafB-BI-CMV-cFos and pT2AL-MafB-BI-CMV-cJun, pBl-CMV1 (Clontech) was digested with Xbal and SalI (for pBSK-CMV1) or with XhoI and SalI (for pBSK-CMV2) and subcloned into the pBSK- vector (pBSK-CMV1 and pBSK-CMV2, respectively). Then, a polyA tail sequence was amplified with specific primers (for pBSK-CMV1, 5'-TAAGAGGCGGGGGCCCACTACATGAGTACATAAGGACGATGAGATAAGATCCGCTCCACAACTGTC-3' and 5'-CCCAAGTCTGACTCATGAG-AGGAAGGTGGTATGGCCGGAGAGCTGAGCATC-3') and inserted into the NotI and SalI sites of the plhHyg-EF2 vector (Nishizawa et al., 2003) via the In-Fusion reaction (Clontech). For pT2AL-MafB-BI-CMV-cFos and pT2AL-MafB-BI-CMV-cJun, pBl-CMV1 (Clontech) was digested with Xbal and SalI (for pBSK-CMV1) or with XhoI and SalI (for pBSK-CMV2) and subcloned into the pBSK- vector (pBSK-CMV1 and pBSK-CMV2, respectively). Then, a polyA tail sequence was amplified with specific primers (for pBSK-CMV1, 5'-TAAGAGGCGGGGGCCCACTACATGAGTACATAAGGACGATGAGATAAGATCCGCTCCACAACTGTC-3' and 5'-CCCAAGTCTGACTCATGAG-AGGAAGGTGGTATGGCCGGAGAGCTGAGCATC-3') and inserted into the NotI and SalI sites of the plhHyg-EF2 vector (Nishizawa et al., 2003) via the In-Fusion reaction (Clontech). For pT2AL-MafB-BI-CMV-cFos and pT2AL-MafB-BI-CMV-cJun, pBl-CMV1 (Clontech) was digested with Xbal and SalI (for pBSK-CMV1) or with XhoI and SalI (for pBSK-CMV2) and subcloned into the pBSK- vector (pBSK-CMV1 and pBSK-CMV2, respectively). Then, a polyA tail sequence was amplified with specific primers (for pBSK-CMV1, 5'-TAAGAGGCGGGGGCCCACTACATGAGTACATAAGGACGATGAGATAAGATCCGCTCCACAACTGTC-3' and 5'-CCCAAGTCTGACTCATGAG-AGGAAGGTGGTATGGCCGGAGAGCTGAGCATC-3') and inserted into the NotI and SalI sites of the plhHyg-EF2 vector (Nishizawa et al., 2003) via the In-Fusion reaction (Clontech).
Then, SLAX12 Nco-cFos and SLAX12 Nco-cJun were digested with HindIII and Clal, and the ORFs of human cFos and cJun were cloned into the pBSK- vector. The ORFs of human cFos and cJun were then subcloned into the NotI and Clal sites of pBSK-CMV1-pA (pBSK-CMV1-cFos-pA and pBSK-CMV2-cJun-pA, respectively). Then, the ORF of chick MafB from pGEMEasy-MafB was digested with EcoRI and HindIII and cloned into the EcoRI and HindIII sites of pBSK-CMV2-pA (pBSK-CMV2-MafB-pA). pBSK-CMV1-cFos-pA and pBSK-CMV2-cJun-pA were digested with Xhol and BglII, and cloned into pTA2L200R150G. pBSK-CMV2-MafB was digested with Xhol and the DNA fragment was cloned into the XhoI site of pTA2L200R150G-CMV1-cFos-pA and pTA2L200R150G-CMV2-cJun-pA (pTA2L-MafB-BI-CMV-cFos and pTA2L-MafB-BI-CMV-cJun, respectively) using the In-Fusion reaction. The genomic regions of the AP-1-binding site peaks were amplified with PCR using primers described in supplementary material Table S1 and were cloned into a mini promoter containing the pGL4.23 [luc2/miniP] vector (Promega).

In ovo DNA electroporation
The plasmid solutions or small interfering RNAs (siRNAs; see below) were coloured with 2.5-5% Fast Green and co-electroporated with pCAGGS-EGFP (a gift from Dr. J. Miyazaki and Dr. T. Ogura) (Niwa et al., 1991) or pCAGGS-RFP (Das et al., 2006) into the presumptive limb mesenchyme as described previously (Ogura, 2002). Briefly, the solution was injected into the lateral plate mesoderm of stage 14-16 chick embryos, and the embryos were then subjected to experimental manipulation was studied after cartilage staining with Alcian Blue. The morphology of the limbs that were subjected to experimental manipulation was studied after cartilage staining with Alcian Blue. The pattern of apoptosis was analysed with vital staining with Nile Blue and LysoTracker Red (Invitrogen) (Mariani et al., 2008).

Co-immunoprecipitation
Co-immunoprecipitation (co-IP) was carried out as described previously (Chen et al., 2004). Briefly, interdigital regions of stage 30 limb buds were dissected from 10 embryos in PBS, and lysed in lysis buffer [10 mM HEPES (pH 7.5); 420 mM NaCl; 0.5% NP-40; 0.2% protease inhibitors (BioVision)] on ice for 25 min with trituration. Lysates were centrifuged at 2500 g for 5 min, and the supernatants were transferred into new tubes. The Maf antibody (Santa Cruz; sc-7866), the Fos antibody (Santa Cruz; sc-52), the Jun antibody (Santa Cruz; sc-1694) or the normal rabbit IgG (PeproTech EC) used for co-IP was covalently cross-linked to Dynabeads (Veritas), and bound proteins were detected on western blots with affinity-purified anti-Maf.

Insertion of beads into limb buds
CM Affi-Gel Blue Gel (Bio-Rad) beads were loaded with 0.1 mg/ml BMP2 (R&D Systems), 1 mg/ml Noggin (R&D Systems) or PBS (Tumpel et al., 2002). AG1-X2 resin beads (Bio-Rad) were loaded with 10 mg/ml all-trans retinoic acid (Sigma) or dimethyl sulfoxide (DMSO). Then a single bead was inserted into the PNZ of stage 21-22 chick wing buds or the interdigital area of stage 28 chick embryos. Embryos were fixed 24 h later in 4% paraformaldehyde and then subjected to in situ hybridization as described above.

Immunohistochemistry
Fluorescent immunostaining was performed on cryosections of limb buds at stage 30 as described previously (Bangs et al., 2011). Primary antibodies used were anti-MafB (Santa Cruz; sc-10022), anti-Fos, anti-Jun and anti-macroage (Acris Antibodies; AM08143PU-N). Confocal images were taken on a LSM780 confocal microscope (Carl Zeiss).

siRNA preparation
siRNAs specific for chicken cFos and cJun and a control siRNA were obtained from Invitrogen (Stealth RNAi). The targeted sequence was 5′-TCAACGACTTCGACTGATGAGGT-3′ (coding region 127-151 of chick cFos; GenBank accession number NM_205508) and 5′-AGAAGCCTAGACTGACCTG-3′ (coding region 615-639 of chick cJun; GenBank accession number NM_001031289).

Cell culture and transfection
COS7 cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Co-transfection of target plasmids (pAc-cFos-GFP or pAc-cJun-GFP) and siRNAs was carried out with Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. To assess the efficiency of the siRNAs on pAc-cFos-GFP or pAc-cJun-GFP expression in COS7 cells, the number of GFP-positive cells was counted 24 h after transfection. Student’s t-test was performed to assess the differences between numbers of cells transfected with siRNA (P<0.00001). For reporter assays, COS7 cells were plated on 24-well plates and transfected using Lipofectamine 2000 and a total of 2.4 μg of DNA. Reporter constructs were co-transfected with 0.8 μg of MafB and/or cJun expression constructs in combination with Renilla vectors. Cells were collected 24 h post-transfection, and luciferase reporter assays were performed using the Dual Luciferase Kit (Promega). Each assay was performed twice.

Chromatin immunoprecipitation sequencing (ChIP-Seq) and qPCR (ChIP-qPCR)
ChIP was performed using leg buds at stage 30. Sixty limb buds were dissected, fixed in 1% formaldehyde for 10 min at room temperature, washed with PBS, and stored at −80°C. ChIP was performed from these tissue samples using antibodies against Maf, Jun or p300 (Santa Cruz; sc-584) as described previously (Visel et al., 2009). DNA samples from the whole-cell extract (WCE) and ChIP fractions were further sheared using an ultrasonic sonicator, ligated to sequencing adapters and amplified according to the manufacturer’s instructions. Gel-purified amplified DNA (100-150 bp) was sequenced on the Applied Biosystems SOLiD 3.5 platform to generate 50 bp reads. Sequence reads were aligned with the Gallus gallus reference genome (galGal3). More than 1 million reads were mapped for each sample. Aligned reads were extended to 100 bp in the 3′ direction. The number of reads was summed up in a 150 bp window with a step size of 10 bp along the chromosome for ChIP and WCE fractions, respectively. After normalization of total reads of the ChIP fraction against the WCE fraction, enrichment values (ChIP/WCE) were calculated as described previously (Lengronne et al., 2004) for each window.

For ChIP-qPCR, DNA samples from the WCE and ChIP fractions were sheared with the ultra sonicator, incubated with p300 antibody and purified. Putative binding site sequences were amplified with specific primers (for negative control, 5′-TCTGATCGTGGTGGTACAAA-3′ and 5′-GCAGATC-CAAAAAGCATCCAT-3′; for BS-p63, 5′-CGCAGATCAAGGTGTCGTCGGT-3′ and 5′-GCCATGTCAGTCCAGGAGAGG-3′; for BS-p73, 5′-TCATG- TGTGGTCACCAACTAGTA-3′ and 5′-GGTGGAGCAAGGTGAGGTTATG-3′). More than 1 million reads were mapped for each sample. Aligned reads were extended to 100 bp in the 3′ direction. The number of reads was summed up in a 150 bp window with a step size of 10 bp along the chromosome for ChIP and WCE fractions, respectively. After normalization of total reads of the ChIP fraction against the WCE fraction, enrichment values (ChIP/WCE) were calculated as described previously (Lengronne et al., 2004) for each window.

Acknowledgements
We thank Koichi Kawakami, Stephen Hughes, Toshihiko Ogura and Junichi Miyazaki for plasmids. We also thank Takayuki Suzuki, Yuji Watanabe, Yuji Yokouchi, Bau-Lin Huang, Hidenori Nishihara, Makiha Hukuda and Tomoya Itoh for technical advice; Satoru Takahashi for sharing unpublished information; and John Fallon for critical comments.

Competing interests
The authors declare no competing financial interests.

Author contributions
N.S. and M.T. designed the project. N.S. performed most of the embryological, cellular and biochemical experiments except those described below. D.S. performed some implantation experiments. M.T. built some DNA constructs. Y.K. performed
sequencing analysis. T.I. and R.N. performed computational analysis. K.K. and C.T. provided materials and advice. M.B. advised on the ChiP analysis. K.S. advised on ChiP analysis and supervised the sequencing analysis. N.S. and M.T. wrote the manuscript.

Funding
This work is supported by a Grant-in-Aid for Scientific Research on Innovative Areas [24113503 to M.T.J.] by the Takeda Science Foundation (M.T.) and by a Saskatchewan Scientific Research Grant (N.S.).

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookupsuppl doi:10.1242/dev.099150/DC1

References


Fig. S1. Global gene expression profiles of 41 AP-1 transcription factors in chick limb buds. Paired images show the expression patterns of the indicated genes in wing buds at stage 26 (left) and in leg buds at stage 31 (right). Although L-Maf was previously shown to be expressed in the interdigital webs of chick embryos using RNA probes synthesized from partial quail L-Maf fragments (Lecoin et al., 2004), L-Maf transcripts recognized by chicken L-Maf probes (Ogino and Yasuda, 1998) were detected only weakly throughout limb buds.
Fig. S2. Apoptosis was not strongly influenced by introduction of RCAS-MafB, pEF-cFos, or pEF-cJun in limb buds. (A,D,G,J) Experimental (right) and control (left) wing buds of the same embryo were stained with Nile blue to show apoptosis 48 hours after electroporation with control RCAS-AP (A), RCAS-MafB (D), pEF-cFos (G), or pEF-cJun (J). (B,E,H,K) Constructs were electroporated together with pCAGGS-EGFP. (C,F,I,L) Fgf8 expression of right experimental wing buds of embryos 48 hours after electroporation with control RCAS-AP (C), RCAS-MafB (F), pEF-cFos (I), or pEF-cJun (L).
Fig. S3. The distribution of MafB, Fos, Jun, and macrophages in interdigital regions of limb buds was examined. (A) A schematic diagram of stage 30 chick leg bud. The red squares represent where the confocal images were taken. (B-E) Horizontal sections of interdigital regions of stage 30 chick leg buds. (B) Confocal images of Fos (red) and MafB (green) and a merged image. Fos and MafB co-localized in interdigital regions (white arrowheads). (C) Confocal images of Jun (red) and MafB (green) and a merged image. Jun and MafB co-localized in interdigital regions (white arrowheads). (D, E) Confocal images of macrophages (red) and MafB (green) and a merged with DAPI staining. MafB proteins were detected in macrophages (white arrowheads in D), and nuclei of several non-macrophage cells (open arrowheads in E).
Fig. S4. Apoptosis was not strongly influenced by knockdown of MafB, cFos, or cJun in limb buds. (A-H) Depletion of cFos or cJun by siRNAs in vitro and in vivo. (A,B) pAc-cFos-GFP with control siRNAs or cFos-siRNAs (A), or pAc-cJun-GFP with control siRNAs or cJun-siRNAs (B) were co-transfected into COS7 cells. At 24 hours after transfection, GFP-positive cells were counted. Transfection of cFos-siRNAs or cJun-siRNAs significantly suppressed the number of GFP-positive cells by 80.6% (A) and 79.0% (B) as compared with control cultures (right panels in A and B; p<0.00001, Student’s t-test). (C-H) Experimental (right) and control (left) wing buds of the same embryo were stained with Nile blue to show apoptosis 48 hours after electroporation with control siRNA (C), cFos-siRNA (E), or cJun-siRNA (G). (D, F, H) Constructs were electroporated together with pCAGGS-EGFP.
Fig. S5. *cmaf, Nfe2l1, Nfe2l2, or Xbp1 were overexpressed in wing buds.* (A,C,E,G) Experimental (right) and control (left) wing buds of the same embryo were stained with Nile blue to show apoptosis 48 hours after electroporation with pEF-cmaf (A), pMSCV-Nfe2l1 (C), pcDNA3-Nfe2l2 (E), or pCMV-Xbp1 (G). (B,D,F,H) Constructs were electroporated together with pCAGGS-EGFP.
Fig. S6. Expression of MafB, cFos, and cJun was examined after implantation of a PBS- or DMSO-soaked bead.

(A-G) Expression of MafB (A,D-G), cFos (B), and cJun (C) 24 hours (A-D,F) or 3 hours (E, G) after implantation of a bead (asterisk) soaked with PBS (A-E), or DMSO (F,G) in the right leg buds at stage 28 (A-C,E,G) or wing buds at stage 21–22 (D,F). (A-G) No detectable changes in the expression pattern of MafB, cFos, or cJun were observed after implantation of a PBS- (A-E) or DMSO- (F,G) soaked bead. Right (experimental) and left (control) limb buds of the same embryo are shown.

Fig. S7. Expression patterns of candidate target gene were examined. (A-B) Expression patterns of DAPK1 in wing buds of chick embryos at stages 26 (A) and in leg buds of chick embryos at stage 31 (B).
Fig. S8. Expression of putative target genes was examined after introduction of RCAS-MafB and pEF-cJun in chick limb buds. (A) Expression of Chk1 in chick wing buds 36 hours after electroporation with a cocktail of RCAS-MafB, pEF-cJun and pCAGGS-RFP. Right (experimental) and left (control) wing buds of the same embryo are shown. (A’) The electroporated sites were visualized with RFP. No obvious changes in expression pattern of Chk1 were observed after electroporation. (B-C) Expression of p63 (B) and p73 (C) in chick wing buds 36 hours after electroporation with control RCAS-AP and pHyg-EF2 vector together with pCAGGS-RFP. (B’-C’) The electroporated sites were indicated by RFP. No obvious changes in expression patterns were observed after misexpression of control constructs.
Fig. S9. p300 bound to the BS-p63 and BS-p73. Occupancy of p300 proteins at BS-p63 and BS-p73 was examined by ChIP-qPCR. (mean±s.d., n=3; *P<0.01, Student's t-test).

Table S1. Primer sets to amplify the cDNAs for in situ probes or the genomic regions of AP-1 binding sites.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crebf</td>
<td>ATCGGCACAGCCCTGACCAC</td>
<td>GAGCGCAGATGGAGAACCAC</td>
</tr>
<tr>
<td>JDP</td>
<td>GAGTCCGACGCCCTGGAGCC</td>
<td>GTCACCCTCAACTGAGCAGCC</td>
</tr>
<tr>
<td>MafF</td>
<td>AGCTGGTACAGCCATTCGACC</td>
<td>CTACAGCTGCTGCTGTTACATG</td>
</tr>
<tr>
<td>MafG</td>
<td>AGCGTACGACTGACTGACC</td>
<td>CTACAGCTGCTGCTGTTACATG</td>
</tr>
<tr>
<td>MafK</td>
<td>AGTGCCTGAGCGATGAACTCG</td>
<td>AATTATGCACTTCTCCAGATGCTC</td>
</tr>
<tr>
<td>TEF</td>
<td>TGACTTTATCCTTTCGAGTGG</td>
<td>TGGTCGTCGACCAGCTGTCAG</td>
</tr>
<tr>
<td>p63</td>
<td>GCTTTCAGTACCAGTACATACC</td>
<td>CTTGGAATCCGAGAGTCAAG</td>
</tr>
<tr>
<td>p73</td>
<td>CAATGGGAGCTGGAATGCTG</td>
<td>AATTATGCACTTCTCCAGATGCTC</td>
</tr>
<tr>
<td>Chk1</td>
<td>ATCGACTCTGCACTTACAG</td>
<td>TGGGTGCTGACCAGCTGTCAG</td>
</tr>
<tr>
<td>DAPK1</td>
<td>GCTTGCCGAATATCACCTAAG</td>
<td>CTGGGAATCCGAGAGTCAAG</td>
</tr>
<tr>
<td>BS-p63</td>
<td>CCGGATATCCTTTGGAGGACACAGTTCAG</td>
<td>CCGGATATCCTTTGGAGGACACAGTTCAG</td>
</tr>
<tr>
<td>BS-p73</td>
<td>CCGGATATCCTTTGGAGGACACAGTTCAG</td>
<td>CCGGATATCCTTTGGAGGACACAGTTCAG</td>
</tr>
</tbody>
</table>
Download Table S2

Download Table S3

Download Table S4

Download Table S5