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CREB phosphorylation at Ser^{133} regulates transcription via distinct mechanisms downstream of cAMP and MAPK signalling

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CREB (cAMP-response-element-binding protein) is an important transcription factor for the activation of a number of immediate early genes. CREB is phosphorylated on Ser^{133} by PKA (protein kinase A), promoting the recruitment of the co-activator proteins CBP (CREB-binding protein) and p300; this has been proposed to increase the transcription of CREB-dependent genes. CREB is also phosphorylated on Ser^{133} by MSK1/2 (mitogen- and stress-activated kinase 1/2) in cells in response to the activation of MAPK (mitogen-activated protein kinase) signalling; however, the relevance of this to gene transcription has been controversial. To resolve this problem, we created a mouse with a Ser^{133} to alanine residue mutation in the endogenous *Creb* gene. Unlike the total CREB knockout, which is perinatally lethal, these mice were viable, but born at less than the expected Mendelian frequency on a C57Bl/6 background. Using embryonic fibroblasts from the S133A-knockin mice we show in the present study that Ser^{133} phosphorylation downstream of PKA is required for CBP/p300 recruitment. The requirement of Ser^{133} phosphorylation for the PKA-mediated induction of CREB-dependent genes was, however, promoter-specific. Furthermore, we show that in cells the phosphorylation of CREB on Ser^{133} by MSKs does not promote strong recruitment of CBP or p300. Despite this, MSK-mediated CREB phosphorylation is critical for the induction of CREB-dependent genes downstream of MAPK signalling.

Key words: CREB (cAMP-response-element-binding protein)-binding protein (CBP), CREB-regulated transcription co-activator 2 (CRTC2), CRTC3, mitogen- and stress-activated kinase 1 (MSK1), p300, protein kinase A (PKA).

INTRODUCTION

The induction of IE (immediate early) genes plays an important role in the cellular response to many signals. CREB (cAMP-response-element-binding protein) is a bZIP (basic leucine zipper) transcription factor, which regulates the expression of many IE genes by binding to the CRE (cAMP-responsive element) present in their promoter regions [1]. Genome-wide studies have shown that CREB can bind to hundreds of gene promoters [2,3], giving CREB the ability to affect many cellular functions. Consequently, roles for CREB have been found in many physiological process, ranging from development, metabolic regulation, neuronal function to innate immunity (reviewed in [4–8]). How the transcriptional activity of CREB is regulated has been the subject of extensive investigation; however, a complete picture of the molecular events controlling CREB is still lacking. Initial studies have suggested phosphorylation to be the primary mechanism by which CREB was controlled [1]. CREB was found to be phosphorylated on Ser^{133} by PKA (protein kinase A), a kinase activated by cAMP in cells [9,10]. Since then, a wide variety of cellular stimuli have been shown to promote CREB phosphorylation, and several additional kinases have been proposed (reviewed in [1,11,12]). For example, MSK1 (mitogen- and stress-activated kinase 1) and MSK2, CaMK (Ca^{2+}/calmodulin-dependent protein kinase) family members, PKD (protein kinase D) and RSK (ribosomal S6 kinase) have all been suggested to phosphorylate CREB on Ser^{133} in cells [13–17].

The phosphorylation of CREB on Ser^{133} by PKA creates a binding site for the KIX domain of the co-activator proteins CBP (CREB-binding protein) and/or p300 [18–20]. Both CBP and p300 possess acetylase activity and are able to stimulate transcription of the promoters to which they are recruited [21]. This gave rise to a model in which PKA-phosphorylated CREB bound to CRE sites in CREB-dependent genes, leading to CBP/p300 recruitment to these promoters. CBP and p300, via their acetylase activity, would then be able to activate transcription. More recently this model has been modified following the discovery of another group of CREB co-activators, the CRTCs (CREB-regulated transcription co-activator) [22]. These proteins can be recruited to CREB independently of Ser^{133} phosphorylation. CRTCs are also regulated via PKA; in unstimulated cells CRTCs are retained in the cytoplasm and the activation of PKA promotes their nuclear translocation [22–24].

Although the phosphorylation of CREB on Ser^{133} has been considered as the classical mechanism by which CREB is activated, it is not clear whether phosphorylation of Ser^{133} itself is sufficient for maximal CREB activation *in vivo*. In particular, it has been proposed that, although PKA-mediated CREB phosphorylation promotes CREB-dependent gene transcription, CREB phosphorylation of Ser^{133} downstream of MAPK (mitogen-activated protein kinase) signalling is not sufficient to promote

Abbreviations: ATF1, activating transcription factor 1; CBP, CREB (cAMP-response-element-binding protein)-binding protein; CRE, cAMP-responsive element; CREB, cAMP-response-element-binding protein; CREM, cAMP-responsive element modulator; CRTC, CREB-regulated transcription co-activator; DMEM, Dulbecco's modified Eagle's medium; DUSP1, dual-specificity phosphatase 1; ERK1/2, extracellular-signal-regulated kinase 1/2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK, human embryonic kidney; IκBα, inhibitor of nuclear factor κBα; IBMX, isobutylmethylxanthine; ICER, inducible cAMP early repressor; IE, immediate early; IL-6, interleukin 6; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; MSK, mitogen- and stress-activated kinase; NF-κB, nuclear factor κB; PKA, protein kinase A; qPCR, quantitative real-time PCR; RSK, ribosomal S6 kinase; SRE, serum-response element; SRF, serum-response factor; TCR, T-cell receptor; TNF, tumour necrosis factor.

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the induction of CREB-dependent genes [1]. Early studies revealed that stimulation of the TCR (T-cell receptor) in Jurkat cells resulted in a strong phosphorylation of CREB, but that this did not stimulate CREB-dependent transcription [25]. TCR stimulation promotes the phosphorylation of CREB on Ser^{133} via the ERK1/2 (extracellular-signal-regulated kinase 1/2) and p38 MAPK pathways. ERK1/2 and p38 do not directly phosphorylate CREB, but instead activate the CREB kinases MSK1 and MSK2 [26]. Double-knockout of MSK1/2 blocks TCR-induced CREB phosphorylation in primary T-cells; however, in line with the earlier study on CREB in Jurkat cells, MSK1/2 knockout did not affect the ability of TCR activation to promote the transcription of classical CREB-dependent genes such as c-*Fos* and *Nur77* [26]. Stimuli such as forskolin or PMA that selectively activate either PKA or ERK1/2 respectively can both induce similar levels of CREB phosphorylation in cells. Intriguingly, using FRET-based reporters it has been found that in cells the phosphorylation of Ser^{133} in CREB by PKA is much more effective at promoting the interaction between CREB and CBP than Ser^{133} phosphorylation downstream of MAPKs [27]. Similarly, MEFs (mouse embryonic fibroblasts) with a deletion of both CBP and p300 result in either little change or even increased transcription of several CREB-dependent genes in response to stimulation with serum [28], a strong activator of ERK1/2. Together this would suggest that the phosphorylation of CREB downstream of MAPKs might not play a role in regulating IE gene transcription. Other data, however, argue against this hypothesis. Although the knockout of the CREB kinases MSK1 and MSK2 does not affect gene induction in T-cells [26], this is not the case in other cell types. For example, double knockout of MSK1 and MSK2 prevents CREB phosphorylation in response to PMA and anisomycin in MEFs [13] and in addition MSK1/2 knockout also reduced the induction of several CREB-dependent genes in response to PMA or anisomycin in these cells [29]. Similar results have also been found following BDNF (brain-derived neurotrophic factor) stimulation of cortical neurons and LPS (lipopolysaccharide) or zymosan stimulation of macrophages [30–32]. Although these findings are consistent with a role for MSK-mediated CREB phosphorylation in gene induction it remains possible that in reality MSKs control the induction of IE genes via the phosphorylation of substrates other than CREB. For example, MSKs also phosphorylate histone H3 on Ser^{10} and Ser^{28} [29,33] and it is therefore possible that MSKs regulate gene induction via directly regulating chromatin structure [34]. Other substrates have also been proposed for MSKs, including Ser^{276} in the RelA NF-κB (nuclear factor κB) subunit. Both NF-κB and CRE sites have been identified in the *Il6* (interleukin 6) promoter; however, it has been proposed that in MEFs MSKs regulate IL-6 transcription via the phosphorylation of the RelA NF-κB subunit and not CREB [35]. Thus it is not clear what role, if any, MSK-mediated CREB phosphorylation plays in regulating gene induction. To resolve this we created a knockin mutation of Ser^{133} to alanine in the endogenous *Creb* gene in mice.

MATERIALS AND METHODS

Mice

Mice with a double knockout of both MSK1 and MSK2 have been described previously [13]. A conditional knockin allele of S133A in CREB has been described previously [36], and to convert this into a constitutive knockin these mice were crossed to a germline expressing Cre transgenic line. Following generation of the constitutive knockin allele, mice were crossed away from the Cre transgene. Unless otherwise stated, both MSK1/2 knockout and CREB S133A mice were backcrossed on to C57Bl/6 mice for a minimum of nine generations before isolation of MEFs. Mice were housed in accordance with U.K. and EU (European Union) regulations and animal work was approved by local ethical review and carried out under an appropriate U.K. Home Office project license.

Cell culture

Primary MEFs were cultured as described [13]. Briefly, day 13.5 embryos were dissected and the head and internal organs removed. The remaining tissue was diced and digested with trypsin. Cells were then centrifuged, resuspended and plated in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % FBS (Sigma), 2 mM L-glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin. Cells were grown for up to five passages. The GloResponse CRE-luc2P HEK (human embryonic kidney)-293 cell line, which expresses a stably incorporated CREB-dependent luciferase gene, were obtained from Promega and maintained in DMEM supplemented with 10 % FBS, 2 mM L-glutamine, 100 units/ml penicillin G, 100 μg/ml streptomycin and 50 μg/ml hygromycin B.

Immunoblotting

Cells were lysed in SDS sample buffer and run on 10 % polyacrylamide gels, and transferred on to nitrocellulose membranes using standard techniques. Antibodies used against phospho-Ser^{133} CREB [which also recognizes ATF1 (activating transcription factor 1) phosphorylated on Ser^{63}], phospho-ERK1/2, phospho-p38, total CREB, total ERK1/2, total p38α, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and total CRTC2 were from Cell Signaling Technology and total CRTC3 was from Abcam. For quantification of total CREB levels, samples were run on a single gel and blotted for CREB and GAPDH. Membranes were imaged using a CCD (charge-coupled-device) camera and quantified using Aida software.

qPCR (quantitative real-time PCR)

Total RNA was isolated using Qiagen RNeasy Micro kit according to the manufacturer's instructions. RNA (0.5–1 μg) was reverse transcribed using iScript (Bio-Rad Laboratories) and qPCR was carried out using SYBR Green-based detection methods (Bio-Rad Laboratories). GAPDH and/or 18S were used as a loading control and fold change was calculated relative to the unstimulated wild-type sample as described previously [41]. Primer sequences are given in Supplementary Table S1 (at http://www.biochemj.org/bj/458/bj4580469add.htm).

Luciferase assays

Cells were serum starved for 16 h prior to stimulation with 400 ng/ml PMA or 20 μM forskolin for 4 h. Luciferase activities were measured by using the dual luciferase assay system (Promega) according to the manufacturer's protocol.

ChIP assay

MEFs were stimulated as indicated in the Figure legends. Cells were then incubated in 1 % formaldehyde in serum-free medium for 10 min at room temperature (20 °C). Cross-linking was stopped by the addition of glycine to a final concentration of 125 mM followed by a further 5 min incubation

at room temperature. Cells were washed twice with ice-cold PBS, detached by scraping and then pelleted by centrifugation at 2403 ***g*** for 10 min at 4 °C. Cells were lysed in buffer containing 1 % SDS, 10 mM EDTA, 50 mM Tris/HCl, pH 8.1, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml aprotinin for 10 min on ice. Chromatin was sheared to an average length of 200–800 bp by nine 15 s bursts of sonication at 4 °C using a VibraCell (Sonics) sonicator at 50 % power. Samples were centrifuged at 13845 ***g*** for 10 min at 4 °C and the soluble chromatin fraction was retained. Fragmented chromatin was then diluted 1:10 in dilution buffer (1 % Triton X-100, 2 mM EDTA, 150 mM NaCl and 20 mM Tris/HCl, pH 8.1) before preclearing with Protein G–Sepharose beads (Generon) and 2 μg of salmon sperm DNA (Invitrogen) for 2 h at 4 °C. At this stage 10 % of the sample was retained for use as an input control. Chromatin fractions were then incubated with 2 μg of antibody with the addition of BRIJ-35 (Merck Biosciences) detergent to a final concentration of 0.1 % overnight at 4 °C. The antibodies used against CBP and p300 were from Santa Cruz Biotechnology, against total CREB and phospho-CREB/ATF1 were from Cell Signaling Technology and the anti-CRTC3 antibody was raised in-house. Mouse IgG (Cell Signaling Technology) and acetyl histone H3 (Millipore) were used as negative and positive controls respectively. To isolate the immunocomplexes, 60 μl of Protein G–Sepharose beads was added to the samples for 1 h at 4 °C. Beads were pelleted by centrifugation and washed once in low-salt buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 150 mM NaCl and 20 mM Tris/HCl, pH 8.0), once in high-salt buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 500 mM NaCl and 20 mM Tris/HCl, pH 8.0), once in 250 mM LiCl, 1 % Nonidet P40, 1 % deoxycholate, 1 mM EDTA and 10 mM Tris/HCl, pH 8.0 and twice in TE buffer (10 mM Tris/HCl, pH 8.0, and 1 mM EDTA). Immunoprecipitated material was eluted in 1 % SDS plus 100 mM $NaHCO_3$ and cross-linking was reversed by the addition of NaCl to a final concentration of 300 mM followed by incubation at 65 °C overnight. Protein was eliminated by treating the samples with Proteinase K (Qiagen) for 1 h at 45 °C and DNA was then purified using the Qiagen PCR purification kit, according to the manufacturer's instructions. Purified immunoprecipitated DNA and input DNA were analysed using the SYBR Green-based qPCR method. The primers were designed in promoter regions covering the CRE site (see Supplementary Table S1). The qPCR data were analysed using the Percentage Input $\{100 \times 2^{[\mathrm{Input}(C_t)-\mathrm{IP}(C_t)]}\}$ method.

RESULTS

Generation of CREB Ser^{133} knockin mouse model

We have previously used gene targeting to create a conditional S133A mutant in the endogenous CREB allele [36]. This was converted into a constitutive S133A mutation by crossing these mice to a Cre recombinase line that deletes in the germline. The resulting constitutive knockin allele was then bred away from the Cre transgene and backcrossed against either C57Bl/6 or CD1 mice for at least six generations. In contrast with CREB-knockout mice, which die shortly after birth [37], live CREB S133A knockin mice were obtained. These mice did not exhibit any apparent adverse welfare effects when maintained under specific pathogen-free conditions, and were fertile. On a C57/Bl6 background the homozygous S133A mice were not obtained at Mendelian frequencies (wild-type 27 %, heterozygous 61.4 % and knockin 11.6 %, $n = 293$ for matings of heterozygous parents), suggesting that some of the knockins were dying *in utero*. When crossed on to a CD1 outbred background this effect was not observed and the S133A knockin mice were obtained at the expected ratio (wild-type 26.2 %, heterozygous 51 % and knockin 22.8 %, $n = 149$ for matings of heterozygous parents).

To examine the effects of the knockin at a cellular level we generated MEFs from wild-type and CREB S133A E13.5 (embryonic day 13.5) embryos. Immunoblotting of lysates from these cells indicated that the expression of CREB was similar in wild-type and CREB S133A knockin cells (Figure 1A). In CREB knockouts, a compensatory increase in the expression of other CREB family members has been reported [37–39]. The expression of CREB, ATF1 and CREM (cAMP-responsive element modulator) mRNA in the CREB S133A knockin MEFs, however, was similar to that seen in wild-type cells (Figure 1B). No consistent difference was seen in the levels of CREB protein between wild-type and CREB S133A MEFs, as determined by immunoblotting (Figure 1C). The stimulation of wild-type cells with forskolin, which activates PKA, promoted the phosphorylation of CREB on Ser^{133} (Figure 1A). As would be expected, no CREB Ser^{133} phosphorylation was detected in the CREB S133A knockin cells. The phosphorylation of ATF1 on the analogous site to Ser^{133} in CREB is also detected by the anti-phospho-CREB antibody and ATF1 phosphorylation was induced to similar levels in wild-type and CREB S133A knockin cells (Figure 1A). Forskolin stimulation also decreased the phosphorylation of the CREB co-activators CRTC2 and CRTC3, as judged by a downwards bandshift on immunoblots. This has been reported previously and correlates with the dephosphorylation of key 14-3-3-binding sites and the nuclear translocation of CRTC2 and CRTC3 [22–24].

As the phosphorylation of Ser^{133} by PKA has been proposed as a key event in the activation of CREB-dependent transcription, we examined the effect of the knockin mutation on forskolin-induced transcription. *Areg*, *Nur77* (NR4A1), *Nurr1* (NR4A2), *Nor1* (NR4A3), *Dusp1* (dual-specificity phosphatase 1) (Mkp1), *JunB* and *ICER* (inducible cAMP early repressor) have all been previously shown to be CREB-regulated genes in MEFs co-stimulated with forskolin and IBMX (isobutylmethylxanthine) [40,41]. IL-6 transcription has also been linked to CREB and has been shown to be regulated in MEFs by MSKs, kinases that can phosphorylate CREB on Ser^{133} [35,41]. In line with previous studies, the mRNA levels of these genes were induced by forskolin in wild-type MEFs; however, only some of these genes were affected by the CREB S133A knockin (Figure 2A and Supplementary Figure S1A at http://www.biochemj.org/bj/458/bj4580469add.htm). Of these, Areg was the most strongly inhibited by the CREB S133A knockin, followed by IL-6 and then Nur77 and Nor1, whose transcription was inhibited by approximately 50 % (Figure 2A). The transcription of Nurr1, DUSP1 and JunB was not significantly reduced by the knockin mutation ($P > 0.05$, Figure 2A and Supplementary Figure 1A). A small, but statistically significant, increase was seen in the induction of ICER mRNA in the CREB S133A knockin macrophages relative to wild-type controls.

The phosphorylation of CREB on Ser^{133} creates a binding site for the CBP/p300 co-activator proteins. ChIP assays were used to determine whether the knockin mutation affects the recruitment of CBP or p300 to CREB at the promoters of CREB-dependent genes. Primers were designed for the *Nur77*, *Nor1*, *Nurr1*, *ICER* and *Il6* promoter region containing the CRE site. In response to forskolin, recruitment of CBP or p300 could be seen to the promoters of *Nur77*, *Nor1*, *Il6* and *ICER*, but not to the promoter of the *Gapdh* gene (Figure 2B). Some recruitment was also seen to the *Nurr1* promoter; however, this was not induced as strongly as for the other CREB-dependent genes tested. The CREB S133A knockin mutation greatly decreased the recruitment

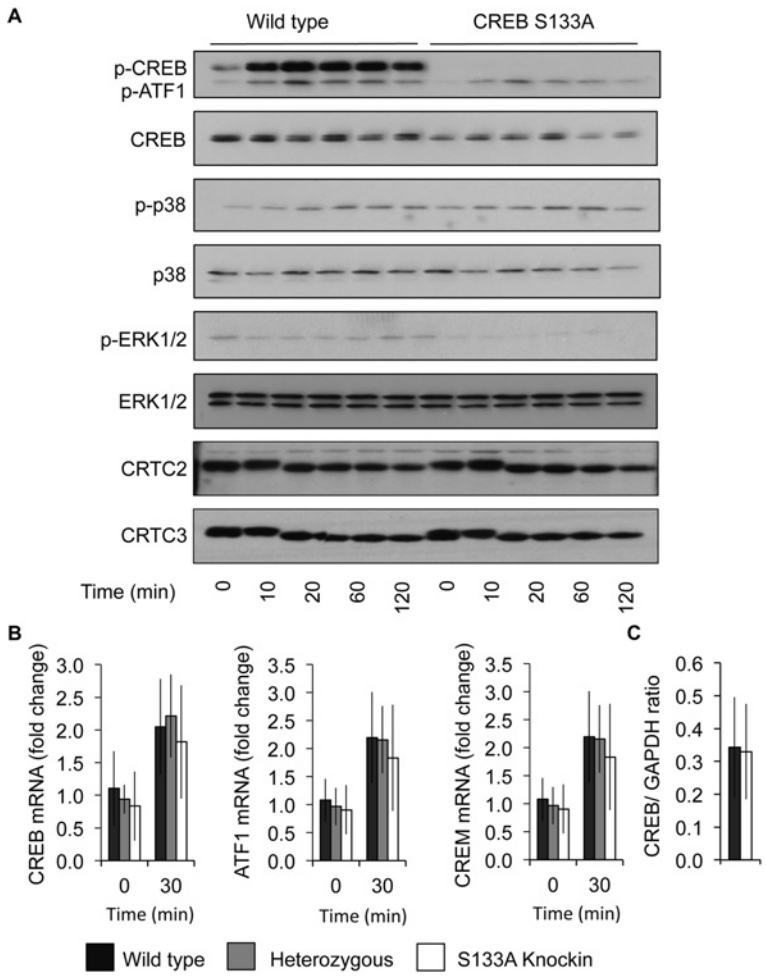

Figure 1 Mutation of Ser[133] in the endogenous *Creb* gene does not result in a compensatory up-regulation of CREB family members

(**A**) MEFs were isolated from wild-type and CREB S133A knockin mice. Cells were serum starved for 16 h and then stimulated for the indicated times with 20 μM forskolin. Following lysis the levels of the indicated proteins were determined by immunoblotting. Both CREB and ATF1 possess a conserved phosphorylation site that is recognized by the same antibody. (**B**) Wild-type, heterozygous or CREB S133A knockin MEFs were serum starved for 16 h and then stimulated for 30 min with 20 μM forskolin. Total RNA was then isolated and the levels of CREB, CREM and ATF1 mRNA were determined by qPCR. Error bars are $\pm$S.D. of three independent stimulations on two different preparations of MEFs per genotype. (**C**) Samples from three independent preparations of wild-type or CREB S133A knockin MEFs were immunoblotted for GAPDH and CREB. The ratio of CREB to GAPDH was determined as detailed in the Materials and methods section and the average $\pm$S.D. ($n = 3$) is shown.

of CBP and p300 to the *Nor1* and *Il6* promoters (Figure 2B). The recruitment of CBP to the *Nur77* promoter was also decreased, although not to the same extent as for Nor1 and IL-6. For the *Nurr1* and *ICER* promoters, the CREB S133A mutation had little or no effect on CBP and p300 levels (Figure 2B).

In order to confirm that CREB is recruited to these promoters we also carried out ChIP with anti-total-CREB and anti-phospho-CREB/ATF1 antibodies. CREB was found to be constitutively bound to the *Nor1*, *Nurr1*, *ICER* and *Il6* promoters, and the levels of CREB at these promoters were not affected by the knockin mutation in unstimulated cells. CREB levels on the *Nur77* promoter in unstimulated cells were low, but increased by forskolin treatment in wild-type, but not CREB S133A knockin, cells. The ChIP signal with the anti-phospho-CREB/ATF1 antibody in unstimulated cells at the five genes tested was similar to that seen with the control antibody, consistent with the low levels of phospho-CREB seen in immunoblotting. However, forskolin stimulation increased the phospho-CREB/ATF1 ChIP signal at the *Nor1*, *Nur77*, *Nurr1*, *ICER* and *Il6* promoters. This signal was reduced, but not completely abolished, by the mutation of Ser[133] in CREB. This reduction was least apparent at the *Nurr1* and *ICER* promoters, which were the two genes whose induction was least affected by the S133A knockin. This could reflect the presence of phosphorylated ATF1 on these promoters. It should also be noted that the phospho-CREB ChIP would not distinguish between an increased recruitment of phosphorylated CREB to a promoter or an increased phosphorylation of CREB that was already constitutively bound to a promoter. The CREB co-activator CRTC3 was also found to be recruited to the promoters of the CREB-dependent genes tested in response to forskolin (Figure 3), in line with its dephosphorylation which would promote its nuclear translocation (Figure 1). On the *Nor1*, *Nur77* and *Il6* promoters, CRTC3 recruitment in response to forskolin was reduced by the CREB S133A knockin. In contrast, the CREB knockin mutation did not affect CRTC3 recruitment to *Nurr1* or *ICER*, an observation that correlated with the presence of phospho-CREB/ATF1 ChIP signal at these promoters.

The above results support a model in which the phosphorylation of CREB on Ser[133] in cells creates a binding site for CBP or p300, and thus increases transcription at target promoters.

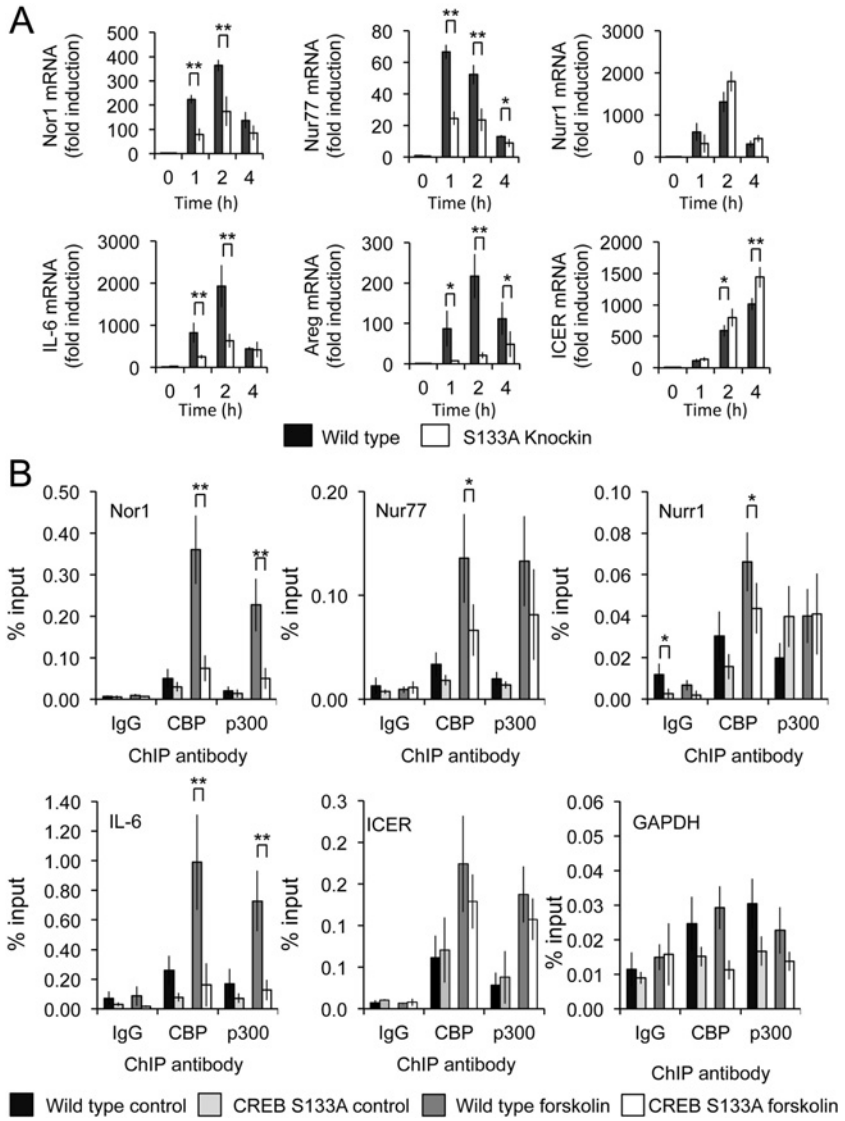

Figure 2 Gene-specific effects of the S133A CREB knockin mutation on forskolin-induced gene induction

(**A**) Wild-type or CREB S133A knockin MEFs were serum staved for 16 h and then stimulated for the indicated times with 20 μM forskolin. Total RNA was then isolated and the induction of the indicated mRNAs was determined by qPCR. Error bars are $\pm$S.D. of independent stimulations of four different preparations of MEFs per genotype. $^{*}P < 0.05$ and $^{**}P < 0.01$ (Student's *t* test) between the two genotypes. (**B**) MEFs from wild-type or CREB S133A knockin MEFs were serum starved for 16 h and then stimulated for 30 min with 20 μM forskolin. ChIP was then carried out as described in the Materials and methods section. Results using antibodies against CBP, p300 or a control IgG are shown. Error bars are $\pm$S.D. of four independent stimulations per genotype. $^{*}P < 0.05$ and $^{**}P < 0.01$ (Student's *t* test) between the two genotypes.

The importance of this phosphorylation for the transcription of endogenous CREB-dependent genes is, however, dependent on the specific promoter analysed.

CREB Ser^{133} phosphorylation is important for the induction of IE genes downstream of MAPK signalling

As discussed above, it has been suggested that the phosphorylation of CREB downstream of MAPKs does not lead to CBP or p300 recruitment and does not stimulate the induction of CREB-dependent reporter genes. Similar results were obtained in HEK-293 cells stably expressing a CREB-dependent luciferase reporter gene. The stimulation of these cells with forskolin resulted in the induction of the CREB-dependent reporter gene, whereas PMA, which is a strong agonist for the ERK1/2 pathway, did not (Figure 4A). In addition to the phosphorylation of CREB on Ser^{133}, PKA also promotes the dephosphorylation of CRTC2 and CRTC3 as shown using SDS/PAGE with a band shift in the forskolin-stimulated cell lysates (Figure 1). The dephosphorylated CRTCs are then translocated to the nucleus [23]. In contrast, ERK1/2 activation does not promote CRTC dephosphorylation (Figure 4B). This is in line with previous studies which found that the activation of MAPKs was insufficient to promote the nuclear localization of CRTCs [24]. These results confirm that the phosphorylation of CREB downstream of MAPK signalling is unable to induce the transcription of CREB-dependent reporter genes. However, it is not clear whether this is also the case for the induction of endogenous genes. Therefore we tested the effect of the CREB S133A knockin mutation on gene induction downstream of MAPK signalling. Both the ERK1/2 and p38 MAPK cascades are able to induce the phosphorylation of CREB

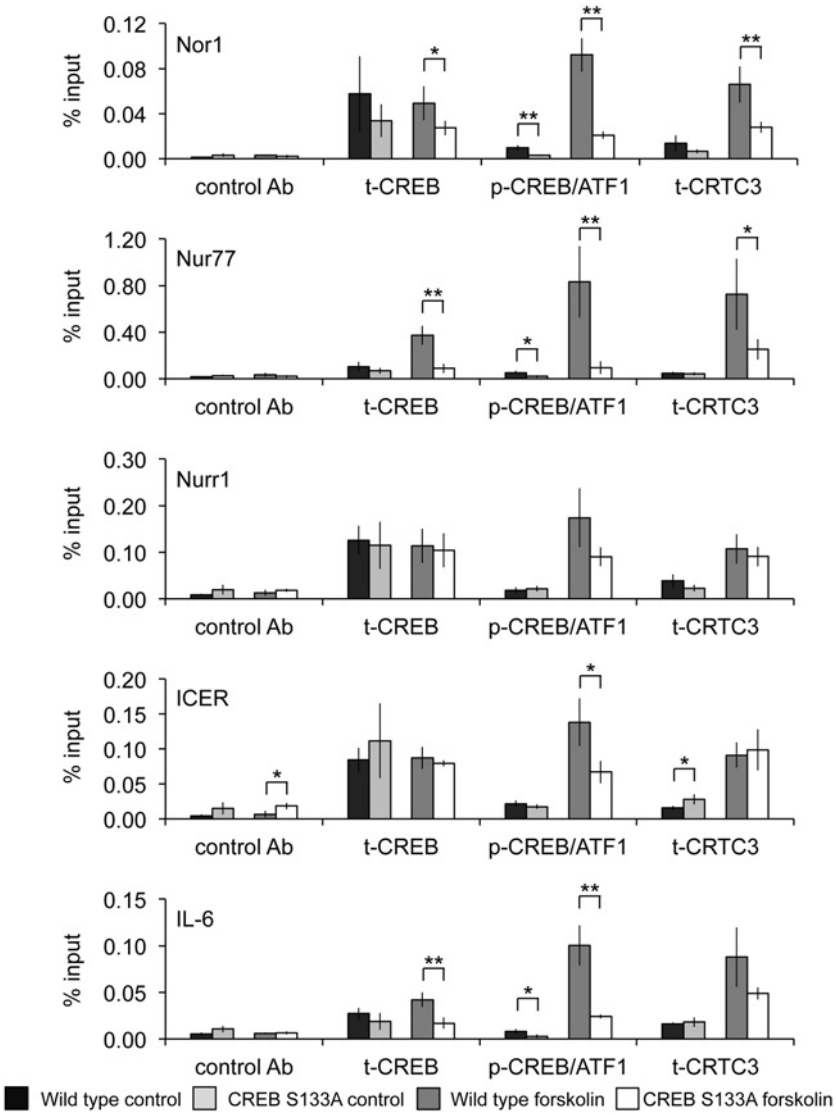

Figure 3 Effect of forskolin on CREB and CRTC levels at CREB-dependent promoters

MEFs from wild-type or CREB S133A knockin MEFs were serum starved for 16 h and then stimulated for 30 min with 20 μM forskolin. ChIP was then carried out as described in the methods using antibodies against total CREB (t-CREB), phospho-CREB/ATF1, total CRTC3 (t-CRTC3) or a control IgG (control Ab). Error bars are $\pm$S.D. of independent stimulations on four preparations of MEFs per genotype. $^{*}P < 0.05$ and $^{**}P < 0.01$ (Student's *t* test) between the two genotypes.

via the activation of MSK1 and MSK2. We have previously shown that in MEFs PMA activates MSKs predominately via ERK1/2, whereas anisomycin acts predominately via p38 [13]. TNF (tumour necrosis factor) activates both ERK1/2 and p38 in MEFs and both MAPK pathways contribute to MSK activation and CREB phosphorylation downstream of TNF [42]. As expected, PMA, anisomycin and TNF all induced CREB Ser^{133} phosphorylation in wild-type, but not CREB S133A knockin, MEFs. The phosphorylation of ATF1 and the activation of ERK1/2 and p38 (as judged by phosphorylation of their activation loops) was unaffected by the knockin mutation (Figures 4B–4D). Similar to forskolin stimulation, PMA induced the transcription of *Areg*, *Nur77*, *Nurr1*, *Nor1*, *Il6* and *ICER* in wild-type MEFs. The induction of all of these genes was greatly decreased in CREB S133A knockin cells relative to wild-type cells (Figure 5A). This was not due to a general deficit in transcription, as the CREB-independent gene c-*Jun* was unaffected by the S133A knockin (Figure 5B). The induction of *Egr1*, a gene we have previously found not to be reduced by knockout of the CREB kinases MSK1 and MSK2 [13], was slightly decreased by the CREB S133A knockin in this experiment. Similar results were obtained for the induction of CREB-dependent genes by anisomycin, although the effect of the knockin mutation on Nur77 transcription was not as great as seen for PMA (Figure 5C). The CREB knockin mutation did not reduce the induction of the CREB-independent genes *Egr1* and *NFKBIA* [IκBα (inhibitor of NF-κBα)] by anisomycin (Figure 5D). Compared with PMA and anisomycin, TNF was a weaker stimulus for the induction of the genes tested. The CREB S133A knockin mutation greatly decreased the induction of CREB-dependent, but only small changes were seen in CREB-independent, genes (Figures 5E and 5F).

Given these results, we tested the ability of PMA to promote CBP or p300 recruitment to CREB-dependent IE gene promoters. As measured by ChIP, PMA induced little or no detectable recruitment of either CBP or p300 to the *Nurr1*, *Nor 1*, *Il6* or *ICER* promoters (Figure 6), although in the same experiments forskolin

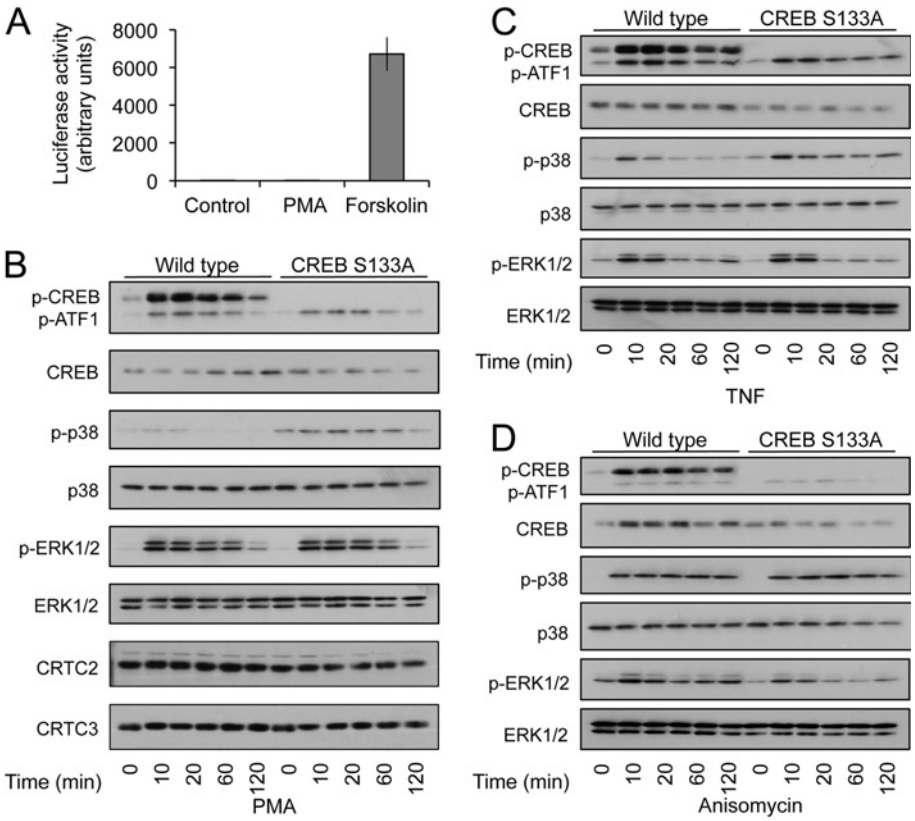

Figure 4 MAPK activation induces CREB phosphorylation, but not the transcription of CREB-dependent luciferase reporters

(**A**) HEK-293 cells with a stably integrated luciferase reporter gene were stimulated with with either 400 ng/ml PMA or 20 μM forskolin for 4 h. Cells were then lysed and luciferase activity was measured as described in the Materials and methods section. Error bar is $\pm$S.D. of three stimulations per condition. (**B**–**D**) Wild-type or CREB S133A knockin MEFs were serum staved for 16 h and then stimulated for the indicated times with either 400 ng/ml PMA (**B**), 10 μg/ml anisomycin (**D**) or 10 ng/ml TNF (**C**) for the indicated times. The levels of phospho-CREB, phospho-ATF1, total CREB, phospho-p38, total p38, phospho-ERK1/2, total ERK1/2, CRTC2 and CRTC3 were determined by immunoblotting.

did induce CBP and p300 recruitment. Some recruitment was observed at the *Nur77* promoter in response to PMA; however, this was less than that obtained in response to forskolin (Figure 6).

DISCUSSION

To examine the role of Ser^{133} phosphorylation in regulating the transcriptional activation of CREB, we generated a knockin mutation of this site in the endogenous *Creb* gene. Previous studies on CREB knockouts have reported that compensation from the related proteins CREM and ATF1 can occur in the absence of the CREB protein [37–39]. The possibility of compensation from other CREB family member is perhaps less likely in the CREB S133A knockin mice, as CREB is expressed at its normal levels and should still able to bind to CRE sites in gene promoters. In MEFs, we did not observe any up-regulation of ATF1 phosphorylation and the expression of both the ATF1 and CREM mRNAs was normal. Despite this, the phenotype of the CREB S133A mice generated in the present study was arguably less severe than that previously reported for total CREB knockout [37]. Unlike the perinatal lethality seen in the knockout, those CREB S133A mice that were born had no apparent adverse phenotype when maintained under specific pathogen-free conditions. This is consistent with the idea that not all CREB functions are dependent on Ser^{133} phosphorylation. In the present study we show that, although CREB phosphorylation is required to promote the transcription of specific CREB-dependent IE genes, this requirement is both promoter- and stimuli-specific. Therefore not all CREB regulated genes require Ser^{133} phosphorylation, whereas for those that do, this requirement is not necessarily seen downstream of all CREB-activating stimuli.

The molecular mechanism of CREB regulation has been most studied in the context of cAMP signalling. Two important mechanisms of CREB regulation have emerged downstream of cAMP signalling; CREB Ser^{133} phosphorylation leading to CBP/p300 recruitment, and the relocalization of CRTCs to the nucleus where they bind CREB independently of Ser^{133} phosphorylation. Both of these events can be regulated by the cAMP-dependent kinase PKA. PKA directly phosphorylates CREB on Ser^{133} [10]. Two genetic models have been used to study the role of CBP/p300 in forskolin-induced gene transcription in MEFs [40,41]. In the first model, a point mutation was introduced into the KIX domains of both CBP and p300 in order to block their binding to phosphorylated CREB [40]. This mutation would also prevent the recruitment of CBP and p300 to CREM and ATF1 phosphorylated on the analogous site to Ser^{133} in CREB. Xu et al. [40] found that blocking the ability of CBP and p300 to bind to CREB reduced the ability of forskolin to induce some CREB-dependent genes, but not others. The importance of CREB family members to the forskolin-induced transcription of the genes tested has been confirmed in MEFs by transfection of A-CREB, a dominant-negative form of CREB that prevents the binding of CREB, ATF1 and CREM

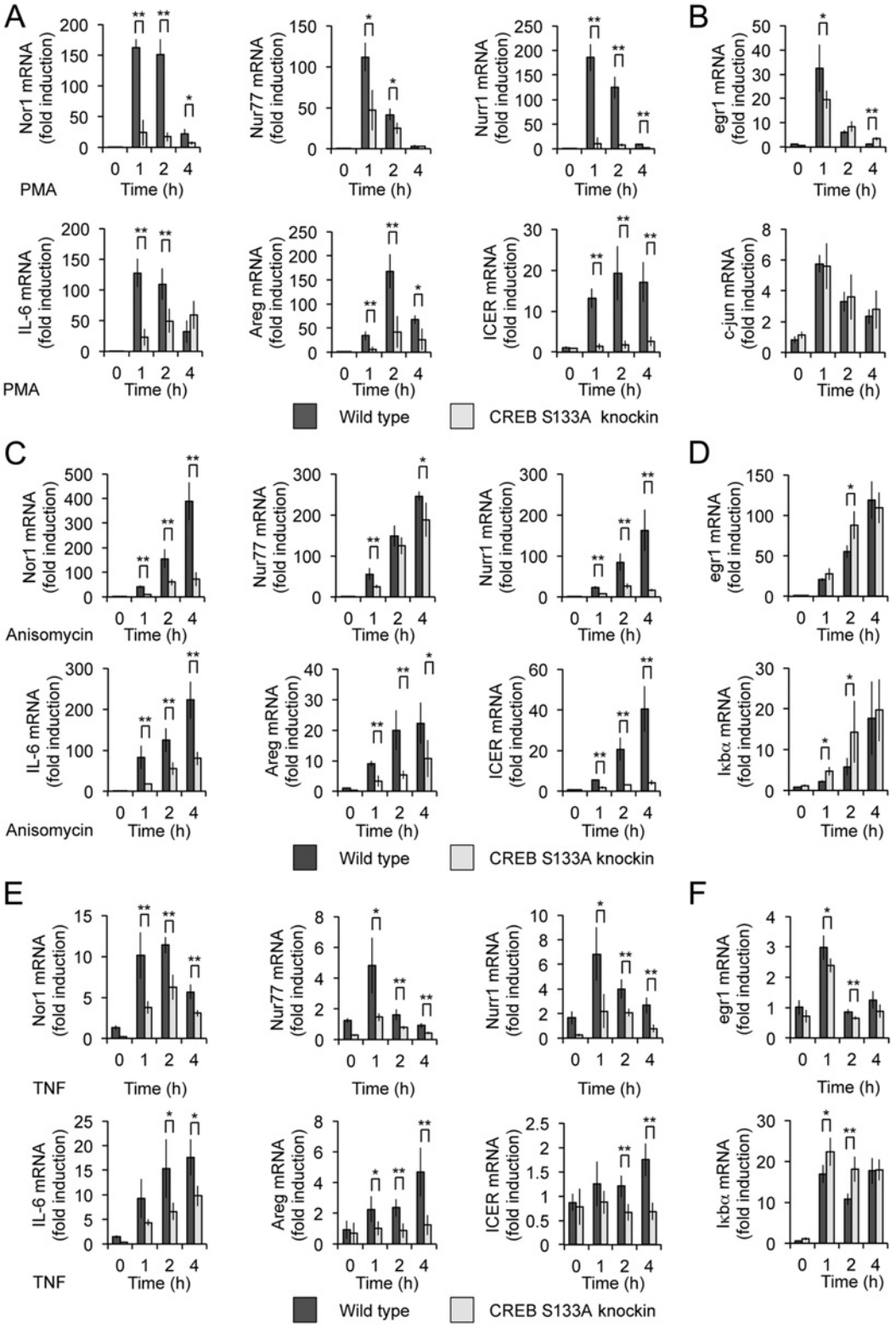

Figure 5 Effect of CREB S133A knockin on IE gene induction downstream of MAPK signalling

Wild-type or CREB S133A knockin MEFs were serum starved for 16 h and then stimulated for the indicated times with either 400 ng/ml PMA (**A** and **B**), 10 μg/ml anisomycin (**C** and **D**) or 10 ng/ml TNF (**E** and **F**) for the indicated times. The induction of a panel of CREB-dependent genes (*Nur77*, *Nurr1*, *Nor1*, *Areg*, *Il6* and *ICER*) were determined by qPCR (**A**, **C** and **E**). As controls the induction of two CREB-independent genes (IκBα, *Egr1* or c-*Jun* depending on the stimuli) was also measured (**B**, **D** and **F**). Error bars are $\pm$S.D. of independent stimulations on four different preparations of MEFs per genotype. $^{*}P < 0.05$ and $^{**}P < 0.01$ (Student's *t* test) between the two genotypes.

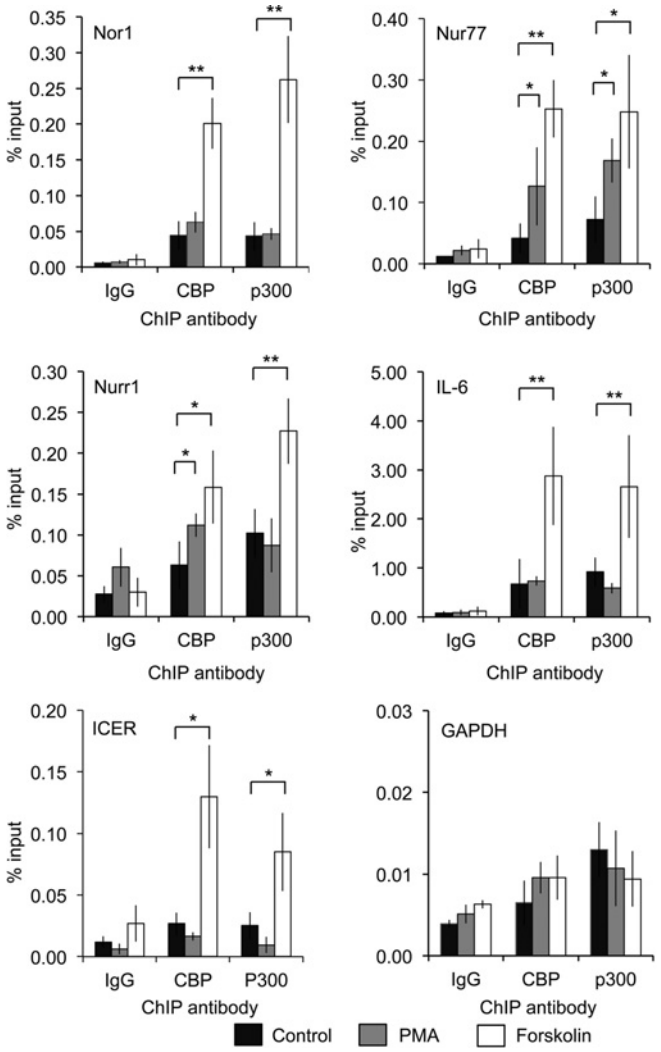

Figure 6 PMA is less effective at recruiting CBP and p300 to CREB-dependent promoters relative to forskolin

Wild-type MEFs were serum starved for 16 h and then stimulated with either 400 ng/ml PMA or 20 μM forskolin for 30 min. ChIP was carried out as described in the Materials and methods section using antibodies against CBP, p300 or a control IgG. Error bars are $\pm$S.D. of independent stimulations on four preparations of MEFs per genotype. $^{*}P < 0.05$ and $^{**}P < 0.01$ (Student's *t* test) between the two genotypes.

to their target promoters [41]. The ability of CREB to act independently of Ser[133] phosphorylation downstream of PKA could be explained by CRTC recruitment. Prior to stimulation, CRTCs are phosphorylated by a member of the SIK (salt-inducible kinase) family on key 14-3-3-binding sites, leading to the tethering of CRTCs in the cytoplasm [23,43]. Activation of PKA promotes the dephosphorylation of these sites, allowing CRTCs to translocate to the nucleus and interact with CREB independently of Ser[133] phosphorylation [23,44]. Although the CREB S133A mutation did not abolish CRTC3 recruitment at the level of endogenous promoters, we did observe that the amount of CRTC3 recruitment, as determined by ChIP, correlated with the phospho-CREB/ATF1 ChIP signal (Figure 3). This would agree with the previous suggestion that CRTCs and CBP may form a complex with CREB in which CRTC and CBP/p300 may stabilize each other's binding [45]. In general there was a good agreement between those genes reported to be affected by the KIX domain mutations in CBP and p300 and the data for the CREB S133A knockin mutation described in the present study (Table 1).

In the second model, both CBP and p300 were knocked out [41]. A more extensive analysis of CREB-dependent genes was reported in [41] and, unexpectedly, although the ability of forskolin to induce some CREB-dependent genes was decreased in the CBP/p300 double-knockin MEFs, the transcription of others was actually increased. Notably Nur77 and Nor1 were increased in the absence of CBP and p300, but reduced by the mutation of Ser[133] in CREB or the KIX domains in CBP and p300. Why this would occur is not clear; however, it may be significant that CBP and p300 are able to interact with multiple other transcription factors independently of their KIX domains. Thus knockout of CBP and p300 will have effects on IE gene transcription that is independent of CREB. Overall these studies support a model in which PKA regulates CREB via at least two independent mechanisms, phosphorylation of Ser[133] and recruitment of CRTCs; however, the relative importance of these two mechanisms is promoter-specific.

As discussed above, although previous studies have indicated that CREB phosphorylation does play a role in mediating CREB activation downstream of cAMP, the role of Ser[133] downstream of MAPK signalling has been less clear. Unexpectedly we show in the present study that the effect of the CREB S133A knockin on CREB-dependent transcription was increased for MAPK-induced transcription compared with that induced by PKA activation for the CREB-dependent genes analysed in the present study (Figures 2 and 5). In addition, different CREB-dependent genes were more uniformly affected downstream of MAPK activation by the S133A knockin mutation compared with PKA activation. This could be explained by the ability of PKA to regulate CREB via promoting the relocalization of CRTCs to the nucleus where they can provide an alternative mechanism to Ser[133] phosphorylation for activating CREB-dependent transcription. As ERK1/2 signalling does not promote the dephosphorylation or nuclear relocalization of CRTCs [24] (Figure 5), this might result in a stronger dependence on CREB Ser[133] phosphorylation. As discussed above, Ser[133] phosphorylation downstream of MAPK signalling in cells does not effectively promote CBP or p300 binding to CREB. In line with this, we show in the present study that PMA, which stimulates CREB phosphorylation via ERK1/2, is a poor stimulus for CBP or p300 recruitment to endogenous CREB-dependent promoters. Some recruitment was seen to the *Nur77* promoter; however, this may be explained by the recruitment of CBP to a SRE (serum-response element) nearby in the *Nur77* promoter. SREs bind a complex of SRF (serum-response factor) and an ETS domain transcription factor such as Elk1 and this SRE-dependent transcription is known to be promoted by ERK1/2 activation [46]. Related to this, Nur77 has been reported to be regulated in part by SRF in T-cells [47], whereas CBP is known to be recruited to SRF [48,49].

Double knockout of CBP/p300 does not affect the induction of CREB-dependent genes in response to serum, a strong activator of ERK1/2 [28]. In contrast, S133A mutation resulted in a major reduction in the induction of CREB-dependent IE genes in response to PMA. Similar results on gene induction were also obtained with the p38 MAPK-activating stimuli anisomycin. In response to PMA and anisomycin, the main Ser[133] kinases are the MAPK-activated kinases MSK1 and MSK2. In accordance with the results reported in the present study for the CREB S133A mutant, double knockout of MSK1 and MSK2 has been shown to reduce the induction of Nur77, Nurr1 and Nor1 in MEFs in response to both PMA and anisomycin [42], whereas Areg and IL-6 were also MSK regulated in MEFs in response to these stimuli (Supplementary Figure S1). The

Table 1 Comparison of the results of the S133A knockin mutation in CREB with knockout or knockin mutations in CBP and p300

n.d., not determined.

Gene	A-CREB transfection [41] 10 μM forskolin plus 100 μM IBMX for 1.5 h	$CREB^{S133A/S133A}$ 20 μM forskolin for 1, 2 and 4 h*	$CBP^{KIX/-}$ $P300^{KIX/KIX}$ [40] 10 μM forskolin plus 100 μM IBMX for 1.5 h	$CBP^{-/-}$ $P300^{-/-}$ [41] 10 μM forskolin plus 100 μM IBMX for 1.5 h
Areg	Reduced	Reduced	Reduced	Reduced
IL-6	Reduced	Reduced	n.d.	Reduced
ICER	Reduced	Unaffected	Unaffected	Unaffected
Nur77 (NR4A1)	Reduced	Reduced	n.d.	Increased
Nurr1 (NR4A2)	Reduced	Unaffected	n.d.	Increased
Nor1 (NR4A3)	Reduced	Reduced	Reduced	Increased
JunB	Reduced	Unaffected	Unaffected	Increased
Dusp1	Reduced	Unaffected	Unaffected	Increased

*Results from the present study

lack of effective CBP or p300 recruitment to CREB-dependent promoters downstream of MAPK signalling, although consistent with previous publications, is perhaps unexpected given the dependence on Ser^{133} phosphorylation for CREB-dependent transcription reported in the present paper. Although there is a possibility that a weak interaction between CBP and CREB could be missed in the ChIP assays, our results are consistent with the finding that double knockout of CBP and p300 did not reduce the induction of Areg or Nur77 by serum [28]. Consequently, this would suggest that, although CREB Ser^{133} phosphorylation by MSKs is critical for the transcriptional activation of CREB, it might regulate CREB activity independently of CBP and p300. This would imply the existence of a further CREB co-activator protein that would be recruited to CREB following Ser^{133} phosphorylation by MSK, but not PKA. Why Ser^{133} phosphorylation has different effects on co-activator recruitment in cells following phosphorylation by MSK or PKA is not clear. One explanation could be that MAPK signalling may in some way inhibit CBP recruitment to CREB. Of note, NGF (nerve growth factor) and insulin have been shown to promote the formation of a complex of the ERK1/2-activated kinase RSK and CBP, and this was able to repress cAMP-induced gene induction [50]. A subsequent report has, however, found that ERK1/2 activation induced the dissociation of CBP and RSK [51]. Further work will be required in order to resolve these issues.

In summary, in the present study we show that the phosphorylation of CREB on Ser^{133} by MSKs is necessary for the maximal induction of CREB-dependent IE genes. In addition, we also show that MSK-mediated CREB phosphorylation has a greater effect on IE gene induction compared with the phosphorylation of the same site by PKA.

AUTHOR CONTRIBUTION

Shaista Naqvi and Kirsty Martin carried out the experimental work. Simon Arthur and Shaista Naqvi analysed data and prepared the paper.

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online data

SUPPLEMENTARY ONLINE DATA

CREB phosphorylation at Ser[133] regulates transcription via distinct mechanisms downstream of cAMP and MAPK signalling

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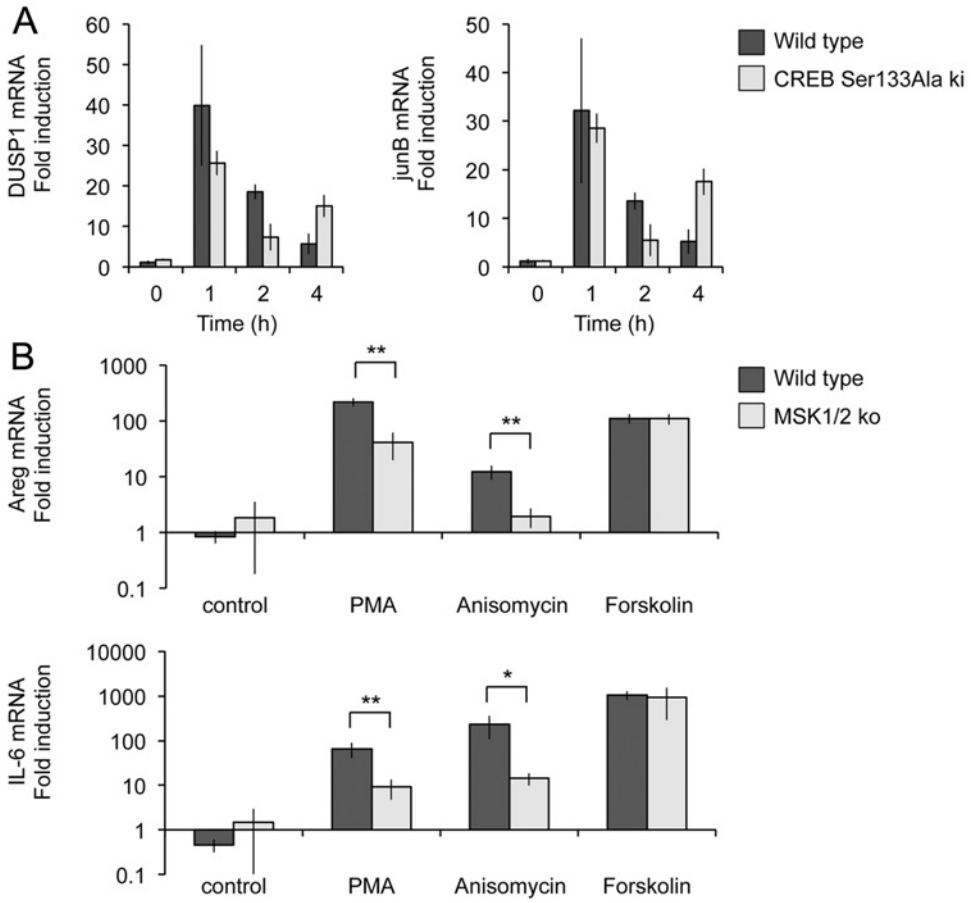

Figure S1 Additional analysis of gene induction in CREB S133A and MSK1/2 knockout MEFs

(**A**) Wild-type or CREB S133A knockin (ki) MEFs were stimulated with 20 μM forskolin for the indicated times. The levels of DUSP1 or JunB mRNA were determined by qPCR as described in the Materials and methods section of the main text. Error bars are $\pm$S.D. of four independent stimulations per genotype. (**B**) Wild-type or MSK1/2 double knockout (ko) MEFs were stimulated with 400 ng/ml PMA, 10 μg/ml anisomycin or 20 μM forskolin as indicated for 2 h. Areg and IL-6 mRNA levels were then determined by qPCR. Error bars are $\pm$S.D. of four independent stimulations per genotype. $^*P < 0.05$ and $^{**}P < 0.01$ (Student's *t* test) between the two genotypes.

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Table S1 Primer sequences used for qPCR

(a) ChIP primers

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
Nor1	GTGACGTAGCGTCCCATGTC	GGCACGTCATTTATGCCACA
Nurr1	CAAGTGGGCTACCAAGGTGA	CGCGCTCGCTTTGGTATATTT
Nur77	GTATGGCCAAAGCTCGCCG	ACATCTTAAGCGCTCCGTGA
IL-6	CATCAAGACATGCTCAAG	AGAATCACAACTAGGAAGG
GAPDH	AGTGCCAGCCTCGTCCCGTAGACAAAATG	AAGTGGGCCCCGGCCTTCTCCAT
ICER	TGCTAGTTCTTTCCTCCTGCC	CTCGGAGCTGACGTCAATGT

(b) Primers for analysis of mRNA induction

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
Areg	CGACAAGAAAACGGGACTG	AACTGGGCATCTGGAACC
Nor1	GCCATCTCCTCCGATCTGTATG	GAGGCCGTCAGAAGGTTGTAG
Nurr1	GAAGAGAGCGGACAAGGAGATC	AAGGCATGGCTTCAGCAGAG
Nur77	CCTGTTGCTAGAGTCTGCCTTC	CAATCCAATCACCAAAGCCACG
IL-6	TTCCATCCAGTTGCCTTCTTG	AGGTCTGTTGGGAGTGGTATC
ICER	ATGGCTGTAACTGGAGATGAAACTG	CACCTTGTGGCAAAGCAGTA
Dusp1	TGGGAGCTGGTCCTTATTTATT	GACTGCTTAGGAACTCAGTGGAA
GAPDH	ACAGTTCTTCATGTGGTGACCC	TGCACCACCAACTGCTTAG

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