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**P/CAF MODULATES THE ACTIVITY OF THE TRANSCRIPTION FACTOR  
p48/Ptf1a INVOLVED IN PANCREATIC ACINAR DIFFERENTIATION**

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Running title: p/CAF modulates p48/Ptf1a activity

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**SUMMARY**

p48, also called Ptf1a, is a tissue-restricted basic helix-loop-helix transcription factor critical for pancreatic commitment during development and for the activation/maintenance of the acinar differentiation program in exocrine pancreas. High-level expression of exocrine digestive enzymes, a hallmark of mature acinar cells, depends largely on the trimeric complex PTF1 (pancreas transcription factor 1), formed by p48, RBP-L and a class A bHLH protein. In addition, p48 induces cell cycle exit by controlling G1-S progression. However, the mechanisms that mediate the PTF1-dependent gene activation are poorly understood. Here, we report that p48 increases transcription through two activation domains located in its N-terminal region by recruiting transcriptional coactivators. The histone acetyltransferase cofactor p/CAF interacts *in vivo* with p48 in acinar cells and is associated with the promoter region of acinar genes targeted by the PTF1 complex. P/CAF potentiates the PTF1 transcriptional activity by selectively enhancing the p48 transactivation activity. P/CAF promotes the nuclear accumulation of p48 and its *in vivo* acetylation in Lys residue K200. The K200R mutation abolishes the transcriptional activity of p48 as well as its capacity to functionally cooperate with RBP-L to ensure an effective PTF1-driven transcription, indicating that p/CAF-mediated acetylation of p48 is required for PTF1 full transcriptional activity. By contrast, p/CAF did not cooperate with p48 in its growth regulatory effects. These results support a critical and selective role of p/CAF in the PTF1-dependent gene activation during acinar differentiation.

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## INTRODUCTION

The basic-helix-loop (bHLH) transcription factor p48 (also designated Ptf1a or Ptf1 $\alpha$ ) exerts critical functions during both early and late pancreatogenesis: it is required for acquisition of pancreatic fate by undifferentiated foregut endoderm [1] and is also necessary for activation/maintenance of the acinar differentiation program in exocrine pancreas. In murine pancreatic buds, p48 expression is detected starting at E9.5 [2,3] in a progenitor population that gives rise to all acinar and ductal cells, as well as the majority of insulin- and glucagon-producing cells, and becomes restricted to the acinar compartment from E14 onward [1,4]. In the absence of p48, progenitors from the dorsal pancreatic bud give rise to the four differentiated intestinal cell types [1]. Inactivation of p48 by homologous recombination in the mouse also results in pancreatic and cerebellar agenesis as well as in defective specification of GABAergic neurons in the spinal cord and horizontal/amacrine cells in developing retina [1,2,5-7]. A similar pancreatic and cerebellar phenotype is observed in patients with permanent neonatal diabetes mellitus harbouring mutations leading to the expression of a truncated form of p48 lacking the C-terminal 32 amino acids [8]. These observations highlight a crucial role of the C-terminal domain of p48 in pancreatic and neuronal specification. Additionally, the C-terminal region of p48 has been shown to be responsible for the antiproliferative activity of the protein [9] and to interact with the mammalian Suppressor of Hairless (RBP-Jkappa) and its paralogue RBP-L [10,3].

The establishment of the acinar differentiation program relies on the formation of transcriptional complexes thought to be responsible for the activation of expression of all acinar digestive enzymes. Thus, the tissue-specific transcription of the acinar genes at high levels is largely controlled by the pancreas-specific transcription complex PTF1 [11,12]. PTF1 is a hetero-oligomeric complex composed of the ubiquitously expressed class A bHLH proteins E2A or HEB, RBP-Jkappa or its paralogue RBP-L [10,13], and p48 [14]. In the adult, RBP-L and Ptf1a are mainly expressed in acinar cells in the pancreas [4;10]. The PTF1 binding site is a bipartite sequence with an E-box and a TC box (TTTCCCA) present in the promoter of all the acinar digestive enzymes genes [11]. An antisense RNA-mediated reduction of p48 synthesis in cultured exocrine pancreatic cells led to inhibition of the exocrine transcription program [4] and *in vitro* acinar-to-ductal transdifferentiation of normal exocrine pancreas is associated with a loss of PTF1 activity and the selective down-regulation of p48 and RBP-L expression ([15], unpublished data). These findings indicate that p48 and RBP-L are essential for maintaining the terminally differentiated state in acinar cells. However, the mechanisms supporting PTF1-mediated gene activation are still unknown.

P/CAF [16] and the closely related GCN5 (also called p/CAF-B) [17] are transcriptional co-activators with intrinsic histone acetyltransferase (HAT) activity which participate in transcriptional regulation by modifying chromatin and transcription factors. Histone acetylation on Lys residues is predominantly associated with gene activation but the precise mechanism by which this post-translational modification augments transcription is not fully understood. It has been proposed that acetylation would weaken the nucleosomal structure and disrupt contacts between nucleosomes, thereby facilitating the access of transcription factors and the progression of RNA polymerase II onto chromatin [18]. In human cells, p/CAF is part of a large multi-subunit complex of more than twenty polypeptides including TATA box-binding protein associated factors [19] and may associate with the HATs CBP/p300 [16]. It is thought that the combination of HATs ensuring the optimal transcription of a given promoter may be determined at least in part by their interaction with specific transcription factors bound to this promoter. In addition to histones, a growing number of transcription factors have been identified as substrates for p/CAF. They include p53 [20], pRb [21] and the bHLH transcription factors MyoD [22,23], Tal1 [24], E2A [25], and Beta-2 [26]. P/CAF-mediated acetylation of these transcription factors is required for their function, illustrating the participation of p/CAF in proliferation, apoptosis or differentiation processes.

Here, we report on the mechanisms through which p48 contributes to transcriptional activation. We demonstrate that the N-terminus of p48 contains activation domains and we show that p/CAF, through its interaction with p48, contributes to the transcriptional activity of

the PTF1 complex. We demonstrate that p48 is acetylated by p/CAF and that p/CAF-mediated acetylation is required for both p48 and PTF1 transcriptional activity. These data reveal a novel mechanism by which p48 function is regulated and support the important contribution of p48 acetylation in the acinar differentiation program.

## EXPERIMENTAL PROCEDURES

**Cells and cell culture.** We used parental AR42J cells, derived from an azaserine-induced pancreatic acinar tumor [27] and acinar pancreatic 266-6 cells, derived from a mouse tumor induced with an elastase I/SV40 T antigen fusion gene [28]. All cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Gaithersburg, MD), supplemented with 10% fetal bovine serum, L-glutamine, non-essential amino acids, penicillin and streptomycin (complete medium).

**Plasmids.** pIRESp48, pIRESN-bHLH, pIRESN+bHLH, pIRESC-bHLH, pIRESC+bHLH expression vectors and 6XA<sub>26</sub>-luc reporter plasmid constructions have previously been described [9]. Plasmids expressing the full-length rat p48/Ptf1a (amino acids 1-326) or the fragments (residues 1-43), (residues 38-108), (residues 104-138), (residues 133-168), N-bHLH (residues 1-168), N+bHLH (residues 1-217), C-bHLH (residues 211-326), C+bHLH (residues 162-326) were constructed by cloning PCR amplification products from pIRES-p48 into the pBXG1 expression vector in frame with the GAL4 DNA-binding domain (Gal4DBD). The pBXG1 expression vector and the Gal4-TKluc reporter vector containing four copies of Gal4 DNA-binding sites upstream of the TK promoter [29] were obtained from P. Muñoz (Centre de Regulació Genòmica, Barcelona, Spain). The p48GFP and pFlagCMV2-- plasmids were constructed by subcloning the rat Ptf1a cDNA into pEGFP-C1 (Clontech Laboratories, Mountain View, CA) and pFlagCMV2 (Sigma, St-Louis, MO), respectively. P48<sup>K196R</sup>, p48<sup>K200R</sup>, p48<sup>K252R</sup>, p48<sup>K286R</sup>, p48<sup>K289R</sup>, p48<sup>K298R</sup>, p48<sup>K307R</sup> and p48<sup>K311R</sup> mutants were generated by PCR-based mutagenesis. Amplification products were cloned into pFlagCMV2, pBXG1 or pIRESneo (Clontech Laboratories, Mountain View, CA). pCx-Flag-pCAF and pCx-Flag-pCAF(HAT-) expression vectors were kind gifts from H. Santos-Rosa (The Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, United Kingdom). pIRES-GFP-p48 and pIRES-GFP-p48<sup>K200R</sup> were obtained by cloning p48 and p48<sup>K200R</sup> PCR products into pIRES-hrGFP-2a (Stratagene, La Jolla CA). pFlagCMV2-GCN5 was obtained from T. Honjo (Graduate School of Medicine, Kyoto University, Kyoto, Japan). The pcDNA<sub>3</sub>RBP-L and the pcDNA<sub>3</sub>E47 plasmids were provided by R. MacDonald (U. Texas Southwestern, Dallas, TX) and A. Cano (Instituto de Investigaciones Biomédicas "Alberto Sols", Madrid, Spain), respectively. All constructs were sequenced and the expression of the corresponding proteins was assessed by western-blotting.

**Antibodies.** The following antibodies were used: affinity-purified rabbit anti-p48 [15], Gal4 DNA-BD monoclonal antibody (mAb) (BD Biosciences, Palo Alto, CA), anti-Flag M2 mAb (Sigma), rabbit polyclonal anti-acetyl-Lys antibody (Chemicon, Temecula, CA) and E2A/E12 rabbit polyclonal antibody (Santa-Cruz Biotechnology, Santa Cruz, CA). The rabbit polyclonal anti-p/CAF and the affinity-purified rabbit anti-RBP-L antisera were kindly provided by Y. Nakatani (Dana-Farber Cancer Institute, Boston, MA) and R. Wagener (Institute for Biochemistry II, University of Cologne, Germany), respectively.

**Transient transfection and reporter gene assays.** AR42J cells were transfected with expression vector and reporter plasmids using Lipofectamine (Invitrogen, Carlsbad, CA). Cells were lysed 24 hours after transfection and luciferase activity was determined using the Dual Luciferase Reporter Assay System (Promega, Madison, WI). The pRL-TK vector (15 ng) was used as an internal control for normalization.

For transactivation assays, cells were cotransfected with 0.2 µg of plasmid expressing p48 or its deletion mutant forms fused to the Gal4DBD and 0.1 µg of Gal4-TKluc reporter plasmid. To determine the coactivation induced by p/CAF, 0.1 µg of p48 and RBP-L expression

vectors or the corresponding empty vectors were transfected with increasing amounts of pCX-Flag-p/CAF or pCX-Flag-p/CAF(HAT-) and 0.1  $\mu\text{g}$  of 6XA<sub>26</sub>-luc. Transcriptional activity assays of wild-type and Lys-mutated forms (p48K-R) of p48 were performed by transfecting 0.2  $\mu\text{g}$  of p48 or p48K-R point mutant expression plasmids, 0.2  $\mu\text{g}$  of RBP-L expression vector and 0.1  $\mu\text{g}$  of 6XA<sub>26</sub>-luc.

**Immunoprecipitation and western-blotting.** HEK293T cells were transfected with 3  $\mu\text{g}$  of pIRESp48 expression vector and 3  $\mu\text{g}$  of Flag-p/CAF or Flag-GCN5 plasmids. After 48 h, cells were lysed in 1 ml of immunoprecipitation (IP) buffer (50 mM Tris pH8, 120 mM NaCl, 0.5% NP40) supplemented with protease inhibitors and lysates were incubated overnight. Endogenous p/CAF was immunoprecipitated from AR42J lysates with anti-p/CAF and protein G-agarose beads (Roche, Basel, Switzerland). Flag-tagged p/CAF and GCN5 were immunoprecipitated with anti-Flag M2 affinity gel (Sigma). P48 and p48<sup>K200R</sup> interaction with RBP-L or E47 was analysed in HEK293T cells transfected with 3  $\mu\text{g}$  of pFlag-p48 or pFlag-p48<sup>K200R</sup> and 3  $\mu\text{g}$  of pcDNA<sub>3</sub>RBP-L or pcDNA<sub>3</sub>E47 expression vectors. Flag-tagged p48 or p48<sup>K200R</sup> were immunoprecipitated with anti-flag M2 affinity gel as described above. The immune complexes were pelleted by gentle centrifugation, washed 3 times with IP buffer, resuspended in loading buffer, and fractionated by SDS-PAGE. The following antibodies were used for western blotting: affinity-purified rabbit p48 (1:800), E2A/E12 rabbit polyclonal antibody (1:500), affinity-purified rabbit anti-RBP-L (1:300) and anti-Flag M2 mAb (1:10000).

**In vitro acetylation assays.** Flag-tagged p48 and N+bHLH proteins were immunoprecipitated from HEK293T transfected cell extracts with anti-Flag M2 affinity gel. After immunoprecipitation, the beads were washed with IP buffer, divided into aliquots and stored at  $-80\text{ }^{\circ}\text{C}$ . For acetylation assays, immunoprecipitated proteins were incubated with 300 ng of recombinant GST-p/CAFHAT domain (Upstate, Temecula, CA) or GST alone and 10 nmol of acetyl-CoA (Sigma) in HAT buffer (25 mM Tris pH8; 2.5% glycerol; 0.5 mM EDTA; 25 mM KCl) at  $30\text{ }^{\circ}\text{C}$  for 1 h. Reactions were stopped by adding sample buffer. Proteins in the input samples were visualised by Coomassie blue staining and acetylated proteins were detected by immunoblotting using an anti-acetyl-Lys antibody.

**In vivo acetylation assays.** HEK293T cells were transfected with 3  $\mu\text{g}$  of Flag-p48 expression vector and 3  $\mu\text{g}$  of pCX-Flag-p/CAF or pCX-Flag-p/CAF(HAT-) plasmids. Acetylation of wild-type and K200R mutated forms of p48 N+bHLH fragment was assessed by transfecting 3  $\mu\text{g}$  of the corresponding expression plasmids and 3  $\mu\text{g}$  of pCX-Flag-p/CAF. Lysates were prepared as described above and immunoprecipitation was performed with 2.5  $\mu\text{g}$  of anti-acetyl-Lys antibody and 20  $\mu\text{l}$  of protein G-agarose beads. For western-blotting, the anti-Flag M2 mAb (1:10000) was used. For reciprocal assay, immunoprecipitation was performed with anti-Flag affinity gel. Acetylated proteins were detected with anti-acetyl-Lys (1:1000). To detect the immunoprecipitated proteins, membranes were stripped and re-probed with anti-Flag mAb.

**Indirect immunofluorescence.** Cos-7 cells were seeded on sterile coverslips and transfected with 0.2  $\mu\text{g}$  of p48GFP expression vector and 0.2  $\mu\text{g}$  of pCX-Flag-p/CAF or pCX-Flag-p/CAF(HAT-) plasmids. Cells were fixed for 10 min with 4% paraformaldehyde and washed. After blocking with 1% BSA, 0.1% saponin, 0.1% Triton X-100 in PBS (blocking buffer) for 30 min, coverslips were incubated with anti-Flag mAb (1:1000), washed, and incubated with rhodamine-conjugated anti-mouse Ig (1:200) (Dako, Glostrup, Denmark). Samples were visualized using a Leica TCS SP2 confocal microscope.

**Chromatin immunoprecipitation.** 266-6 cells were transfected with lipofectamine with either 3  $\mu\text{g}$  Flag-p/CAF or with 3  $\mu\text{g}$  Flag-tagged p48. Forty-eight h later, cells were cross-linked with 1% formaldehyde. Nuclear extracts were prepared by lysis in 50 mM Tris (pH 8), 2 mM EDTA, 0.1% NP40, 10% glycerol with protease inhibitors. After centrifugation, the pellet was resuspended in SDS lysis solution (50 mM Tris (pH8), 10 mM EDTA, 1% SDS) with protease

inhibitors. Lysates were sonicated and precleared with protein G-agarose beads for 3 h at 4 °C. Half of the chromatin was immunoprecipitated overnight at 4 °C with pre-blocked agarose beads coupled to anti-Flag M2 mAb, the other half with pre-blocked agarose coupled to anti-HA mAb (Sigma, St-Louis, MO). After washing, bound chromatin was eluted in 100 mM Na<sub>2</sub>CO<sub>3</sub> with 1% SDS and proteinase K (1mg/ml) (Sigma). Cross-linking was reversed at 65 °C. After treatment with RNase A (0.04mg/ml) (Roche, Mannheim, Germany) and proteinase K, the immunoprecipitated DNA was purified by the GFX PCR DNA purification kit (Amersham, Buckinghamshire, UK). Quantitative SYBR Green PCR (Applied Biosystems, Foster City, CA) was performed comparing the Ct values for anti-HA versus anti-Flag precipitates. Results were expressed as fold increase of Flag-immunoprecipitated DNA over HA-immunoprecipitated DNA. Two independent experiments were performed and all samples were analyzed in triplicate. Primer sequences were as follows: chymotrypsinogen B1 promoter: 5'-GCTGGCCACTACCAATGTTC-3' and 5'-CTGAGGCTCTTTTATGTCCC-3'; carboxypeptidase A1 promoter: 5'-CCATGGTCAAGGGTGAAAGC-3' and 5'-TCTGGGGCCTTTTTAAACAC-3'. Transfection efficiency of 266-6 cells was <10%, as determined using a p48GFP plasmid and counting of GFP positive cells.

#### Quantitative RT-PCR analysis of elastase, carboxypeptidase and chymotrypsinogen.

AR42J cells were transfected using lipofectamine with 6 µg of pIRES-GFP-p48, pIRES-GFP-p48<sup>K200R</sup> or pIRES-GFP empty vector and 20 h later GFP positive cells sorted by FACSvantage SE (Becton Dickinson, San Jose, CA) were isolated. To examine the effects of glucocorticoids, AR42J cells were treated with 100 nM dexamethasone for 72 h. RNA was extracted and treated with DNase I using the DNA free kit (Ambion, Austin, TX). cDNA generated by Taqman reverse transcription was analysed by quantitative PCR (Applied Biosystems). The relative Ct values were calculated as previously described [8]. Primer sequences used were as follows: HPRT: 5'-GTTCTTTGCTGACCTGCTGGA-3' and 5'-TTATGTCCCCCGTTGACTGGT-3'; p48 or p48<sup>K200R</sup>: 5'-GCTCCTGGAGCATTTCCTCCG-3' and 5'-CTGAGGAACTCTACCTCCGC-3'; elastase: 5'-TCCGTGAAGACCAACATGGTG-3' and 5'-CCAAAGCTCACAATACCGTGC-3'; carboxypeptidase A1: 5'-TCCAGATCGGCAACACCTTT-3' and 5'-TCCCTAGAATGGATGCCAGTG-3'; chymotrypsinogen B: 5'-GCAAGACCAAATACAATGCCC-3' and 5'-TGCGCAGATCATCACATCG-3'. HPRT mRNA was used as internal control and total p48 mRNA was used as reference to normalize for transfection efficiency. For comparison of RNA levels in cells expressing transfected p48 or p48<sup>K200R</sup>, Mann-Whitney test was used.

## RESULTS

### The N-terminal region of p48 functions as a transactivation domain

To identify the p48 transcriptional activation domain, a series of deletion derivatives of p48 fused to the yeast Gal4-DBD was expressed in AR42J cells. Their transcriptional activity on a Gal4-driven promoter was measured using the Gal4-TKluc reporter (Figure 1). Full-length p48 (residues 1-326) and its N-terminal region (residues 1-168) exhibited an 87- and 55-fold activation of luciferase activity, respectively, whereas the p48 N-terminal region including the bHLH domain (residues 1-217) and the p48 C-terminal region (residues 211-326) did not transactivate the Gal4 promoter (Figure 1A). These results indicate that p48 activates transcription through its N-terminal region and that the transactivation of the N-terminal region is negatively modulated by the bHLH domain. The comparison between the activities of the full-length p48 and its N-terminal region suggests that the C-terminus, inactive by itself, may potentiate the transactivation of the N-terminal region.

To further define the domains responsible for transcriptional activation, fine deletion analysis of the p48 N-terminal region was performed (Figure 1B). The products of a construct coding for residues 1-43 of p48 and, to a lesser extent, a construct coding for residues 38-108 efficiently activated the Gal4-promoter. By contrast, residues 104-138 exhibited minimal activity and the construct coding for residues 133-168 was inactive. From these results we

conclude that p48 activates transcription through two activation domains located in its N-terminal region between residues 1-43 and 43-138.

#### **p48 interacts *in vivo* with p/CAF and GCN5**

The primary sequence of the most N-terminal region of p48 is particularly rich in acidic amino acids, a feature previously reported for several transcriptional activating domains that recruit coactivators possessing an intrinsic HAT activity. We therefore tested whether p48 could interact *in vivo* with coactivators of the p/CAF family using co-immunoprecipitation experiments. Protein extracts from HEK293T cells cotransfected with pIRESp48 vector in combination with Flag-p/CAF or Flag-GCN5 were immunoprecipitated with the anti-Flag antibody. The presence of p48 in the immunoprecipitates was assessed by western-blotting with anti-p48 antibodies. As shown in Figure 2A, an efficient *in vivo* interaction of p48 was observed with p/CAF (left panel) and with its homologue GCN5 (right panel). A similar experiment performed with a CBP expression vector failed to reveal any interaction between p48 and CBP (data not shown). To determine whether endogenous p48 and p/CAF did interact in acinar cells, protein extracts from AR42J cells were immunoprecipitated with an anti-p/CAF antibody and p48 was assayed in the immunocomplexes by western-blot. As shown in Figure 2B, p48 and p/CAF interact *in vivo* in acinar cells.

#### **p/CAF potentiates the transcriptional activity of p48**

In acinar cells, p48 is part of the PTF1 complex which also includes RBP-L. Both proteins have been shown to physically interact and functionally cooperate to ensure the high transcriptional activity of PTF1 on a reporter driven by tandem repeats of a PTF1 binding sites (6XA<sub>26</sub>luc) [10]. To determine the effect of p/CAF on the transcriptional activity of p48 and RBP-L, AR42J cells were cotransfected with increasing amounts of p/CAF (Figure 3, left panel), p/CAF(HAT-) plasmid (Figure 3, right panel), or the corresponding empty vector, in combination with expression vectors for p48, RBP-L, and the 6XA<sub>26</sub>luc construct as a PTF1-responsive reporter. As expected for a cofactor, p/CAF alone did not activate the PTF1-responsive promoter (not shown) whereas p48-mediated activation increased in a dose-dependent manner from 3.5 to 16.4-fold upon p/CAF overexpression (Figure 3, left panel). In contrast, p/CAF did not enhance RBP-L mediated activation. Transfection with the p/CAF expression plasmid induced an 8-fold increase in the activity of the PTF1-responsive promoter upon co-transfection of p48 and RBP-L cDNAs. P/CAF coactivation was selective for p48/RBP-L as it was not observed when RBP-J kappa, the RBP-L paralogue, was expressed in the same conditions (data not shown). In addition, the plasmid coding for a p/CAF mutant lacking HAT activity failed to cooperate with p48 (Figure 3, right panel). These results show that p/CAF functions as a coactivator of p48 on a PTF1-responsive promoter and suggest that p/CAF coactivation of p48/RBP-L transcriptional activity is mediated by p48 as RBP-L activity is not affected by p/CAF.

Glucocorticoids have been shown to increase the mRNA levels of digestive enzymes [31]. We analyzed whether these effects were accompanied by an induction of Ptf1a, p/CAF, or Gcn5 using quantitative RT-PCR: treatment with dexamethasone for 72 h induced a 3-fold increase in amylase mRNA levels (p=0.013) but had no significant effects in the expression of Ptf1a, PCAF, or Gcn5 transcripts.

#### **p/CAF enhances nuclear accumulation of p48**

To determine whether p/CAF could affect the subcellular distribution of p48, Cos-7 cells were co-transfected with a p48GFP fusion protein plasmid and Flag-p/CAF or Flag-p/CAF(HAT-) plasmids. p48GFP distribution was quantified in cells expressing, or not, exogenous p/CAF or p/CAF(HAT-) and the latter proteins were detected using rhodamine-labeled secondary antibodies. In the absence of exogenous p/CAF, p48GFP was distributed in either the nucleus or both the nucleus and the cytoplasm in the vast majority of the cells (Figure 4A). As expected, p/CAF and p/CAF(HAT-) were predominantly found in the nucleus. Importantly, p/CAF co-localized with nuclear p48GFP. As shown in Figure 4B, exogenous p/CAF expression strongly enhanced the proportion of cells with exclusively nuclear

distribution of p48GFP (from 36.7% to 81%) and concomitantly reduced the proportion of the cells with cytoplasmic p48GFP. HAT deficient p/CAF mutant, which retains the capacity to interact with p48 (not shown), also increased the nuclear distribution of p48GFP although to a lesser extent. Thus, p/CAF promotes nuclear accumulation of p48 through a mechanism that is partially dependent of its acetyltransferase activity.

#### **p48 is acetylated by p/CAF *in vitro* and *in vivo*.**

We next investigated whether p48 is a target of p/CAF acetyltransferase activity. Immunoprecipitated Flag-tagged p48 protein was incubated with either GST or recombinant GST-p/CAF HAT domain in HAT buffer containing acetyl-CoA. After SDS-PAGE, proteins were stained by Coomassie blue and acetylated proteins were detected with anti-acetyl-Lys antibodies. Figure 5A shows that addition of p/CAF HAT domain, but not GST, results in the detection of a band corresponding to p48 by anti-acetyl-Lys antibodies, indicating that p48 is acetylated by p/CAF *in vitro*. For *in vivo* acetylation assay, a plasmid coding for Flag-tagged p48 was expressed in HEK293T cells alone or in combination with plasmids coding for p/CAF-Flag or p/CAF(HAT-)-Flag proteins. Lysates from transfected cells were immunoprecipitated with anti-acetyl-Lys antibodies and Flag-tagged acetylated proteins were detected by western-blot with anti-Flag antibodies (Figure 5B). P48-Flag was not immunoprecipitated with anti-acetyl-Lys antibodies in the absence of co-transfected p/CAF, indicating that p48 is not acetylated or that p48 acetylation level is too weak to be detected in this assay in HEK293T cells. Upon expression of p/CAF-Flag, both p48 and p/CAF were detected in the immunoprecipitates using anti-acetyl-Lys antibodies. Auto-acetylation of p/CAF has been previously reported [30] and confirmed the functionality of its HAT domain in this assay. By contrast, acetylated p48 was not detected when p48 was co-expressed with the HAT-deficient p/CAF demonstrating the critical role of its enzymatic activity. These results were confirmed by the reciprocal assay where, as shown in Figure 5C, the two Flag-tagged proteins p48 and p/CAF were exclusively detected by anti-acetyl-Lys antibodies upon p/CAF expression.

#### **The K200 residue of p48 is essential for PTF1 transcriptional activity and is a target of p/CAF acetylation.**

To identify the p48 residues targeted by p/CAF acetylation and study the functional relevance of p48 acetylation, we generated K-R mutants at each Lys residue of p48. The eight Lys residues of p48 are conserved through species. The first two Lys are in the bHLH domain while the six other Lys residues are all located in the C-terminal region of the protein (Figure 6A). After confirming by western-blot the correct expression of the K-R mutant forms of p48 in HEK293T transfected cells, we tested their transcriptional activity and their capacity to functionally cooperate with RBP-L on the PTF1-responsive promoter. As shown in Figure 6B, all single Lys-mutated forms of p48, with the exception of the p48<sup>K200R</sup> mutant, exhibited a transcriptional activity similar to that of the wild-type protein. In addition, they were able to cooperate with RBP-L, although to variable extents, for an efficient activation of the PTF1-responsive promoter. By contrast, The K200R mutation completely abolished the transcriptional activity of p48 as well as its cooperation with RBP-L. Moreover, this mutant exhibited a normal capacity to interact with p/CAF (Figure 6C), RBP-L (Figure 6D, upper part) and E47 (Figure 6D, lower part) indicating that loss of p48/RBP-L transcriptional activity due to K200R mutation does not apparently result from an alteration of p48 interaction with RBP-L, E47 or p/CAF.

To determine whether the K200 residue of the bHLH domain is a target of p/CAF-mediated acetylation, we performed *in vitro* and *in vivo* acetylation assays using the wild-type or the K200R mutated form of the N+bHLH region of p48. The N+bHLH<sup>K200R</sup> fragment is neither detected nor immunoprecipitated by anti-acetyl-Lys antibodies in both *in vitro* and *in vivo* acetylation assays (Figure 7 A and 7B, left panel). By contrast, the N+bHLH wild-type fragment is acetylated *in vitro* by p/CAF HAT domain (Figure 7A). Acetylation of the wild-type p48 fragment is also observed *in vivo* when it is coexpressed with exogenous p/CAF (right part of Figure 7B), thus demonstrating that the K200 residue is a target of p/CAF acetylation. Taken together, these data indicate that the K200 residue of p48 is essential for its transcriptional

activity and strongly suggest that its acetylation by p/CAF is required for full transcriptional activity of p48 and PTF1 complex.

### The K200 mutation alters the expression of the endogenous PTF1 target genes.

The importance of K200 residue acetylation for p48 function was assessed by comparing the expression of the three PTF1 target genes *carboxypeptidase A1* (*Cpa1*), *chymotrypsinogen B* (*Ctrb*) and *elastase* in cells overexpressing the wild-type or the mutated form of p48. Protein extracts and RNA were prepared from AR42J cells overexpressing p48 or p48<sup>K200R</sup> and expression of the three PTF1 target genes was measured by quantitative RT-PCR. Both wild-type and mutated forms of exogenous p48 were similarly expressed as assessed by western-blot (data not shown). Figure 8 shows that p48<sup>K200R</sup> expression results in a decrease of 42.2%, 47.35% and 44.2% of the RNA levels of *Cpa1*, *Ctrb* and *elastase*, respectively. Thus, substitution of K200 for a residue that cannot be acetylated severely compromises the p48 capacity to sustain a high level expression of the three PTF1 target genes.

### p/CAF is associated with the promoter of PTF1 target genes

To determine whether p/CAF is associated with the promoter region of acinar genes targeted by the PTF1 complex, ChIP experiments were performed using acinar 266-6 cells transfected with Flag-p/CAF or Flag-p48; for these experiments transfected p48 was used as control. Chromatin immunoprecipitation with anti-Flag from p48 and p/CAF transfected cells lysates showed an enrichment of the promoter region of *chymotrypsinogen B1* of 4.3±0.9-fold and 2.6±0.6-fold, respectively. Similar enrichment levels were demonstrated at the promoter of *carboxypeptidase A1*: 3.5±1.3-fold (p48-Flag) and 1.9±0.1-fold (p/CAF-Flag), indicating that p/CAF and p48 are associated with the same region of the *chymotrypsinogen B1* or the *carboxypeptidase A1* promoter.

## DISCUSSION

Cell differentiation relies on coordinated transcriptional regulation as well as on changes in cellular organization leading to functional maturation. Tissue-specific gene expression depends on the expression of tissue-specific transcription factors and on changes in chromatin structure. Histone acetylation has been predominantly correlated with gene activation [18;32] and the activity of HAT appears to be critical for gene expression. Association of HAT cofactors with site-specific DNA binding factors allows to direct acetylation-dependent regulation to specific loci. In addition to core histones, HATs may also acetylate Lys residues of architectural DNA binding factors, basal transcription factors and tissue-specific DNA binding factors [18].

In pancreatic acinar cells, expression of genes coding for digestive enzymes is largely controlled by PTF1 [10-12]. However, the mechanisms responsible for the activation of gene expression by PTF1 remain to be deciphered. Here, we show that p/CAF interacts *in vivo* with p48 in acinar cells and potentiates PTF1 transcriptional activity through p48 acetylation at K200. P48 interacts with Class A bHLH proteins through its bHLH domain [14] and with RBP-J kappa and RBP-L through its carboxy-terminal region [10,3]. Recently, a new model of the PTF1 complex has been proposed according to which recruitment of RBP-L, in place of RBP-Jkappa, by a DNA binding dimer formed by p48/Class A bHLH protein provides high transcriptional activity in the adult pancreas [10,13]. The recruitment of RBP-L would result in ensuring the formation of a PTF1 complex with highest transcriptional activity by excluding RBP-Jkappa [10], leading to full acinar cell differentiation. Here, we show that mutation of the p/CAF-acetylated residue K200 results in the loss of p48 transcriptional activity and of its functional cooperation with RBP-L without affecting its capacity to interact with RBP-L and the class A bHLH protein E47 (Fig. 6C), or p/CAF (Fig. 6D). We propose that p/CAF-mediated acetylation of p48 on K200 residue could, by favouring the selective cooperation between RBP-L and p48, help to confer to the PTF1 complex the high and specific activity required for full differentiation of acinar cells.

p/CAF has been reported to interact with, and to regulate the function of, several bHLH transcription factors required for cell fate specification or terminal differentiation. For example, MyoD interacts with, and is acetylated by, p/CAF and both effects are necessary for its transcriptional activity on chromatin-associated templates and for triggering terminal myogenic differentiation [22,33]. BETA2/NeuroD, essential for the differentiation of pancreatic islet and neural cells, is an *in vivo* target of p/CAF acetylation in pancreatic  $\beta$  cells. Acetylation of BETA2 affected its transcriptional activity on the insulin gene promoter supporting the idea that gene activation by BETA2 is regulated by acetylation [26]. The close relation between p/CAF-mediated bHLH transcriptional regulation and cell differentiation is further illustrated by TAL1, a critical bHLH regulator of hematopoietic and vascular development. Inhibition of p/CAF-mediated TAL1 acetylation in erythroleukemia cells resulted in an inhibition of TAL1 transcriptional activity and compromised differentiation [24]. As reported for BETA2 and TAL1, p48 is acetylated *in vivo* by p/CAF on a Lys residue located in the bHLH domain. Several Lys residues are found at conserved positions in the helix 2 region of bHLH factors and, in a few cases, have been demonstrated to be acetylated by p/CAF and essential for transcriptional activation (underlined):

p48	KRLSK <u>V</u> DTLRLA
TAL1	<u>K</u> KL <u>S</u> KNEILRLA
BETA2/NeuroD	<u>Q</u> KL <u>S</u> KIETLRLA
Math1	KKLSKYETLQMA
MASH2	KKLSKVETLRSA
Mist1	KKLSKIETLTLA
Neurogenin 3	AKLTKVETLRFA

A comparison of these sequences indicates that p/CAF acetylation takes place in a highly conserved region of the bHLH proteins. It is conceivable that the activity of other bHLH factors required for neuronal and/or pancreatic differentiation such as Mash2 [34], Mist1 [35,36], or the Neurogenin-3 [37], could be similarly regulated by p/CAF acetylation. Whether HAT-mediated acetylation of p48 is required for its function in pancreas and cerebellar organogenesis remains to be determined.

Despite the fact that the K200R mutation impairs the acetylation of the N-terminal region of p48, the p48<sup>K200R</sup> mutant is immunoprecipitated by anti-acetyl-Lys antibodies upon p/CAF co-transfection (data not shown), supporting the notion that additional Lys residues are targeted by p/CAF in the full-length protein. Interestingly, five out of the eight potentially acetylated Lys residues are in the most C-terminal part of p48, close to the interaction domains with RBP-Jkappa and RBP-L [10], within a region shown to be crucial for pancreatic and cerebellar organogenesis [8] and for p48 antiproliferative activity [9]. We have previously described that p48-mediated inhibition of proliferation is associated with the up-regulation of p21 mRNA, the down-regulation of Cyclin D2 mRNA levels, and increased p27 protein levels. Up-regulation of p21 mRNA levels by MyoD has been shown to depend on MyoD/p/CAF interaction and p/CAF-mediated acetylation in differentiated muscle cells [38,39]. Up-regulation of p21 mRNA levels by the Krüppel-like Factor 6 (KLF6) also required KLF6 acetylation by p/CAF and CBP cofactors [40]. To determine the contribution of p/CAF to the effects of p48 on cell proliferation, we used cell cycle analysis, p21 promoter reporter assays, and p21 qRT-PCR. p/CAF did not cooperate with p48 in any of these assays (see Supplementary data). We cannot rule out a contribution of endogenous p/CAF; however, these findings indicate a distinct role of p/CAF in enhancing transcriptional activation of acinar genes and other p48-mediated cellular effects. Whether p/CAF plays a role in a p48-dependent recovery from injury upon induction of experimental acute pancreatitis also merits additional study [41].

Regarding the mechanisms through which p/CAF contributes to enhanced transcriptional activity, we show that - in Cos-7 cells - p/CAF promotes the nuclear accumulation of p48, an effect that has been reported for other transactivators [42]. Should HAT activity be required for nuclear targeting or retention of p48, expression of the p/CAF(HAT-)

mutant would not modify the subcellular distribution of p48 or might compete with endogenous p/CAF resulting in an increase of cytoplasmic p48. However, we observed that p/CAF(HAT-) also leads, though to a lesser extent, to nuclear p48 accumulation indicating that HAT activity is not fully required for this effect. We therefore propose that the p/CAF-mediated effect on the subcellular distribution of p48 relies mainly on its interaction with p48 rather than on its capacity to acetylate it. However, we cannot exclude that modulation of p48 distribution results from an indirect effect of p/CAF. It is thought that E2A could, by interacting with p48, be responsible for its nuclear import [43]. Obata et al. reported that, in Cos-7 cells, overexpression of E47 (produced from E2A gene) shifted the predominant localization of a myc-tagged p48 from the cytoplasm to the nucleus [3]. However, using a different tag, we have found that the distribution of the GFP-p48 in Cos-7 cells is predominantly nuclear in 80% of the cells. Additionally, overexpression of E47 or HEB in acinar and non-acinar cultured cells had minor effects on p48 subcellular localization (M. Lichtenstein, unpublished data). The importance of analyzing the mechanisms responsible for the nuclear targeting of p48 is underlined by the study of acinar tumors. We have reported that in some acinar pancreatic tumours, endogenous p48 was found exclusively in the cytoplasm and was associated with low expression of amylase [15]. p48 is also partially redistributed to the cytoplasm in *ElasCCK2* transgenic mice in association with defects in acinar cell differentiation [44]. These findings suggest that altered subcellular distribution of p48 may contribute to reduced expression of acinar genes and compromised exocrine function. Additional experiments need to be performed, using p/CAF null mice [45], to determine the p/CAF contribution to p48 nuclear localization and transcriptional activity.

To further assess the role of P/CAF in cell differentiation, knock-down RNA interference experiments were conducted in cultured acinar cells. However, we could not detect any effect, possibly because of redundancy with GCN5, a HAT protein, as has been suggested to be the case in p/CAF null mice [45]. GCN5 displays a very high amino acid identity with p/CAF and is also expressed in the pancreas (unpublished data). Therefore, it is possible that the results reported for P/CAF may be extensive to GCN5.

Altogether, the results presented here provide new insights into the mechanisms through which PTF1 complex mediates acinar gene expression during exocrine differentiation. Deciphering the mechanisms by which p/CAF and/or other HAT cofactors may regulate p48 functions is an important step towards the understanding of acinar differentiation and fate determination in the pancreas and in the nervous system. In addition, it may contribute to a better understanding of the role of acinar transcriptional regulators in disease processes such as chronic pancreatitis and pancreatic cancer [46].

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## FOOTNOTES

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Abbreviations: bHLH, basic helix-loop-helix; PTF1, pancreas-specific transcription factor 1; HAT, histone acetyltransferase; Gal4DBD, Gal4 DNA binding domain; IP, immunoprecipitation; mAb, monoclonal antibody; SDS, Sodium dodecyl sulphate.

Keywords: pancreas, p48/PTF1a, p/CAF HAT, acinar, differentiation, RBP-L

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## FIGURE LEGENDS

**Figure 1.** *p48 activates transcription through its N-terminal region.* AR42J cells were transiently co-transfected with pBXG1 vector containing the Gal4DBD fused to the full-length or deleted forms of p48, the Gal4-TKluc reporter and the pRL-TK vector. pRL-TK was included as an internal control for normalization. Fold activation values were calculated by dividing the luciferase activity of each construct by the luciferase activity of pBXG1 empty vector. Fold activation values are the mean of 3 independent experiments, each performed in triplicate. (A) Transcriptional activity of the full-length p48 (1-326), the N-bHLH (1-168), the N+bHLH (1-217) and the C-bHLH (211-326) fragments of p48. (B) Transcriptional activity of the p48 N-terminal region including the N-bHLH (1-168), p48 (1-43), p48 (38-108), p48 (104-138) and p48 (133-168) fragments.

**Figure 2.** *p48 interacts in vivo with p/CAF and GCN5.* (A) Whole-cell extracts from HEK293T cells expressing p48 and Flag-p/CAF (left panel) or p48 and Flag-GCN5 (right panel) were immunoprecipitated with anti-Flag or mouse IgG antibodies. Immune complexes were analysed by western blotting using anti-p48 (top) or anti-Flag (bottom) antibodies. (B) AR42J cell lysates were immunoprecipitated with rabbit preimmune (PI) or anti-p/CAF antiserum. Endogenous p48 and p/CAF were detected by western blotting with affinity purified anti-p48 (top) and anti-p/CAF (bottom). Input corresponds to 10% of the amount of extracts used for immunoprecipitation.

**Figure 3.** *p/CAF enhances p48-mediated transcription.* AR42J cells were transiently transfected with increasing amounts (0, 50, and 100 ng) of pCX-Flag-p/CAF (left panel) or pCX-Flag-p/CAF(HAT-) (right panel) together with 0.1  $\mu$ g of pIRESp48 (white bars), 0.1  $\mu$ g of pcDNA<sub>3</sub>RBP-L (light-grey bars), or 0.1  $\mu$ g of both (dark-grey bars) and 0.1  $\mu$ g of 6XA<sub>26</sub>-luc reporter plasmid. pRL-TK was included as an internal control for normalization. The results are displayed as –fold activation over the transfection of reporter with equivalent amounts of empty vectors and represent an average of two experiments performed in triplicate.

**Figure 4.** *p/CAF promotes nuclear accumulation of p48.* Cos-7 cells were transfected with p48GFP plasmid and pCX-Flag-p/CAF, pCX-Flag-p/CAF(HAT-) or empty vector. Twenty hours after transfection, cells were stained with an anti-Flag antibody followed by rhodamine-conjugated secondary antibody and analysed for either GFP fluorescence or rhodamine staining. (A) Subcellular distribution of p48GFP upon p/CAF or p/CAF(HAT-) co-expression. Representative fields are shown. (B) Quantitative analysis of the p48 distribution. The percentage of cells with GFP fluorescence exclusively nuclear (N), predominantly nuclear (N>C), or homogeneously distributed in both nucleus and cytoplasm (N=C), in the presence of empty vector or the indicated p/CAF expression plasmids, is shown. Results are the average percentages with their standard deviation derived from three independent experiments. For each experiment, more than 300 cells were scored.

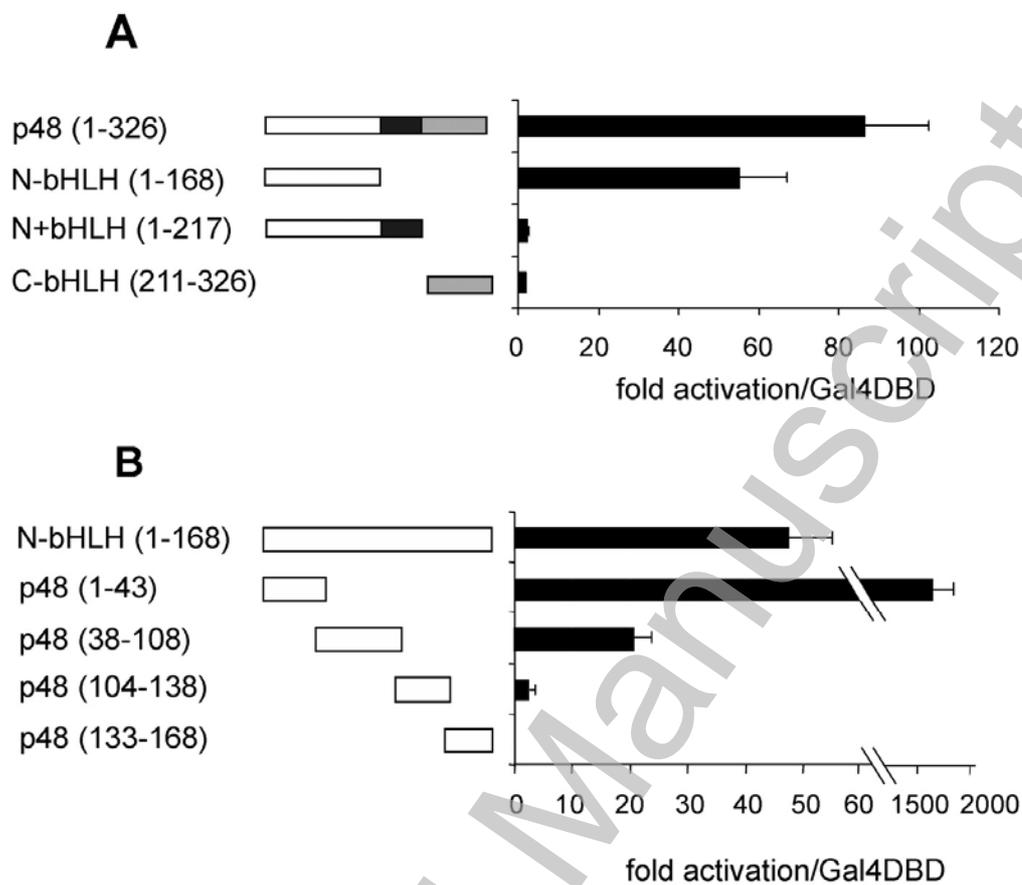
**Figure 5.** *p48 is acetylated by p/CAF.* (A) *In vitro* acetylation assay of p48. Immunoprecipitated p48-Flag was incubated with GST or with the recombinant GST-p/CAFHAT domain. Acetylated proteins were analysed by SDS-PAGE and western blotting with anti-acetyl-Lys antibody. Coomassie blue staining indicates the input protein bands. (B) and (C) *In vivo* acetylation assays. Total extracts from HEK293T cells transfected with 3  $\mu$ g of pCMVFlag-p48 and 3  $\mu$ g of pCx-Flag-p/CAF or pCx-Flag-p/CAF(HAT-) were immunoprecipitated with anti-acetyl-Lys or control rabbit IgG. Immune complexes were analysed by western blotting using anti-Flag antibody (panel B). In reciprocal experiment, extracts from HEK293T transfected cells were immunoprecipitated with anti-Flag or mouse IgG and immune complexes were analysed by western blotting using anti-acetyl-Lys (C, upper panel). The anti-acetyl-Lys blot was stripped and re-probed with anti-Flag (C, lower panel) to confirm that the bands corresponding to p48 and p/CAF-Flagged proteins overlap with the acetylated proteins shown in the upper panel.

**Figure 6.** *The K200 residue of p48 is essential for transcriptional activity.* (A) Comparison of the amino acid sequences of mouse, rat, human, *Xenopus* and zebrafish p48. The bHLH domain is represented in bold and Lys residues are marked with asterisks. (B) Transcriptional activity of wild-type and K-R p48 mutants on a PTF1-responsive promoter. AR42J cells were transiently transfected with pIRESp48 or pIRESp48<sup>K-R</sup>, in the presence (black bars) or in the absence (white bars) of pcDNA<sub>3</sub>RBP-L, 6XA<sub>26</sub>-Luc reporter construct, and pRL-TK. pRL-TK vector was included as an internal control for normalization. The results are displayed as –fold activation over the transfection of reporter with equivalent amounts of empty vectors and represent an average of three experiments performed in triplicate. (C) p48<sup>K200R</sup> mutant interacts with p/CAF. Whole-cell extracts from HEK293T cells expressing p48<sup>K200R</sup> mutant and Flag-p/CAF were immunoprecipitated with anti-Flag or mouse IgG antibodies. The immunoprecipitates were assessed by western-blot using anti-p48 antibodies. (D) p48 and p48<sup>K200R</sup> interact with RBP-L and E47. Total extracts from HEK293T cells expressing Flag-p48 or the Flag-p48<sup>K200R</sup> mutant in association with RBP-L or E47 were immunoprecipitated with either anti-Flag antibody or mouse IgG. Immunoprecipitates were tested for RBP-L (upper panel) or E47 (lower panel) by western blotting using anti-E2A.E12 and anti-RBP-L, respectively.

**Figure 7.** *The K200 residue of p48 is acetylated by p/CAF.* (A) *In vitro* acetylation of N+bHLH. Wild-type N+bHLH or N+bHLH<sup>K200R</sup> fragments of p48 were incubated with GST or recombinant p/CAFHAT domain in HAT buffer containing acetyl-CoA. After incubation, acetylated proteins were analysed by SDS-PAGE and western-blotting with anti-acetyl-Lys antibody. Coomassie staining of the gel indicates the input protein bands. (B) *In vivo* acetylation of N+bHLH. Total extracts from HEK293T cells expressing flagged wild-type N+bHLH or N+bHLH<sup>K200R</sup> p48 fragments in association with Flag-p/CAF were immunoprecipitated with rabbit anti-acetyl-Lys antibody or rabbit IgG. Immune complexes were analysed by western blotting using anti-Flag. Results of acetylation assays with N+bHLH<sup>K200R</sup> and N+bHLH fragments are shown in the left and right panels, respectively.

**Figure 8.** Expression of endogenous PTF1 target genes is reduced by the K200 mutation of p48. RNA from AR42J cells overexpressing p48 (white columns) or p48<sup>K200R</sup> (black columns) was extracted and analysed by quantitative RT-PCR for carboxypeptidase A1 (CPA1), chymotrypsinogen B (CTRB) and elastase expression. RNA levels were calculated using HPRT as internal control and normalized by dividing by the total p48 RNA value. Each value represents the mean of three independent experiments performed in triplicate. Statistical analysis: \**P*<0.001 (Mann-Whitney test).

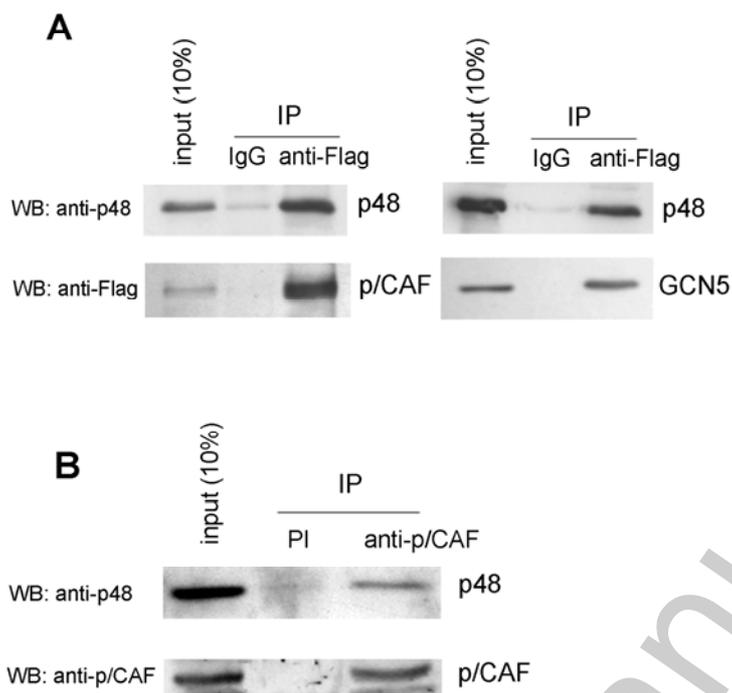
**Figure 1**



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**Figure 2**



**Figure 3**

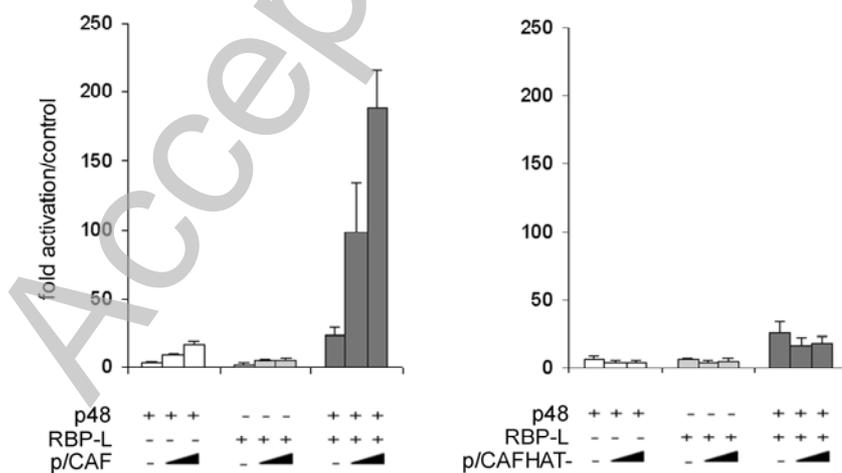
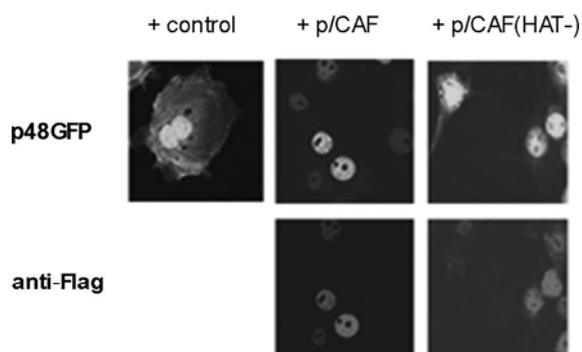


Figure 4

**A**



**B**

p48GFP distribution (% cells) in cells transfected with:

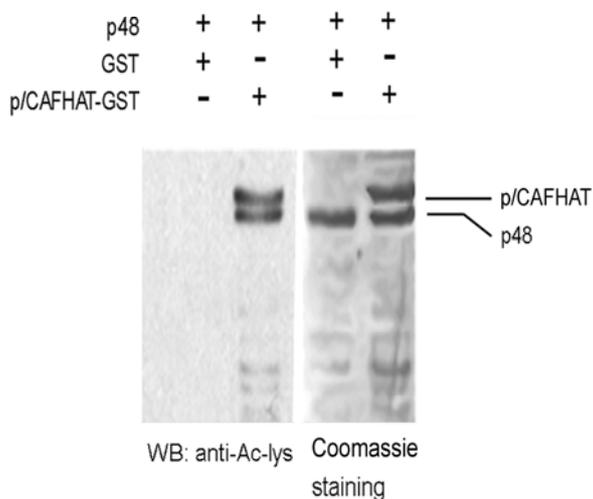
	empty vector	p/CAF	p/CAF(HAT-)
N	36.7 ± 3.5	81 ± 3.6	67.9 ± 11.7
N>C	44 ± 5	13.3 ± 2.9	23.2 ± 7.8
N=C	19.3 ± 2.1	5.7 ± 0.9	8.9 ± 4.8

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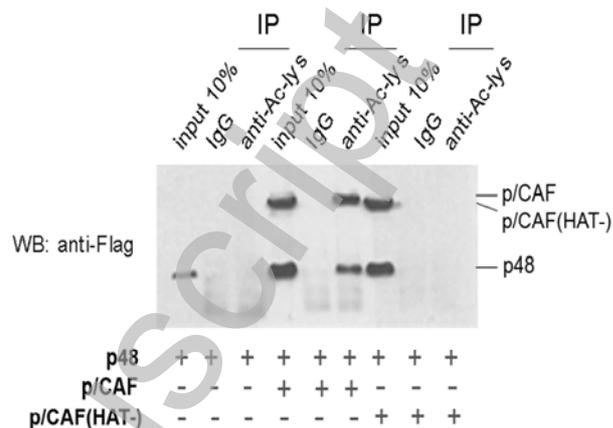
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Figure 5

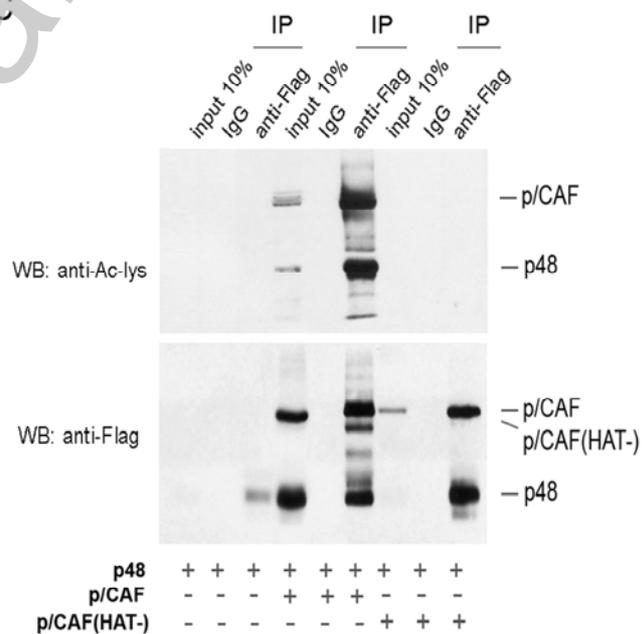
**A**



**B**



**C**

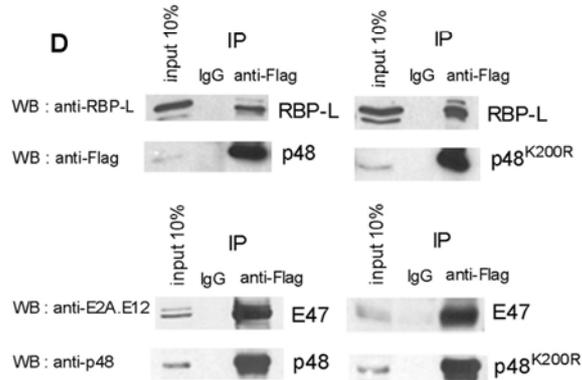
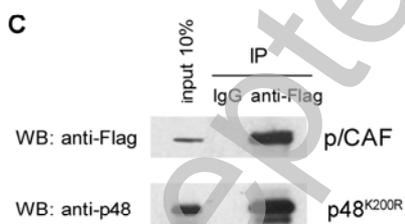
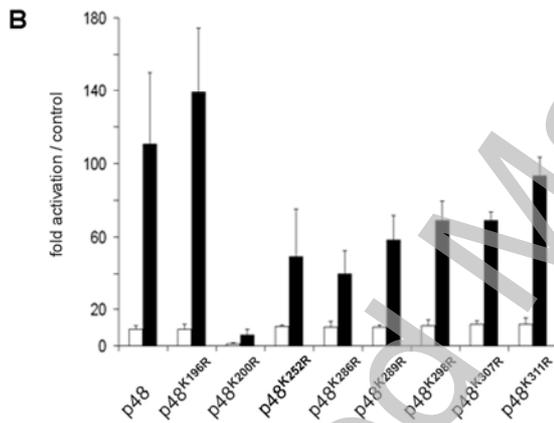


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Figure 6

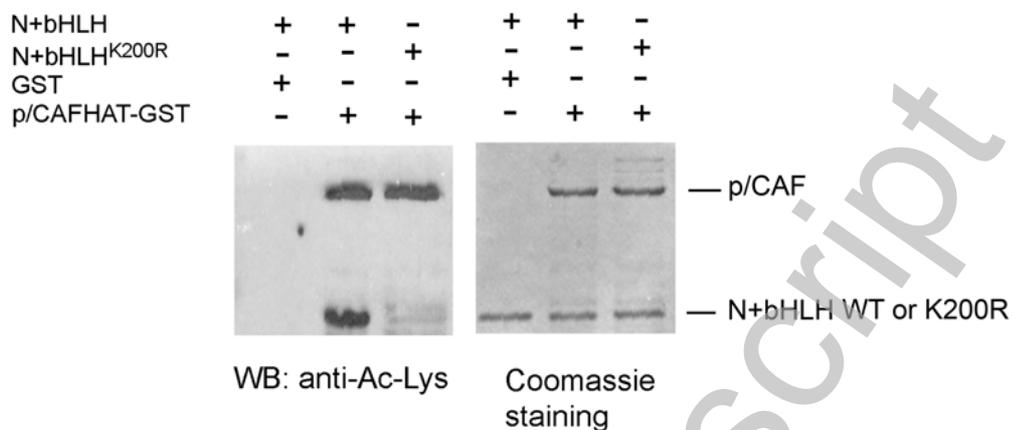
**A**

<b>Mouse</b>	MDAVLLEHFPGLDTPFPSPYFDEEDFFTDQSSRDPLEDSDELLGDEQAEVEFLSHQLHEY
<b>Rat</b>	MDAVLLEHFPGLDTPFPSPYFDEEDFFTDQSSRDPLEDSDELLGDEQAEVEFLSHQLHEY
<b>Human</b>	MDAVLLEHFPGLDTPFPSPYFDEEDFFTDQSSRDPLEDSDELLGDEQAEVEFLSHQLHEY
<b>Xenopus</b>	METVLEQLA--GLESFSPYFDEEDFFTDHSSRDAL--DADDFLEDD--VDFLAGQIQDY
<b>Zebrafish</b>	MDTVLDPF--GLDSFSSSYFDDDDFFTDHSSRDHL--DTDDFLDED--VDFLTNIQIY
<b>Mouse</b>	CYRDGACLLLPAPSAAPHALAPPLGDPGEPEDNVS--YCCDAGAPLAFFYSPGSPPS
<b>Rat</b>	CYRDGACLLLPAPSAAPHALAPPLGDPGEPEDSGS--YCCDAGAPLAGFPYSPGSPPS
<b>Human</b>	CYRDGACLLLPAPSAAPLALAPPSSGGLGEPDGGGGGVCETGAPPGGFPYSPGSPPS
<b>Xenopus</b>	-YRDSRVLHT-----DDDYCDAGN-----FSFSSSSG-
<b>Zebrafish</b>	-YKDSRISQ-----DGDYCDVGN-----FSFSSSSS--
<b>Mouse</b>	CLAYPCA---AVLSPGARLGLN--GAAAAAARRRRRSEAEQLQLRQAANVRERRRM
<b>Rat</b>	CLAYPCT---AVLSPGTRLRGLNGAAAAAARRRRRSEAEQLQLRQAANVRERRRM
<b>Human</b>	CLAYPCAQA---AVLSPGARLGLS---GAAAAAARRRRRSEAEQLQLRQAANVRERRRM
<b>Xenopus</b>	GFPEYCGDGGCDLSPGM--KGGSL-----VMKRRRRLSDAEMQQLRQAANVRERRRM
<b>Zebrafish</b>	TFSYGCADSTSELSPHR--DGG-----LLKRRRRRSEVENMQQLRQAANVRERRRM
<b>Mouse</b>	QSNDAFEGLRSHIPTLPYEKRLSKVDTLRLAIGYINFLSELVQADLPLHGSGAGCGGP
<b>Rat</b>	QSNDAFEGLRSHIPTLPYEKRLSKVDTLRLAIGYINFLSELVQADLPLRSGTGCGGP
<b>Human</b>	QSNDAFEGLRSHIPTLPYEKRLSKVDTLRLAIGYINFLSELVQADLPLRGGGAGCGGP
<b>Xenopus</b>	QSNDAFEGLRSHIPTLPYEKRLSKVDTLRLAIGYINFLSEHVQSDPLR-----NP
<b>Zebrafish</b>	QSNDAFEGLRSHIPTLPYEKRLSKVDTLRLAIGYINFLAELVQSDNPIR-----NP
<b>Mouse</b>	GGSRHLGEDSPGNQAQKVIICHRGTRSPSPSPDPDYGLPPLAGHLSWTDEKQLKEQNIIR
<b>Rat</b>	GGSRHLGGDSPGNQAQKVIICHRGTRSPSPSPDPDYGLPPLAGHLSWADEKQLKEQNIIR
<b>Human</b>	GGGRLGGDSPGSQAQKVIICHRGTRSPSPSPDPDYGLPPLAGHLSWTDEKQLKEQNIIR
<b>Xenopus</b>	NS-----DS--GNQPKKVIICHRGTRSPSPSPDPDYGLPPLAGHLSWTDEKQLRDNQVIR
<b>Zebrafish</b>	HS-----DA--LNQPKKVIICHRGTRSPSPNDPDYGLPPLAGHLSWTDEKQLKDNQIIR
<b>Mouse</b>	TAKVWTPEDPRKLNKSKS--FDNIENEPPEFVS
<b>Rat</b>	TAKVWTPEDPRKLNKSKS--FDNIENEPPEFVS
<b>Human</b>	TAKVWTPEDPRKLNKSKSFNINIENEPPEFVS
<b>Xenopus</b>	TAKVWTPEDPRKLN--KSPFSNIENEPPEFV-
<b>Zebrafish</b>	TAKVWTPEDPRKHLKSSINNIENEPFNFI



**Figure 7**

**A**



**B**

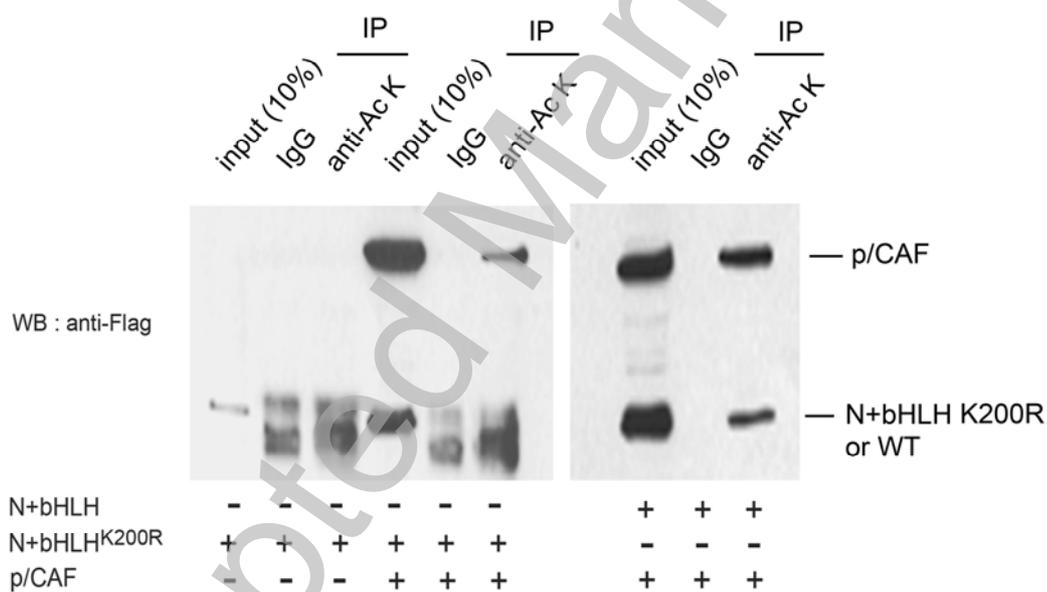
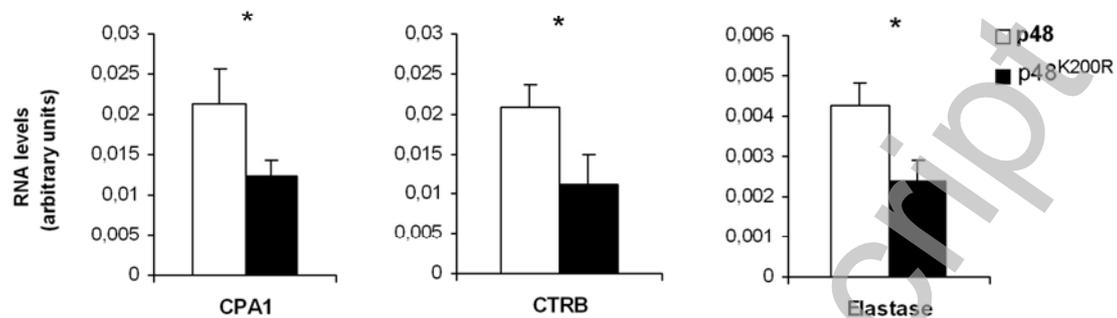


Figure 8



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