The EMBO Journal Vol.18 No.12 pp.3392–3403, 1999

Net, a negative Ras-switchable TCF, contains a second inhibition domain, the CID, that mediates repression through interactions with CtBP and de-acetylation

Paola Criqui-Filipe, Catherine Ducret, Sauveur-Michel Maira and Bohdan Wasylyk[1]

Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, 1 Rue Laurent Fries, BP 163, 67404 Illkirch cedex, France

[1]Corresponding author
e-mail: boh@igbmc.u-strasbg.fr

Signalling cascades are integrated at the transcriptional level by the interplay between factors such as the ternary complex factors (TCFs) that interact with serum response factor (SRF) and the serum response element (SRE) of the *fos* promoter. Net is a negative TCF that is switched to a positive regulator by the Ras signal. To understand the mechanisms of repression by Net, we used a yeast two-hybrid screen to identify factors that interact with its inhibitory domain. We isolated mCtBP1, the murine homologue of huCtBP1, a factor implicated in negative regulation of transformation by E1A plus Ras. We show that mCtBP1 interacts strongly with Net both *in vitro* and *in vivo*. The CtBP interaction domain of Net, the CID, mediates repression independently of the previously identified negative element, the NID. The CID inhibits by recruiting the co-repressor mCtBP1. The CID and mCtBP1 need to use de-acetylase activity for repression, whereas the NID apparently represses by other mechanisms. Finally, we provide evidence that CtBP and de-acetylation repress the c-*fos* SRE in low serum when it is inactive, but not in high serum when it is active. These results provide insights into the cross-talk between pathways that inhibit and stimulate transformation at the level of Net, a regulator of gene expression.

Keywords: c-*fos*/E1A/NID/transcription

Introduction

Transcriptional repressors in many circumstances are as important as activators in the regulation of gene expression (for reviews, see Gray and Levine, 1996; Hanna-Rose and Hansen, 1996; Ashraf and Ip, 1998; Fisher and Caudy, 1998a,b; Gregory and Horz, 1998; Torchia *et al*., 1998). Repressors act by a variety of mechanisms, including direct interactions with the basal transcriptional machinery or activators, thereby blocking their activity, and competition for *cis*-regulating elements leading to exclusion of activators from the promoter. Another mechanism is the recruitment of co-repressors that bridge repressors with their targets. The composition of co-repressor complexes and the manner in which they mediate repression are being studied intensively. A number of co-repressors have histone de-acetylase activity that generates a repressed chromatin structure with decreased accessibility to the transcription machinery. Histone acetyl transferase activity found in some co-activators counterbalances the effects of co-repressors, generating active chromatin. Local alterations of chromatin structure on promoters is emerging as a mechanism of precise regulation of transcription (for reviews, see Ashraf and Ip, 1998; Gregory and Horz, 1998; Torchia *et al*., 1998).

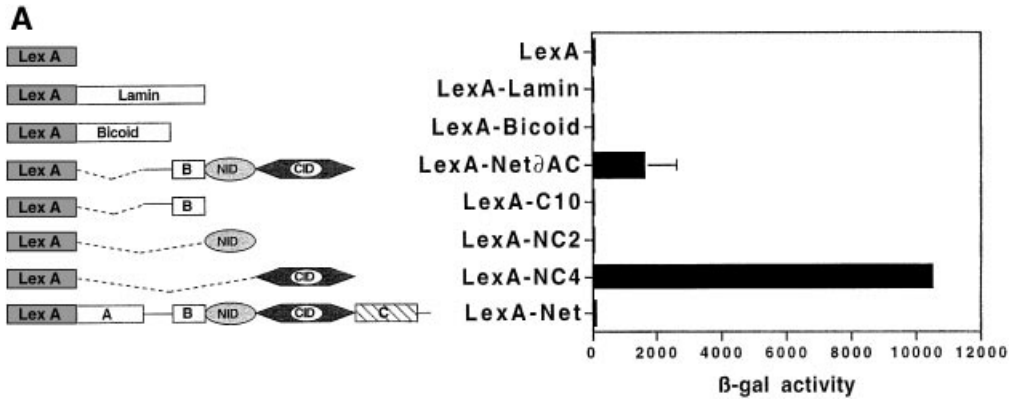
Net is a transcriptional repressor (Giovane *et al*., 1994) that belongs to the Ets family of oncogenes (for reviews see Treisman, 1996; Sharrocks *et al*., 1997; Wasylyk and Nordheim, 1997; Dittmer and Nordheim, 1998; Graves and Petersen, 1998; Wasylyk *et al*., 1998). Net as well as Elk1 and Sap1 are called ternary complex factors (TCFs) from their ability to form complexes with SRF on the c-*fos* serum response element (SRE). The TCFs have three similar domains, A, B and C, respectively involved in DNA binding, interaction with SRF and activation of transcription when phosphorylated by MAP kinases. The TCFs are co-expressed in many cell types and are highly conserved from mouse to man. Net differs from the other TCFs in its ability to strongly repress transcription. Net has an inhibitory domain, the NID, that is thought to form a helix–loop–helix (HLH) protein–protein interaction motif similar to myogenic factors such as MyoD (Maira *et al*., 1996). The NID is C-terminal to the B domain (Figure 1A) and is part of the sequences that differ between the TCFs.

We investigated the mechanisms of repression by Net by searching for interacting proteins with the yeast two-hybrid screen. We isolated mCtBP1, the murine homologue of huCtBP, an E1A C-terminal-binding protein. Binding of CtBP to E1A has been shown to restrict the tumorigenic activity of E1A. CtBP is a negative regulator of Ras-dependent E1A transformation (Schaeper *et al*., 1995). We show that CtBP is a co-repressor that interacts with a newly identified inhibitory domain of Net, the CID (CtBP inhibition domain). This second inhibitory domain lies in the region of Net which has not previously been attributed a function, and which lies between the NID and the C domain (Figure 1A). Net is a link between the opposing activities of CtBP and Ras at the level of transcriptional regulation of gene expression.

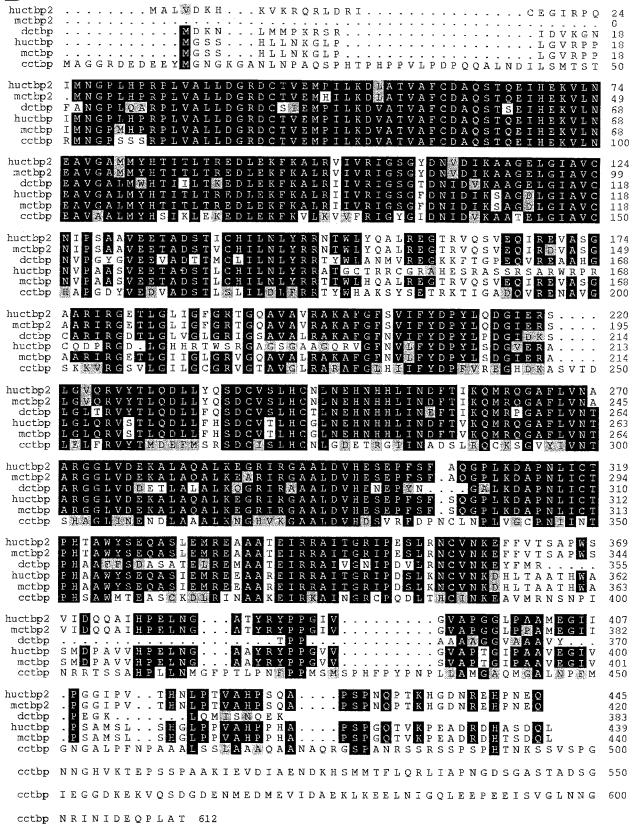
Results

Cloning of the murine CtBP1 homologue in a yeast screen for proteins that interact with Net

We used the yeast two-hybrid system to identify proteins that interact with the central region of Net, which contains the NID and adjacent regions but lacks the Ets (A, DNA binding) and transactivation (C) domains (Figure 1A, LexA–Net∂AC). We screened a library prepared from



B



C

CtIP	489	PLDLSDRF	497
dHairy	375	PLSLVIKK	365
dKnirps	371	PMDLSMKT	379
dSnail1	40	PQDLSLKR	48
dSnail2	185	PEDLSVRN	193
dKruppel	481	PEDLSMHS	489
Net	274	PLNLSSGS	282
E1A ade2	232	PLDL CKR	240
E1A ade41	239	PLNLSLKR	247

Fig. 1. In a yeast two-hybrid assay, Net interacts with mCtBP1 through a specific element found in different proteins. (**A**) The L40 yeast strain was transformed with 1 µg of pASV3-clone 45 encoding the VP16 activation domain fused to full-length mCtBP1 and 1 µg of various pBTM116–LexA recombinants. The LexA fusion proteins are illustrated on the left and β-gal activity on the right. (**B**) Sequence alignment of mCtBP1 (mctbp) and 2 (mctbp2), huCtBP1 (huctbp) and 2 (huctbp2), dCtBP and cCtBP. The DDBJ/EMBL/GenBank accession No. of mCtBP1 is AJ010483. (**C**) Alignment of CtBP interaction domains located at the indicated amino acid positions. The alignments were generated with the ClustalW program and Prettyplot.

pooled mouse embryos of 9.5, 10.5, 11.5 and 12.5 days, during which time Net was verified to be expressed (data not shown). Out of 2×10^7 transformants screened, 223 positives clones were obtained and analysed. Clone 45 was chosen for further studies for different reasons: (i) it was picked up relatively frequently (34/223); (ii) it interacted specifically with the bait and not the control proteins, in contrast to several other clones that were somewhat less specific (data not shown); and (iii) sequence analysis showed that clone 45 is homologous to human CtBP1 (86% similarity at the nucleotide level and 93% similarity at the peptide level; Figure 1B). The high degree of similarity suggests that clone 45 encodes mCtBP1, the murine homologue of human CtBP1. Clone 45 encoded the complete coding sequence of mCtBP1. huCtBP1 is implicated in transcription repression and Ras function (Schaeper *et al*., 1995; Sollerbrant *et al*., 1996), as is Net.

We studied the interactions of mCtBP1 with Net using the two-hybrid assay. We initially showed that the interaction is specific, in that mCtBP1 interacts with LexA–NetδAC but not with a number of control baits (LexA–lamin and LexA–bicoid, Figure 1A; LexA–Myc, LexA–Max, LexA–cyclin C and LexA–CDC2, not shown). Using Net deletion mutants (Figure 1A, LexA–C10, LexA–NC2 and LexA–NC4), we found that the region downstream of the NID mediates the interaction with mCtBP1 (LexA–NC4). Full-length Net did not interact detectably with mCtBP1, even though the fusion proteins were expressed at similar levels (data not shown). However, the full-length proteins interacted in other assays (see

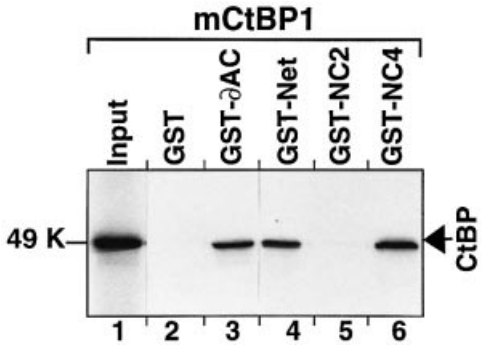


Fig. 2. Net interacts with mCtBP1 *in vitro*. GST, GST–Net and GST–Net deletion mutants were incubated with *in vitro* translated mCtBP1 and analysed by the GST pull-down assay. 49K: size marker.

below). Six amino acids in the C-terminus of E1A are important for the interaction with huCtBP1. We found that Net has a similar sequence. The sequence is also found in other proteins that recently have been shown to interact with CtBP (Figure 1C).

Net interacts with mCtBP1 in vitro

We used an *in vitro* assay to study the interaction between Net and mCtBP1. GST, GST–Net or GST–Net deletion mutants were expressed in bacteria, immobilized on glutathione–agarose beads and incubated with *in vitro* translated mCtBP1. Almost 40% of the input mCtBP1 was retained by the fragment of Net used as a bait (GST–Net∂AC) and none by GST alone (Figure 2, compare lane 3 and 2), showing that mCtBP1 interacts specifically with Net∂AC *in vitro*. The interactions with different deletion mutants were as expected from the yeast assay, in that GST–NC4 interacted with mCtBP1 (lane 6), but not GST–NC2 containing the NID (lane 5). Interestingly, in contrast to the result in yeast, full-length Net fused to GST interacted with mCtBP1 (lane 4) with an efficiency similar to the original bait, showing that full-length Net interacts with mCtBP1 *in vitro*.

Net interacts with mCtBP1 in Cos cells

Net–mCtBP1 interactions in mammalian cells were studied by co-precipitation, using either GST-tagged proteins or untagged full-length proteins and specific antibodies. mCtBP1 was retained specifically by GST–Net∂AC (Figure 3A, lanes 1 and 3), as well as by GST–E1A (the positive control, lane 4). Using full-length proteins, GST–Net did not interact detectably with mCtBP1 (lane 2). However, in the converse experiment, GST–CtBP interacted with Net (lanes 6 and 7) and with E1A (lanes 8 and 9). A lower level of expression of GST–Net apparently does not account for the absence of a detectable interaction with mCtBP1 because increasing the amount of expressed fusion protein did not change the results (data not shown). However, using a mutant in the NID, Net_{elk}, known to modify the three-dimensional structure of Net (Maira *et al*., 1996), we detected a strong interaction with mCtBP1 (lane 5). The absence of interaction between GST–Net and mCtBP1 might result from a 'closed' conformation of Net which decreases the accessibility of the CtBP interaction domain to CtBP. This conformation may be favoured somehow by fusing heterologous sequences (GST or LexA) to the N-terminus of Net.

To determine if the conserved sequence in Net (Figure 1C) is the binding site for mCtBP1, we adopted the mutational strategy used for E1A by Schaeper *et al*. (1995). In c1 and c2, the first and second pairs of amino acids, respectively, are mutated to Ala–Ser (Figure 3B). GST–∂AC with the c1 or c2 mutations did not retain mCtBP1 (Figure 3C, lanes 10–13) and, conversely, Net c1 and Net c2 did not interact with GST–CtBP (lanes 14–19). The GST fusion proteins and all the unfused proteins (Net, Net mutants and mCtBP1) were expressed at comparable levels (see lower panels and data not illustrated), showing that differences in protein levels could not account for the results.

We attempted to detect a complex between endogenous proteins using co-immunoprecipitation experiments with non-transfected Cos cell extracts. However, we could not detect precipitated Net nor co-precipitated CtBP (Figure 3D, lanes 1 and 3). When both proteins were co-expressed by transfection, immunoprecipitation of Net using an anti-Net serum resulted in co-precipitation of mCtBP1 (Figure 3D, lanes 2 and 4). Around 10% of exogenous Net was immunoprecipitated and 6% of exogenous CtBP was bound to Net. The lack of detection of endogenous proteins and their interaction may be due to the low levels of the proteins or the selectivity of the available antibodies.

Deletion mutants of mCtBP1 (Figure 3E, top panel) were used to localize its interaction domain. The central region of mCtBP1 (amino acids 100–315) is highly homologous to the family of 2-hydroxy dehydrogenases, whereas the N- and C-terminal regions are unique. Using GST–CtBP deletion mutants and full-length Net, we found that interactions were unaffected by the loss of sequences from the C-terminus to 315 or 100 (Figure 3E, bottom panel, lanes 1–4). However, the interaction was lost by removing 100 amino acids from the N-terminus (lane 5). These results show that residues 1–100 of mCtBP1 are sufficient and necessary for the interaction with Net. The same sequences were also required for the interaction with E1A (Figure 3E, bottom panel, lanes 6–8). Significantly, in these experiments, the complexes were detected, even though they were washed with RIPA buffer, indicating that mCtBP1 and Net interact strongly.

Net delocalizes mCtBP1 from the cytoplasm to the nucleus

We investigated the cellular localization of mCtBP1 by immunofluorescence confocal microscopy. Exogenously expressed mCtBP1 was found to be exclusively cytoplasmic in Cos-7 cells and mainly nuclear in Saos2 cells (Figure 4). This result raised the possibility that interactions with nuclear proteins could relocate mCtBP1. Since Net is mainly nuclear when expressed in Cos-7 cells (Figure 4), we tested whether Net could recruit mCtBP1. When the two proteins were co-expressed, mCtBP1 was found to be mainly nuclear (Figure 4). The recruitment was observed at all levels of Net tested (50-fold range), showing that Net can recruit mCtBP1 to the nucleus efficiently. Endogenous CtBP was barely detectable in different experiments with the available antibodies (data not shown), preventing an analysis of the localization of endogenous CtBP in the presence of transfected Net. These results indicate that mCtBP1–Net

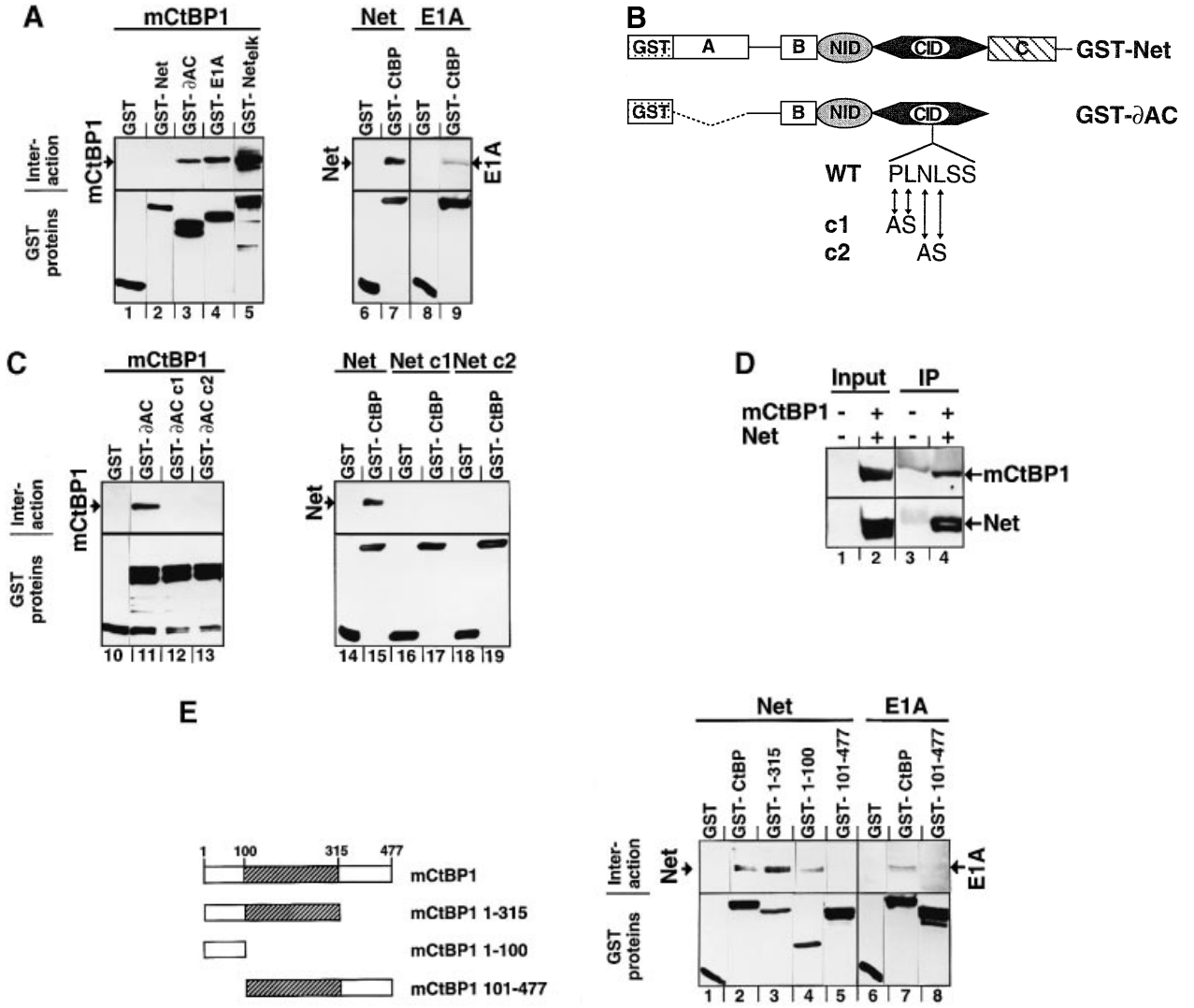


Fig. 3. Net interacts with mCtBP1 in eukaryotic cells. (**A**) mCtBP1 interacts with Net and E1A. Cos cells were co-transfected with expression vectors for GST, GST–Net, GST–∂AC, GST–E1A, GST–Net$_{elk}$, mCtBP1, Net and E1A as indicated (10 μg for GST–Net, 5 μg for the others). Cell extracts were analysed by the GST pull-down assay. Western blotting was used to detect the proteins retained on the beads by protein–protein 'interaction' (upper panels) with the 'GST proteins' (lower panels). (**B**) Schematic representation of GST–Net, GST–∂AC and the c1 and c2 mutants. (**C**) Mutation of the Net sequence that resembles known CtBP-binding sites inhibits its interaction with mCtBP1. A GST pull-down assay was performed as described in (A) using 5 μg of GST–∂AC c1, c2 expression vectors or 5 μg of pTL2-Net c1, c2. (**D**) Co-immunoprecipitation of CtBP with Net. Cos cell extracts not transfected or transfected with 5 μg of CtBP and Net expression vectors were used for immunoprecipitation with an anti-Net polyclonal serum 375. One-third of the input was loaded. The antibodies used for Western blotting were anti-CtBP 1123 (top panel) and anti-Net 375 (low panel). Net migrates as a double band, probably due to phosphorylation. The secondary antibody we used for immunoblotting, was protein A coupled to peroxidase. The shadow above the band corresponds to the heavy chains. (**E**) The non-conserved N-terminal region of mCtBP1 mediates the interaction with Net. The deletion mutants of mCtBP1 expressed as GST fusion proteins are represented on the top. GST pull-down assays were performed as described above with 5 μg of expression vector for the GST fusion protein, Net and E1A 12S. Hatched boxes represent the signature domains of the dehydrogenase protein family, with which mCtBP1 has significant homology.

interactions can be detected in fixed cells as well as after cell disruption in extracts.

Interaction with mCtBP1 affects Net transcriptional activity

To study the functional significance of the Net–mCtBP1 interaction, we used transfection assays to compare the activity of wild-type Net with mutants that cannot interact with mCtBP1 (Net c1 and c2). Net expression has been shown to repress the basal activity of a reporter containing multimerized Ets-binding sites from the stomelysin 1 promoter (Maira *et al*., 1996; and Figure 5A, lanes 2, 5, 8 and 11). The Net mutants clearly had impaired inhibitory activity when limiting quantities of expression vectors were used (Figure 5A, lanes 2–7). Under conditions where Net inhibited by ~60%, the c1 mutant inhibited by ~20% (lanes 2 and 3) and c2 by <10% (lanes 2 and 4). However, with saturating quantities of expression vectors, the mutants inhibited to similar extents as the wild-type (lanes 8–13), as expected from the presence of another inhibitory domain in Net, the NID (see below). Western blots showed that similar amounts of proteins were expressed [Figure 5B, the results were corrected for variations in transfection efficiency using co-expressed green fluorescent protein (GFP)], excluding the possibility that the results were due to a difference in expression levels. These results suggest that Net represses transcription in part by recruiting CtBP.

Ha-Ras expression switches Net from a repressor to an

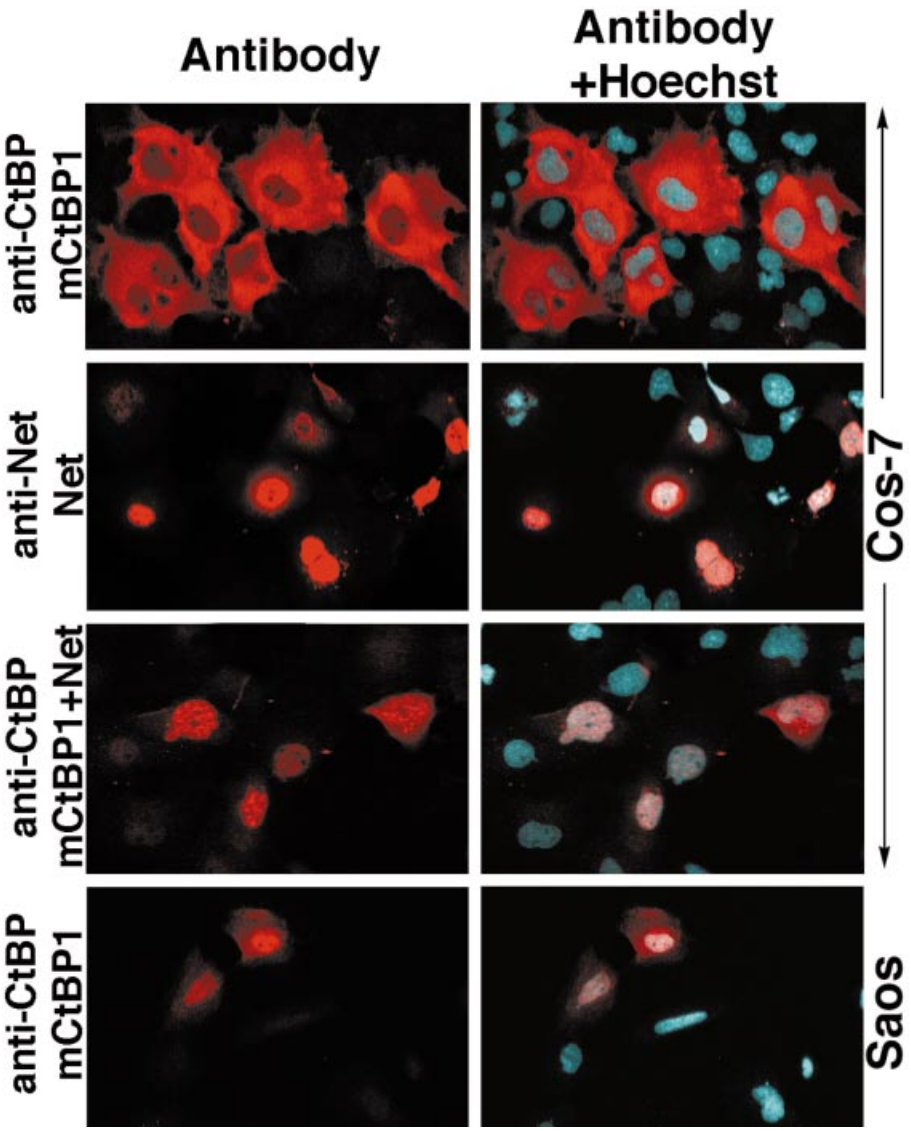


Fig. 4. mCtBP1 is relocated from the cytoplasm to the nucleus in the presence of Net. Cos cells were transfected with 20 ng of pTL1-mCtBP1, 20 ng of pTL2-Net or 20 ng of pTL1-mCtBP1 and 1 μg of pTL2-Net. Saos2 cells were transfected with 100 ng of pTL1-mCtBP1. The cells were fixed, incubated with the indicated antibodies and fluorescence-labelled secondary antibodies, and analysed by confocal microscopy. Immunofluorescence is shown on the left and immunofluorescence plus Hoechst staining on the right.

activator of transcription (Giovane *et al*., 1994; Figure 5C, lanes 2, 5, 8 and 11). We investigated how mutating the CtBP interaction domain affected Ras activation. The Net mutants co-transfected with activated Ras were up to five times more active than the wild-type (lanes 1–13), indicating that inhibition through the CID dampens Ras activation. We found that other Ets-responsive reporters responded similarly, including Py4B-Luc, which contains the Ras-responsive element of the polyoma enhancer, and SRE-Luc and Fos-Luc, containing the SRE in different contexts (data not shown). These results show that several Ets-responsive reporters are repressed by a CID-dependent mechanism, whether or not Net is activated by Ras.

The CID is a new repression domain of Net

The CID is sufficient for strong interaction with mCtBP1, yet its mutation has only a partial effect on repression, raising the possibility that it functions independently of the NID (Maira *et al*., 1996). To test this hypothesis, we used Gal–NC4 (Figure 6A), a fusion protein between the Gal4 DNA-binding domain and the smallest region of Net known to interact with mCtBP1. Gal4–NC4 efficiently inhibited transcription from a Gal4-UAS-Luc reporter (Figure 6B, lanes 2–4), similarly to Gal4–NC2 containing the NID (lanes 5–7). As expected, a Gal4 fusion with both domains also efficiently inhibited transcription (data not shown). We tested whether CtBP mediates repression by the CID, using either mutant proteins or competition assays with a specific E1A competitor. Gal–NC4 c1, c2 and c3, containing mutations in the PLNLSS sequence that are homologous to other CtBP interaction sequences (Figure 6A), did not significantly repress the basal transcription activity of the reporter (Figure 6C, lanes 4–9), in contrast to the wild-type fusion protein (Figure 6C, lanes 1–3). The expression levels of the differents mutants were similar (Figure 6D). These results show that the sequence that is homologous to other CtBP-binding sites is important for repression by the CID. A specific compet-

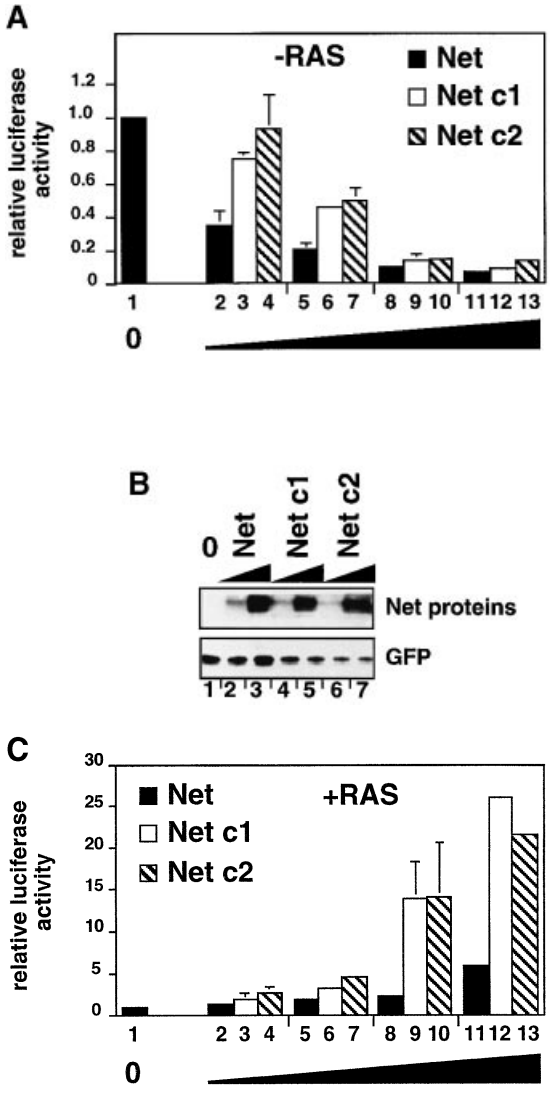


Fig. 5. Mutation of the CID of Net partially relieves inhibition and increases activation in the presence of Ras. CHO cells were co-transfected with 1, 10, 20 or 100 ng of expression vectors for either wild-type, c1 and c2 mutant Net, 0.5 μg of pCMV-LacZ, 1.5 μg of Pal×8-Luc (containing eight ets-binding sites) and 0.2 μg of either pΔRasCTB×2 (control vector) (**A**) or pRasCTB×2 encoding Ha-Ras-Val 12 (**C**). Luciferase values, normalized with β-gal, are presented relative to the basal activity of the reporter. (**B**) Western blot with anti-Net 375 or anti-GFP of CHO cell extracts transfected with 100 ng or 1 μg of Net expression vectors and 1 μg of pEGFP-C1. Relative values, after scanning the autoradiograms and correcting for GFP expressed levels were, for 100 ng of expression vectors for wild-type, c1 and c2: respectively 1, 1.2 and 1.3; for 1 μg transfected: 1, 1.3 and 2.

itor for CtBP derived from E1A (ΔE1A) and an equivalent control lacking the CtBP interaction domain (m ΔE1A; Sollerbrant *et al*., 1996) were used to show that CtBP is implicated in CID-mediated inhibition. Repression by Gal4–NC4 was decreased by the specific competitor (from 90 to 35%, Figure 6E, lanes 1–5), but not by the control (lanes 6–8). Repression by Gal4–NC2 was not affected (lanes 9–15), suggesting that the CID and the NID inhibit by different mechanisms. These results show that the Net sequence that resembles other CtBP-binding elements is a key component of a new repression domain of Net, and they raise the possibility that it functions by recruiting a co-repressor, CtBP.

mCtBP1 is a co-repressor

To test whether CtBP is a co-repressor, we investigated whether direct recruitment of mCtBP1 to a promoter by fusion with a heterologous DNA-binding domain would suffice for repression. A fusion protein between the Gal4 DNA-binding domain and mCtBP1 efficiently inhibited the basal activity of a Gal4-responsive reporter (Figure 7A, lanes 1, 3 and 5). These results confirm that mCtBP1 acts as a repressor when it is recruited to DNA.

Repression by mCtBP1 and the CID involve histone de-acetylation

Recent results show that huCtBP1 interacts with HDAC1, a histone de-acetylase (Sundqvist *et al*., 1998). To investigate whether repression by mCtBP1 and the CID involves histone de-acetylation, we used the specific inhibitor, trichostatin A (TSA). Gal4–CtBP repression was strongly decreased by treating cells with TSA (Figure 7A, lanes 1–6), in keeping with a role for histone de-acetylation in repression by CtBP. As expected, Gal4–NC4 repression was also relieved by TSA, from 90 to 50% (Figure 7B, lanes 1–6). We reproducibly found that TSA was more efficient in relieving repression by Gal4–CtBP than by Gal–NC4, raising the possibility that there might be some difference in their modes of repression. However, the results clearly show that histone de-acetylation is involved in repression by both CtBP and the CID.

Regulation of the c-fos SRE by mCtBP1 and histone de-acetylation

The c-*fos* SRE is tightly regulated by SRF and several TCFs, including Net. CtBP may be recruited to the SRE to inhibit its activity under low serum conditions, and activation may involve loss of CtBP inhibition. To investigate the role of CtBP in SRE activity in CHO cells, we used the E1A competitor for CtBP and an SRE-Luc reporter. Under low serum conditions [0.05% fetal calf serum (FCS)], the specific competitor increased SRE activity ~3-fold (Figure 8A, lanes 1, 3, 5 and 7), whereas the control competitor had little effect (lanes 9, 11 and 13). The connection between CtBP and histone de-acetylation led us to investigate the effect of TSA on the activity of the SRE reporter. As predicted, TSA increased the activity of the SRE 3-fold under basal conditions (Figure 8A, lanes 15 and 17). Serum stimulated the SRE-Luc reporter ~20-fold (not shown) and greatly decreased the effects of the competitor and the TSA (data not shown). These results implicate CtBP and histone de-acetylation in the repression of the c-*fos* SRE under low serum conditions. To investigate whether TCFs were involved in these effects, we used two different approaches, mutation in the ets motif of the SRE and expression of *trans*-dominant Net mutant proteins. We reproducibly observed a decrease in activation by the competitor due to the mutation (Figure 8A, lanes 2, 4, 6 and 8; compare lane 6 with 5 and 8 with 7). Furthermore, the effect of TSA is strongly diminished by the mutation (compare lane 18 with lane 17). The *trans*-dominant Net mutant C10 contains both the ets domain and the SRF interaction domain, whereas C12 has just the ets domain (Figure 8B). The *trans*-dominant mutants lack the CID and are expected to replace Net on the ets-binding site of the SRE reporter and thereby inhibit recruitment of CtBP. We reproducibly

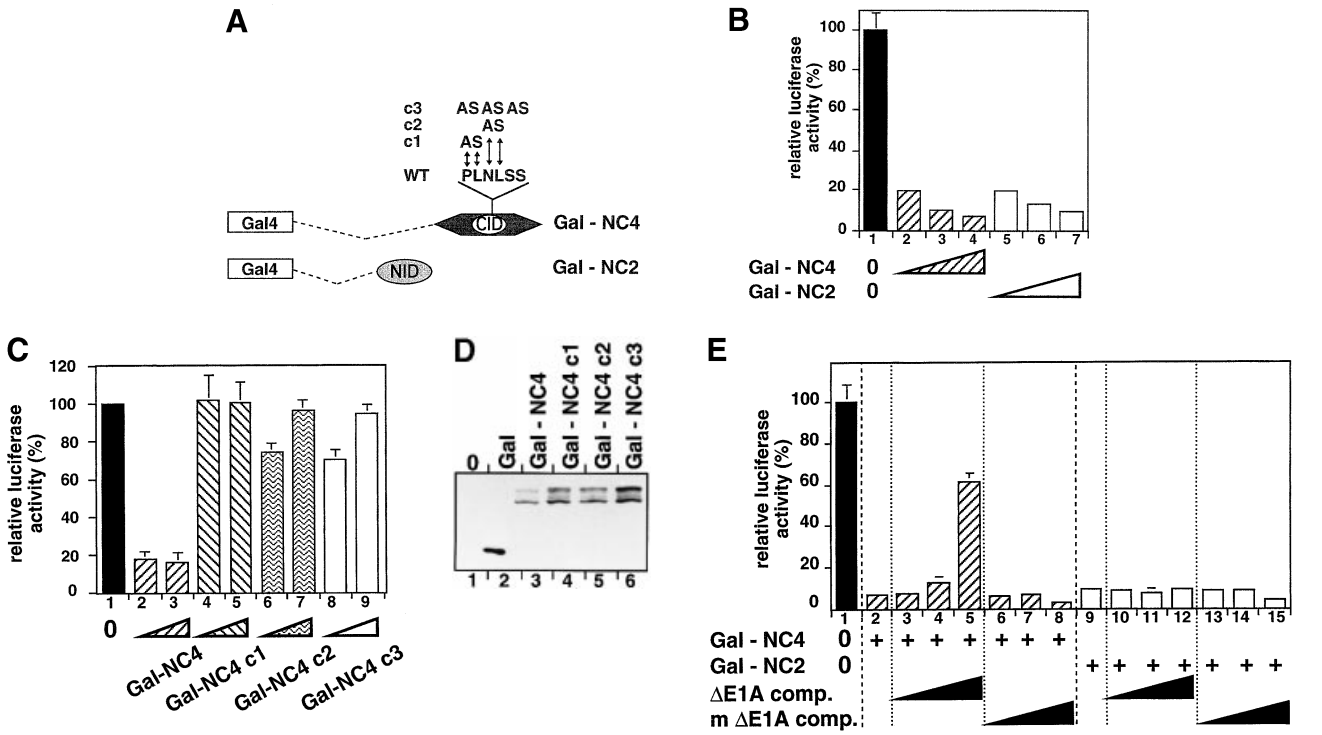


Fig. 6. Repression by the CID domain of Net. (**A**) Structure of the Gal4 fusion proteins. (**B**) NC4 inhibits transcription. CHO cells were co-transfected with 1, 10 and 100 ng of either Gal–NC4 or Gal–NC2 expression vectors, 0.5 μg of pCMV-LacZ and 2 μg of UAS-Luc (containing five Gal4-binding sites). (**C**) Mutations in the CtBP-binding site remove repression. CHO cells were transfected with 10 or 100 ng of DNA for each fusion protein. (**D**) Expression levels of the fusion proteins. One microgram of DNA was transfected in CHO cells and extracts were analysed by Western blotting using a monoclonal anti-Gal4 antibody. (**E**) Competition for CtBP relieves repression by the CID but not the NID. CHO cells were transfected with 10 ng of expression vectors for either Gal–NC4 or Gal–NC2 and 10 ng, 100 ng or 1 μg of either pML00512S-ΔCR1 (ΔE1A comp.) or pML00512S-ΔCR1Δ225–238 (m ΔE1A comp.). Luciferase values are expressed as percentages of the basal activity of the reporter. One representative experiment of three, with each point in triplicate, is shown.

observed that both C10 and C12 attenuated the increase in SRE activity due to the competition (Figure 8B; lanes 6–10 for C10; lanes 11–15 for C12; compare with the control, lanes 1–5). These results show that the ets-binding site of SRE mediates to some extent negative regulation of the c-*fos* gene through CtBP and de-acetylation. However, they do not exclude that other TCF-independent mechanisms are involved in this regulation.

Discussion

We have shown that mCtBP1 interacts strongly with Net *in vitro* and under *in vivo* conditions. mCtBP1 binds to a second repressor domain, the CID, which is distinct from the previously identified inhibitory domain, the NID. The CID is the minimal functional domain defined in this study, corresponding to the NC4 deletion mutant that contains an essential short sequence homologous to the other CtBP-binding domains. The CID and the NID appear to mediate repression by distinct mechanisms. mCtBP1 acts as a co-repressor for Net through a mechanism involving de-acetylation. CtBP1 is also involved in negative regulation of the c-*fos* SRE, a transcription element implicated in Net-mediated repression. These studies raise the possibility of a link between Net repression, histone de-acetylation and altered chromatin structure.

Net contains two repression domains

The NID and the CID are distinct domains that appear to inhibit by different mechanisms. They have unrelated sequences; the NID probably forms an HLH structure (Maira *et al*., 1996), whereas the CID has a crucial short sequence found in a number of proteins that interact with CtBP (Figure 1C). The two repressor domains function independently. Inactivation of either element in full-length Net is apparently insufficient to relieve repression fully in CHO cells. They both efficiently repress transcription when fused separately to a heterologous DNA-binding domain. Only repression by the CID is inhibited by a competitor for CtBP. Finally, a de-acetylase inhibitor relieves repression by the CID much more efficiently than by the NID (Results; and data not shown). The presence of two inhibitory domains is a feature of a number of repressors, including several factors recently shown or implicated in interactions with CtBP. These associated inhibitory domains may have independent or complementary functions.

The *Drosophila* Krüppel gene encodes a repressor required for segmentation and at later stages of development. The protein contains two conserved domains that repress transcription by distinct mechanisms and have different biological functions. The N-terminal domain forms an α-helix and represses activators that the C-terminal domain cannot inhibit. The C-terminal domain has a different sequence, and, intriguingly, point mutations that decrease repression alter a putative CtBP-binding element (Hanna-Rose *et al*., 1997). *Drosophila* Hairy is a bHLH protein required for embryonic segmentation. Hairy and E(sp1)m∂ (a Hairy-related protein) have two repression domains that independently recruit the co-repressors

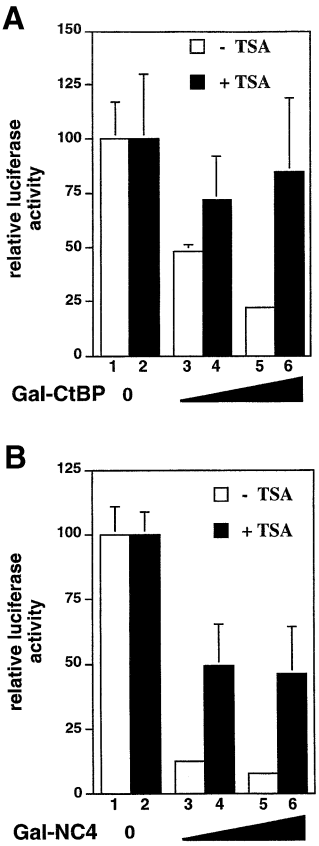


Fig. 7. Evidence for the role of histone de-acetylation in repression by mCtBP1 and NC4. (**A**) mCtBP1 inhibition is alleviated by a histone de-acetylase inhibitor. CHO cells were co-transfected with 5 or 10 ng of the expression vector for Gal–CtBP1, 1.5 μg of UAS-Luc and 0.5 μg of pCMV-LacZ, and treated or not with 300 nM TSA. (**B**) NC4 inhibition is alleviated by a de-acetylase inhibitor. CHO cells were transfected as in (A) with 10 or 100 ng of the expression vector for Gal–NC4. Luciferase activities, normalized with β-gal, are presented as percentages of the basal activity of the reporter.

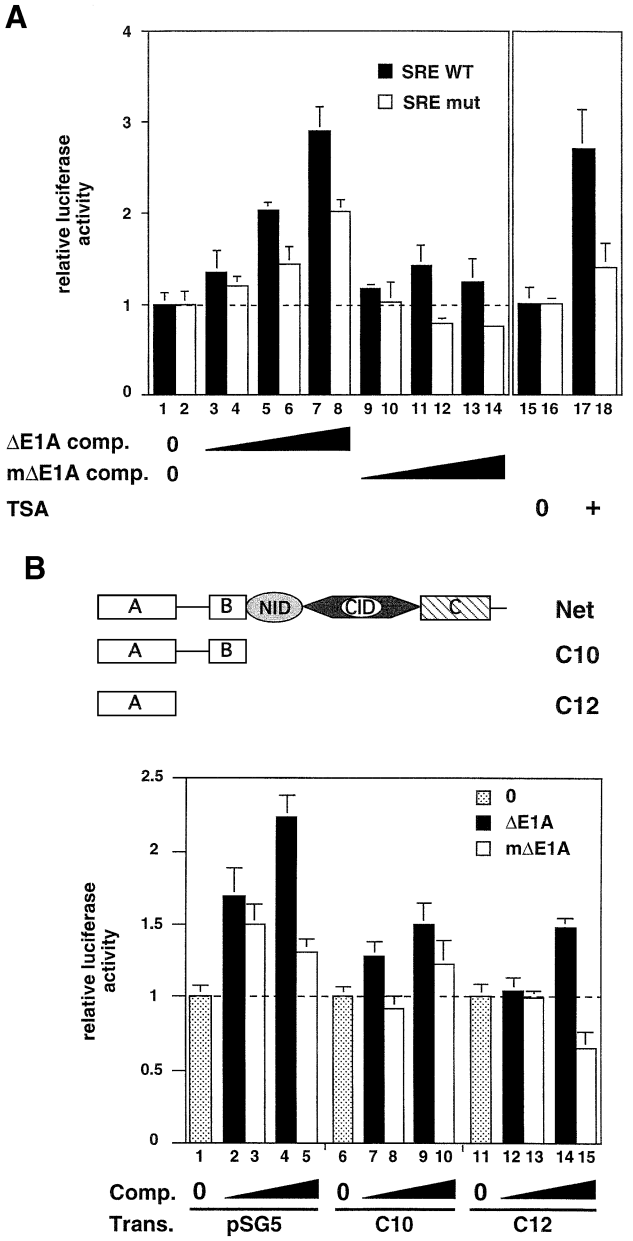


Fig. 8. The SRE element of the c-*fos* promoter is regulated by mCtBP1 and histone de-acetylation. (**A**) Regulation of the SRE by mCtBP1 and histone de-acetylation, and effect of mutating the ets-binding site. CHO cells were transfected in low (0.05%) serum with 0.5 μg of pCMV-LacZ and 0.5 μg of SRE-Luc or SRE mut-Luc. The competition experiments contained 10 ng, 100 ng or 1 μg of pML00512S-ΔCR1 (ΔE1A comp.) or pML00512SΔCR1Δ225–238 (m ΔE1A comp.). For TSA treatment, 150 nM TSA (+) or the equivalent amount of ethanol (0) were added 12 h before scraping the cells. (**B**) *Trans*-dominant mutant Net proteins affect mCtBP1-dependent regulation of the SRE. One microgram of SRE-Luc reporter was transfected with 2 μg of pSG5, C10 or C12 and 0.2 or 1 μg of competitor expression vectors.The results are presented as fold activation relative to the basal levels in the absence of competitors.

CtBP and Groucho (Jimenez *et al*., 1997; Fisher and Caudy, 1998a,b; Parkhurst, 1998; Poortinga *et al*., 1998). CtBP interacts with the E1A-related sequence PXDLS (Poortinga *et al*., 1998), whereas Groucho binds to the C-terminal WRPW sequence. CtBP and Groucho have different roles during development but the mechanisms of repression by either domain are not completely elucidated. Mutation of the dCtBP motif has no obvious effect on Hairy-mediated repression. A Hairy protein containing an optimal CtBP-binding site prevented Hairy-mediated repression. Hairy and CtBP appear to mediate separate pathways and might function antagonistically (Zhang and Levine, 1999). Groucho is not always associated with CtBP in transcription repressors. For example, both Engrailed (Jimenez *et al*., 1997; Tolkunova *et al*., 1998) and Dorsal (Lehming *et al*., 1994) interact with Groucho and have other inhibitory domains. In certain cases, repressor domains appear to confer complementary functions that tighten inhibition. For example, the thyroid hormone receptor (TR) interacts with the basal transcription machinery [TFIIB (Baniahmad *et al*., 1993; Fondell *et al*., 1993) and TBP (Fondell *et al*., 1996)] and also recruits the co-repressors proteins SMRT or N-CoR (Chen and Evans, 1995; Hörlein *et al*., 1995) that affect chromatin structure. It remains to be seen why there are two repression domains in Net and whether they have complementary, distinct or antagonistic activities.

CtBP interacts tightly with different repressors

CtBP has now been shown to interact with a diverse set of factors with no obvious common function (Schaeper *et al*., 1995; Nibu *et al*., 1998; Poortinga *et al*., 1998;

Schaeper *et al*., 1998) including: (i) E1A, an essential viral protein that does not interact directly with DNA; (ii) Net, an Ets domain protein that binds to ets motifs and responds to Ras signals; (iii) CtIP, which may be involved in tumour suppression through protein–protein interactions with BRCA1 (Yu *et al*., 1998); and (iv) the *Drosophila* proteins, Hairy, E(sp1)m∂, Snail and Knirps, that are important for different aspects of development (Boulay *et al*., 1987; Nauber *et al*., 1988; Paroush *et al*., 1994; Gray and Levine, 1996; for a review, see Fisher and Caudy, 1998a). They have different DNA-binding domains: Hairy and E(sp1)m∂ have bHLH sequences, Snail has zinc fingers and Knirps has a nuclear receptor domain. CtBP is widely expressed (Katsanis and Fisher, 1998; Poortinga *et al*., 1998), raising the possibility that CtBP mediates a general mechanism of repression in many cell types and that specificity results from recruitment by particular transcription factors.

CtBP, Net and histone de-acetylase complexes

huCtBP1 has been shown to interact with the histone de-acetylase HDAC1 both *in vitro* and *in vivo* (Sundqvist *et al*., 1998). We have strengthened the connection by showing that the de-acetylase inhibitor TSA decreases the inhibitory activities of mCtBP1 and Net CID. These results raise the possibility that Net and mCtBP1 might recruit one of the recently described multi-protein complexes that contain HDACs (Alland *et al*., 1997; Hassig *et al*., 1997; Laherty *et al*., 1997; Nagy *et al*., 1997; Zhang *et al*., 1997). The composition and number of complexes are not well defined, but they contain uncharacterized subunits in addition to HDAC1, HDAC2, NCoR, SMRT, Sin3, RbAp46, RbAp48 and SAP30 (Ashraf and Ip, 1998; Davie, 1998; Kuo and Allis, 1998; Laherty *et al*., 1998; Luger and Richmond, 1998; Torchia *et al*., 1998; Zhang *et al*., 1998). Net interacts with the N-terminal region of mCtBP1 that is so far unique to CtBP family members. The conserved central domain of CtBP might be involved in protein–protein interactions. It is possible that CtBP acts as a linker between specific factors and general repression complexes. Transcription factors recruit de-acetylase complexes to specific promoter elements, resulting in local histone de-acetylation and transcription repression (Kadosh and Struhl, 1998; Rundlett *et al*., 1998). This raises the possibility that Net might induce local repressive chromatin conformations on promoters and in particular the c-*fos* promoter. Moreover, the SRE has been shown to have a centrally positioned nucleosome adjacent to the SRE (Herrera *et al*., 1997), which could be a target for Net/TCF- and CtBP-mediated repression of the c-*fos* SRE. However, repression by Net and CtBP may involve substrates other than histones, such as transcription factors that are regulated by acetylation (Gu and Roeder, 1997; Imhof *et al*., 1997; Zhang and Bieker, 1998). Mutating the ets-binding site of the *fos* SRE altered the response to the CtBP competitor and TSA treatment. Net *trans*-dominant proteins, which are expected to displace the Net/CtBP repressor, also decreased the effect of competitor. However, both the mutation and the *trans*-dominants had a partial effect, suggesting that other factors are also involved (Alberts *et al*., 1999).

The prevailing model for a number of factors that switch from negative to positive is that there is a flip from the recruitment of histone de-acetylase to acetylase complexes. These switches are induced by ligands binding to hormone repressors, the replacement of Mad–Max by Myc–Max complexes, and phosphorylation and release of pRb from E2F (Brehm *et al*., 1998; Ferreira *et al*., 1998; Magnaghi-Jaulin *et al*., 1998; Torchia *et al*., 1998). Phosphorylation of the ternary complex factor SAP1a in response to MAP kinase cascades leads to the recruitment of the histone acetyl transferase CBP (Janknecht and Hunter, 1996a,b; Janknecht and Nordheim, 1996a,b). These comparisons raise the possibility that Net switches from a negative to a positive factor by differential recruitment of de-acetylase and acetylase complexes. However, stimulation of Net by Ras does not eliminate inhibition by CID, suggesting that Net does not simply conform to the de-acetylation/acetylation switch mechanism. CtBP-mediated repression may be relieved by other mechanisms, such as cellular localization. CtBP is nuclear in both Saos2 cells and normal *Drosophila* embryos (Nibu *et al*., 1998), but cytoplasmic when expressed in Cos-7 cells. Co-expression of Net, a nuclear protein, results in the relocation of mCtBP1 to the nucleus. This raises the possibility that interactions with Net and other factors could change the cellular localization of CtBP and consequently its effects on transcription.

The link to transformation and cancer

Chromatin structure appears to be important for transformation since proteins that modify nucleosomes are often found to be altered in cancer (Futamura *et al*., 1995; Dhordain *et al*., 1997; Brehm *et al*., 1998; David *et al*., 1998; DePinho, 1998; Giles *et al*., 1998; Grignani *et al*., 1998; Lin *et al*., 1998; Magnaghi-Jaulin *et al*., 1998; Versteege *et al*., 1998; Wang *et al*., 1998). CtBP, through its interactions with de-acetylases, appears to be a link between chromatin structure and transformation. E1A mutants unable to interact with CtBP have both an increased transforming ability in combination with Ras (Boyd *et al*., 1993; Sollerbrant *et al*., 1996) and a higher metastatic potential due to the loss of repression of protease expression (Frisch *et al*., 1990; Linder *et al*., 1992). We have shown that CtBP and Ras affect the activity of Net, providing a link between inhibitors and activators of transformation, and repression and activation of gene expression.

Materials and methods

Plasmids

LexA–Net fusions. (pLexA–Net, –∂AC, –C10, –NC2 and –NC4). *Eco*RI-flanked PCR fragments were cloned in the *Eco*RI site of pBTM116 (Hollenberg *et al*., 1995) in-frame with the full-length LexA DNA-binding domain. Clones were screened for the correct orientation and sequenced. The LexA–lamin and LexA–bicoid fusions are a generous gift from R.Brent.

Prokaryotic GST fusion proteins. Net or ∂AC *Eco*RI-flanked PCR fragments were cloned in the *Eco*RI site of pGEX2TK in-frame with the GST moiety. For NC2 and NC4 constructs, *Eco*RI–*Bam*HI-flanked PCR fragments were cloned in pGEX2TK digested by *Eco*RI and *Bam*HI.

Eukaryotic GST fusion proteins. pBC-Net and pBC-Net_{elk} were described previously (Maira *et al*., 1996), pBC-∂AC, -∂AC c1 and c2 were obtained by PCR cloning in the *Eco*RI–*Kpn*I sites of the pBC vector (Chatton *et al*., 1995). The c1 and c2 point mutations were generated by the two-round PCR mutagenesis strategy. GST–CtBP and deletion mutants of

mCtBP1 were cloned by PCR. pBC-E1A is a generous gift from Dr B.Chatton and encodes full-length E1A 12S.

pTL2 expression vectors. pTL2-Net was described elsewhere (Giovane *et al*., 1994), pTL2-Net c1, c2 and c3 were obtained by the two-round PCR mutagenesis strategy. pKOZ-C10 and pKOZ-C12 were described in Maira *et al*. (1996). pTL1-mCtBP1 was generated by cloning the Klenow-filled *Sfi*I fragment from the pASV3-clone 45 isolated by the yeast two-hybrid screen, in the *Sma*I site of pTL1. pML00512SΔCR1 (ΔE1A comp.) and pML00512SΔCR1Δ225–238 (m ΔE1A comp.) are a generous gift from Dr C.Svenson (Sollerbrant *et al*., 1996). pEGFP-C1 was provided by Clontech.

Gal4 fusion proteins. Gal4–NC2 was described elsewhere (Maira *et al*., 1996). Gal4–NC4 was constructed by the same strategy as Gal4–NC2. Mutants Gal4–NC4 c1, c2 and c3 were obtained by cloning *Asp*718-digested PCR fragments in the *Asp*718 site of pG4mpolyII. Gal4–CtBP was obtained by cloning a *Cla*I PCR fragment corresponding to the full-length cDNA of CtBP in the *Cla*I site of pG4mpolyII (Webster *et al*., 1988).

PCR fragments were verified systematically by sequencing on both strands.

Reporters

Pal×8-TK-Luc, SRE-TK-Luc and SREmut-TK-Luc were generated by transferring, into *Sma*I–*Bgl*II-digested pGL2 (Promega), the *Pvu*II–*Bgl*II fragments encoding respectively Pal×8-TK, SRE-TK and SREmut-TK from pBL-Pal×8-TK-CAT4, pBL-SRE-TK-CAT4 and pBL-SREmut-TK-CAT4 (described in Giovane *et al*., 1994; Maira *et al*., 1996). UAS-TK-Luc, generously provided by Dr A.Bradford and Dr A.Gutierrez-Hartman, contains five Gal4-binding sites in pGL2-Luc.

Yeast two-hybrid screen

The two-hybrid screen was performed as described previously (Hollenberg *et al*., 1995). pLexA-∂AC, a deletion mutant of Net lacking the Ets DNA-binding domain and the transactivation domain, was used to screen a VP16-tagged mouse cDNA library generated from combined 9.5, 10.5, 11.5 and 12.5 day embryos (vom Baur *et al*., 1996). Transformants were analysed by the liquid β-galactosidase activity and histidine prototrophy assays. His^+ $LacZ^+$ recombinants were isolated and used for direct two-hybrid assays with related and unrelated fusion proteins. The nucleotide sequence of mCtBP1 was submitted to the DDBJ/EMBL/GenBank database and has the accession No. AJ010483.

In vitro protein–protein interactions

GST fusion proteins were expressed in bacteria and mCtBP1 was synthesized *in vitro* using rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions. The interaction assays were done as described previously, except that they were performed in Eppendorf tubes for 2 h at 4°C. Ten percent of the interaction assay and 10% of the input were analysed on 10% SDS–PAGE gels.

Cell culture and transfections

Cos-7 cells were maintained in Dulbecco's modified Eagle's medium (Sigma Chemical, St Louis, MO) suplemented with 5% FCS. CHOD cells were cultured in αMEM1900 (Sigma) medium plus 10% FCS and 20 mM glutamine. Cells were transfected by the BBS calcium phosphate method (Chen and Okayama, 1987) in either 90 mm dishes with 20 μg of DNA for Cos-7 cells, or in 6-well plates with 4 μg of DNA for CHOD cells. At 16–18 h after transfection, the cells were washed twice with FCS-free medium and incubated for 24 h in 0.05% FCS medium (or 10% FCS medium where indicated). The cells were scraped in 1 ml of phosphate-buffered saline (PBS) and processed for protein–protein interactions (Cos-7 cells) or luciferase assays (CHO DHFR– cells). TSA (Sigma-Aldrich chimie), 150 or 300 nM, was added to media of the cells 12 h before harvesting. Luciferase assays were performed with the Luciferase Assay System from Promega according to the manufacturer's instructions and analysed with a Luminometer (EG&G Berthold). At least two independently prepared DNAs were tested. Each experiment was performed at least three times, either in duplicate or in triplicate, and one representative experiment is shown. Luciferase activity was corrected for the transfection efficiency using β-gal activity as an internal control.

Western blots and antibodies

Western blots. Proteins were fractionated by SDS–PAGE and transferred to nitrocellulose membranes. After 1 h of saturation in PBSTM (PBS with 0.05% Tween-20 and 3% milk), the membranes were incubated either overnight at 4°C or for 1 h at room temperature with specific primary antibodies in PBSTM (0.5% milk), washed four times for 5 min with PBST, incubated for 1 h at room temperature with secondary antibodies coupled to peroxidase (diluted 1/5000) in PBSTM (0.5% milk), washed four times for 5 min with PBST and revealed with the ECL detection kit (Amersham, Ref RPN 2106).

Antibodies. Anti-Net, anti-Gal, anti-GST and secondary antibodies are described in Maira *et al*. (1996). Anti-mCtBP is rabbit polyclonal PAb1123 or 1128 raised against the ovalbumin-coupled peptide corresponding to amino acids 452–477 or 352–374 of murine CtBP1 respectively. Anti-GFP is a monoclonal antibody from Clontech Lab (ref. 8362-1). Anti-E1A is the rabbit polyclonal antibody described in Chatton *et al*. (1993). Protein A, peroxidase-linked, is a secondary antibody from Amersham (ref. NA 9120).

In vivo protein–protein interactions

The eukaryotic GST pull-down assay (Maira *et al*., 1996) was modified as follows. Transfected Cos-7 cells were lysed in 100 μl of lysis buffer [0.4 M KCl, 20 mM Tris–HCl pH 7.5, 20% glycerol, 5 mM dithiothreitol (DTT), 0.4 mM phenylmethylsulfonyl fluoride (PMSF)] by freezing in liquid nitrogen and thawing at 37°C once. Cell lysates were cleared by centrifugation (10 min at 10 000 *g*) and 80 μl aliquots were incubated for 2 h at 4°C in 1 ml of either low-stringency buffer Ls (50 mM Tris–HCl, pH 7.8, 0.1% NP-40, 250 mM NaCl) or RIPA buffer (PBS with 0.1% SDS, 0.5% sodium deoxycholate, 0.5% NP-40) with 40 μl of glutathione–agarose beads (Sigma chemicals; 50% suspension in 0.1% gelatine, 0.01% sodium azide). The beads were washed once with 1 ml of Ls buffer containing 1 M NaCl, twice with Ls buffer containing 0.5% NP-40 or twice with RIPA buffer, resuspended in 20 μl of SDS loading buffer and boiled for 8 min. Proteins were fractionated by SDS–PAGE, transferred to nitrocellulose membranes and analysed by immunoblotting (see above).

Co-immunoprecipitation

Cos-7 cells were lysed in IP lysis buffer (50 mM Tris pH 8, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40 and 1 mM PMSF) by freezing in liquid nitrogen and thawing at 37°C. Cell lysates were cleared by centrifugation (10 min at 10 000 *g*) and 200 μg of protein was pre-incubated for 30 min at 4°C with 40 μl of protein G–Sepharose (Sigma chemicals; 50% suspension in 0.1% gelatine). The beads were removed and extracts incubated for 2 h at 4°C with 10 μl of polyclonal anti-Net 375 in a final volume of 1 ml, followed by 1 h at 4°C with 40 μl of protein G–Sepharose. The beads were washed four times with SNNTE (5% sucrose, 1% NP-40, 0.5 M NaCl, 50 mM Tris pH 7.4 and 5 mM EDTA). Proteins were fractionated by SDS–PAGE, transferred to nitrocellulose membranes and analysed by immunoblotting (see above).

Immunofluorescence

Cells were cultured on cover slips, transfected as described above and processed at room temperature. They were washed three times with PBS, fixed for 30 min in 4% paraformaldehyde, washed three times in PBS, saturated for 40 min in PBS + 3% bovine serum albumin (BSA), incubated for 40 min with primary antibody diluted 1/500, washed three times for 10 min with PBS, incubated for 45 min with the secondary antibody diluted 1/250 in PBS + 0.5 mg/ml BSA and washed three times for 10 min in PBS. The cover slips were incubated for 20 s in Hoechst dye (5 μg/ml in PBS), washed three times in PBS and placed inverted on a drop of mounting solution (80% glycerol, 20% 1× PBS with 5% propylgallate) on a slide. The slides were stored in the dark at 4°C and visualized under fluorescence or confocal microscopes.

Acknowledgements

We thank R.Brent, B.Chatton, J.M.Garnier, N.Messadeq, J.-L.Vonescht, F.Jeanmougin, C.Svensson, A.Bradford and A.Gutierrez-Hartman for the gift of recombinants, cell lines, antibodies and advice with computing, and the IGBMC facilities staff for invaluable help. The following establishments are thanked for fellowships: the MRT and the Ligue Nationale Française contre le Cancer to P.C.-F., the MRT to C.D., and the MRT, the Ligue Nationale Française contre le Cancer and BioAvenir to M.-S.M. Financial assistance was received from BioAvenir, the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Hôpital Universitaire de Strasbourg, the Association pour la Recherche sur le Cancer, the Fondation pour la

Recherche Médicale, the Ligue Nationale Française contre le Cancer, the Ligue Régionale (Haut-Rhin) contre le Cancer and the Ligue Régionale (Bas-Rhin) contre le Cancer (the Legs Meyer).

References

Alberts,A.S., Geneste,O. and Treisman,R. (1998) Activation of SRF-regulated chromosomal templates by Rho-family GTPases requires a signal that also induces H4 hyperacetylation. *Cell*, **92**, 475–487.

Alland,L., Muhle,R., Hou,H.,Jr, Potes,J., Chin,L., Schreiber-Agus,N. and De Pinho,R.A. (1997) Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. *Nature*, **387**, 49–55.

Ashraf,S.I. and Ip,Y.T. (1998) Transcriptional control: repression by local chromatin modification. *Curr. Biol.*, **8**, R683–R686.

Baniahmad,A., Ha,I., Reinberg,D., Tsai,S., Tsai,M.J. and O'Malley,B.W. (1993) Interaction of human thyroid hormone receptor beta with transcription factor TFIIB may mediate target gene derepression and activation by thyroid hormone. *Proc. Natl Acad. Sci. USA*, **90**, 8832–8836.

Boulay,J.L., Dennefeld,C. and Alberga,A. (1987) The *Drosophila* developmental gene *snail* encodes a protein with nucleic acid binding fingers. *Nature*, **330**, 395–398.

Boyd,J.M., Subramanian,T., Schaeper,U., La Regina,M., Bayley,S. and Chinnadurai,G. (1993) A region in the C-terminus of adenovirus 2/5 E1a protein is required for association with a cellular phosphoprotein and important for the negative modulation of T24-ras mediated transformation, tumorigenesis and metastasis. *EMBO J.*, **12**, 469–478.

Brehm,A., Miska,E.A., McCance,D.J., Reid,J.L., Bannister,A.J. and Kouzarides,T. (1998) Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature*, **391**, 597–601.

Chatton,B., Bocco,J.L., Gaire,M., Hauss,C., Reimund,B., Goetz,J. and Kedinger,C. (1993) Transcriptional activation by the adenovirus larger E1a product is mediated by members of the cellular transcription factor ATF family which can directly associate with E1a. *Mol. Cell. Biol.*, **13**, 561–570.

Chatton,B., Bahr,A., Acker,J. and Kedinger,C. (1995) Eukaryotic GST fusion vector for the study of protein–protein associations *in vivo*: application to interaction of ATFa with Jun and Fos. *Biotechniques*, **18**, 142–145.

Chen,C. and Okayama,H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.*, **7**, 2745–2752.

Chen,J.D. and Evans,R.M. (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature*, **377**, 454–457.

David,G., Alland,L., Hong,S.H., Wong,C.W., DePinho,R.A. and Dejean,A. (1998) Histone deacetylase associated with mSin3A mediates repression by the acute promyelocytic leukemia-associated PLZF protein. *Oncogene*, **16**, 2549–2556.

Davie,J.R. (1998) Covalent modifications of histones: expression from chromatin templates. *Curr. Opin. Genet. Dev.*, **8**, 173–178.

DePinho,R.A. (1998) Transcriptional repression. The cancer–chromatin connection. *Nature*, **391**, 533–536.

Dhordain,P., Albagli,O., Lin,R.J., Ansieau,S., Quief,S., Leutz,A., Kerckaert,J.P., Evans,R.M. and Leprince,D. (1997) Corepressor SMRT binds the BTB/POZ repressing domain of the LAZ3/BCL6 oncoprotein. *Proc. Natl Acad. Sci. USA*, **94**, 10762–10767.

Dittmer,J. and Nordheim,A. (1998) Ets transcription factors and human disease. *Biochim. Biophys. Acta*, **1377**, F1–F11.

Ferreira,R., Magnaghi-Jaulin,L., Robin,P., Harel-Bellan,A. and Trouche,D. (1998) The three members of the pocket proteins family share the ability to repress E2F activity through recruitment of a histone deacetylase. *Proc. Natl Acad. Sci. USA*, **95**, 10493–10498.

Fisher,A.L. and Caudy,M. (1998a) Groucho proteins: transcriptional corepressors for specific subsets of DNA-binding transcription factors in vertebrates and invertebrates. *Genes Dev.*, **12**, 1931–1940.

Fisher,A.L. and Caudy,M. (1998b) The function of hairy-related bHLH repressor proteins in cell fate decisions. *BioEssays*, **20**, 298–306.

Fondell,J.D., Roy,A.L. and Roeder,R.G. (1993) Unliganded thyroid hormone receptor inhibits formation of a functional preinitiation complex: implications for active repression. *Genes Dev.*, **7**, 1400–1410.

Fondell,J.D., Brunel,F., Hisatake,K. and Roeder,R.G. (1996) Unliganded thyroid hormone receptor alpha can target TATA-binding protein for transcriptional repression. *Mol. Cell. Biol.*, **16**, 281–287.

Frisch,S.M., Reich,R., Collier,I.E., Genrich,L.T., Martin,G. and Goldberg,G.I. (1990) Adenovirus E1A represses protease gene expression and inhibits metastasis of human tumor cells. *Oncogene*, **5**, 75–83.

Futamura,M., Monden,Y., Okabe,T., Fujita-Yoshigaki,J., Yokoyama,S. and Nishimura,S. (1995) Trichostatin A inhibits both ras-induced neurite outgrowth of PC12 cells and morphological transformation of NIH3T3 cells. *Oncogene*, **10**, 1119–1123.

Giles,R.H., Peters,D.J. and Breuning,M.H. (1998) Conjunction dysfunction: CBP/p300 in human disease. *Trends Genet.*, **14**, 178–183.

Giovane,A., Pintzas,A., Maira,S.M., Sobieszczuk,P. and Wasylyk,B. (1994) Net, a new ets transcription factor that is activated by Ras. *Genes Dev.*, **8**, 1502–1513.

Graves,B.J. and Petersen,J.M. (1998) Specificity within the ets family of transcription factors. *Adv. Cancer Res.*, **75**, 1–55.

Gray,S. and Levine,M. (1996) Short-range transcriptional repressors mediate both quenching and direct repression within complex loci in *Drosophila*. *Genes Dev.*, **10**, 700–710.

Gregory,P.D. and Horz,W. (1998) Chromatin and transcription—how transcription factors battle with a repressive chromatin environment. *Eur. J. Biochem.*, **251**, 9–18.

Grignani,F. *et al*. (1998) Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. *Nature*, **391**, 815–818.

Gu,W. and Roeder,R.G. (1997) Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell*, **90**, 595–606.

Hanna-Rose,W. and Hansen,U. (1996) Active repression mechanisms of eukaryotic transcription repressors. *Trends Genet.*, **12**, 229–234.

Hanna-Rose,W., Licht,J.D. and Hansen,U. (1997) Two evolutionarily conserved repression domains in the *Drosophila* Krüppel protein differ in activator specificity. *Mol. Cell. Biol.*, **17**, 4820–4829.

Hassig,C.A., Fleischer,T.C., Billin,A.N., Schreiber,S.L. and Ayer,D.E. (1997) Histone deacetylase activity is required for full transcriptional repression by mSin3A. *Cell*, **89**, 341–347.

Herrera,R.E., Nordheim,A. and Stewart,A.F. (1997) Chromatin structure analysis of the human c-fos promoter reveals a centrally positioned nucleosome. *Chromosoma*, **106**, 284–292.

Hollenberg,S.M., Sternglanz,R., Cheng,P.F. and Weintraub,H. (1995) Identification of a new family of tissue-specific basic helix–loop–helix proteins with a two-hybrid system. *Mol. Cell. Biol.*, **15**, 3813–3822.

Hörlein,A.J. *et al*. (1995) Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature*, **377**, 397–404.

Imhof,A., Yang,X.J., Ogryzko,V.V., Nakatani,Y., Wolffe,A.P. and Ge,H. (1997) Acetylation of general transcription factors by histone acetyltransferases. *Curr. Biol.*, **7**, 689–692.

Janknecht,R. and Hunter,T. (1996a) Versatile molecular glue. Transcriptional control. *Curr. Biol.*, **6**, 951–954.

Janknecht,R. and Hunter,T. (1996b) Transcription. A growing coactivator network. *Nature*, **383**, 22–23.

Janknecht,R. and Nordheim,A. (1996a) MAP kinase-dependent transcriptional coactivation by Elk-1 and its cofactor CBP. *Biochem. Biophys. Res. Commun.*, **228**, 831–837.

Janknecht,R. and Nordheim,A. (1996b) Regulation of the c-fos promoter by the ternary complex factor Sap-1a and its coactivator CBP. *Oncogene*,**12**, 1961–1969.

Jimenez,G., Paroush,Z. and Ish-Horowicz,D. (1997) Groucho acts as a corepressor for a subset of negative regulators, including Hairy and Engrailed. *Genes Dev.*, **11**, 3072–3082.

Kadosh,D. and Struhl,K. (1998) Targeted recruitment of the Sin3–Rpd3 histone deacetylase complex generates a highly localized domain of repressed chromatin *in vivo*. *Mol. Cell. Biol.*, **18**, 5121–5127.

Katsanis,N. and Fisher,E.M. (1998) A novel C-terminal binding protein (CTBP2) is closely related to CTBP1, an adenovirus E1A-binding protein, and maps to human chromosome 21q21.3. *Genomics*, **47**, 294–299.

Kuo,M.-H. and Allis,C.D. (1998) Roles of histone acetyltransferases and deacetylases in gene regulation. *BioEssays*, **20**, 615–626.

Laherty,C.D., Yang,W.M., Sun,J.M., Davie,J.R., Seto,E. and Eisenman,R.N. (1997) Histone deacetylases associated with the mSin3 corepressor mediate mad transcriptional repression. *Cell*, **89**, 349–356.

Laherty,C.D. *et al*. (1998) SAP30, a component of the mSin3 corepressor complex involved in N-CoR-mediated repression by specific transcription factors. *Mol. Cell*, **2**, 33–42.

Lehming,N., Thanos,D., Brickman,J.M., Ma,J., Maniatis,T. and Ptashne,M. (1994) An HMG-like protein that can switch a transcriptional activator to a repressor. *Nature*, **371**, 175–179.

Lin,R.J., Nagy,L., Inoue,S., Shao,W., Miller,W.H.,Jr and Evans,R.M. (1998) Role of the histone deacetylase complex in acute promyelocytic leukaemia. *Nature*, **391**, 811–814.

Linder,S., Popowicz,P., Svensson,C., Marshall,H., Bondesson,M. and Akusjarvi,G. (1992) Enhanced invasive properties of rat embryo fibroblasts transformed by adenovirus E1A mutants with deletions in the carboxy-terminal exon. *Oncogene*, **7**, 439–443.

Luger,K. and Richmond,T.J. (1998) The histone tails of the nucleosome. *Curr. Opin. Genet. Dev.*, **8**, 140–146.

Magnaghi-Jaulin,L., Groisman,R., Naguibneva,I., Robin,P., Lorain,S., Le Villain,J.P., Troalen,F., Trouche,D. and Harel-Bellan,A. (1998) Retinoblastoma protein represses transcription by recruiting a histone deacetylase. *Nature*, **391**, 601–605.

Maira,S.M., Wurtz,J.M. and Wasylyk,B. (1996) Net (ERP/SAP2) one of the Ras-inducible TCFs, has a novel inhibitory domain with resemblance to the helix–loop–helix motif. *EMBO J.*, **15**, 5849–5865.

Nagy,L., Kao,H.Y., Chakravarti,D., Lin,R.J., Hassig,C.A., Ayer,D.E., Schreiber,S.L. and Evans,R.M. (1997) Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell*, **89**, 373–380.

Nauber,U., Pankratz,M.J., Kienlin,A., Seifert,E., Klemm,U. and Jackle,H. (1988) Abdominal segmentation of the *Drosophila* embryo requires a hormone receptor-like protein encoded by the gap gene knirps. *Nature*, **336**, 489–492.

Nibu,Y., Zhang,H. and Levine,M. (1998) Interaction of short-range repressors with *Drosophila* CtBP in the embryo. *Science*, **280**, 101–104.

Parkhurst,S.M. (1998) Groucho: making its Marx as a transcriptional co-repressor. *Trends Genet.*, **14**, 130–132.

Paroush,Z., Finley,R.L.,Jr, Kidd,T., Wainwright,S.M., Ingham,P.W., Brent,R. and Ish-Horowicz,D. (1994) Groucho is required for *Drosophila* neurogenesis, segmentation, and sex determination and interacts directly with hairy-related bHLH proteins. *Cell*, **79**, 805–815.

Poortinga,G., Watanabe,M. and Parkhurst,S.M. (1998) *Drosophila* CtBP: a Hairy-interacting protein required for embryonic segmentation and hairy-mediated transcriptional repression. *EMBO J.*, **17**, 2067–2078.

Rundlett,S.E., Carmen,A.A., Suka,N., Turner,B.M. and Grunstein,M. (1998) Transcriptional repression by UME6 involves deacetylation of lysine 5 of histone H4 by RPD3. *Nature*, **392**, 831–835.

Schaeper,U., Boyd,J.M., Verma,S., Uhlmann,E., Subramanian,T. and Chinnadurai,G. (1995) Molecular cloning and characterization of a cellular phosphoprotein that interacts with a conserved C-terminal domain of adenovirus E1A involved in negative modulation of oncogenic transformation. *Proc. Natl Acad. Sci. USA*, **92**, 10467–10471.

Schaeper,U., Subramanian,T., Lim,L., Boyd,J.M. and Chinnadurai,G. (1998) Interaction between a cellular protein that binds to the C-terminal region of adenovirus E1A (CtBP) and a novel cellular protein is disrupted by E1A through a conserved PLDLS motif. *J. Biol. Chem.*, **273**, 8549–8552.

Sharrocks,A.D., Brown,A.L., Ling,Y. and Yates,P.R. (1997) The ETS-domain transcription factor family. *Int. J. Biochem. Cell Biol.*, **29**, 1371–1387.

Sollerbrant,K., Chinnadurai,G. and Svensson,C. (1996) The CtBP binding domain in the adenovirus E1A protein controls CR1-dependent transactivation. *Nucleic Acids Res.*, **24**, 2578–2584.

Sundqvist,A., Sollerbrant,K. and Svensson,C. (1998) The carboxy-terminal region of adenovirus E1A activates transcription through targeting of a C-terminal binding protein–histone deacetylase complex. *FEBS Lett.*, **429**, 183–188.

Tolkunova,E.N., Fujioka,M., Kobayashi,M., Deka,D. and Jaynes,J.B. (1998) Two distinct types of repression domain in engrailed: one interacts with the groucho corepressor and is preferentially active on integrated target genes. *Mol. Cell. Biol.*, **18**, 2804–2814.

Torchia,J., Glass,C. and Rosenfeld,M.G. (1998) Co-activators and co-repressors in the integration of transcriptional responses. *Curr. Opin. Cell Biol.*, **10**, 373–383.

Treisman,R. (1996) Regulation of transcription by MAP kinase cascades. *Curr. Opin. Cell Biol.*, **8**, 205–215.

Versteege,I., Sevenet,N., Lange,J., Rousseau-Merck,M.F., Ambros,P., Handgretinger,R., Aurias,A. and Delattre,O. (1998) Truncating mutations of hSNF5/INI1 in aggressive paediatric cancer. *Nature*, **394**, 203–206.

vom Baur,E. *et al*. (1996) Differential ligand-dependent interactions between the AF-2 activating domain of nuclear receptors and the putative transcriptional intermediary factors mSUG1 and TIF1. *EMBO J.*, **15**, 110–124.

Wang,J., Hoshino,T., Redner,R.L., Kajigaya,S. and Liu,J.M. (1998) ETO, fusion partner in t(8;21) acute myeloid leukemia, represses transcription by interaction with the human N-CoR/mSin3/HDAC1 complex. *Proc. Natl Acad. Sci. USA*, **95**, 10860–10865.

Wasylyk,B. and Nordheim,A. (1997) Ets transcription factors: partners in the integration of signal responses. In Papavassiliou,A.G. (ed.), *Transcription Factors in Eucaryotes*. Landes Bioscience, Georgetown, pp. 253–286.

Wasylyk,B., Hagman,J. and Gutierrez-Hartmann,A. (1998) Ets transcription factors: nuclear effectors of the Ras–MAP-kinase signaling pathway. *Trends Biochem. Sci.*, **23**, 213–216.

Webster,N., Jin,J.R., Green,S., Hollis,M. and Chambon,P. (1988) The yeast UASG is a transcriptional enhancer in human HeLa cells in the presence of the GAL4 trans-activator. *Cell*, **52**, 169–178.

Yu,X., Wu,L.C., Bowcock,A.M., Aronheim,A. and Baer,R. (1998) The C-terminal (BRCT) domains of BRCA1 interact *in vivo* with CtIP, a protein implicated in the CtBP pathway of transcriptional repression. *J. Biol. Chem.*, **273**, 25388–25392.

Zhang,H. and Levine,M. (1999) Groucho and dCtBP mediate separate pathways of transcriptional repression in the *Drosophila* embryo. *Proc. Natl Acad. Sci. USA*, **96**, 535–540.

Zhang,W. and Bieker,J.J. (1998) Acetylation and modulation of erythroid Krüppel-like factor (EKLF) activity by interaction with histone acetyltransferases. *Proc. Natl Acad. Sci. USA*, **95**, 9855–9860.

Zhang,Y., Iratni,R., Erdjument-Bromage,H., Tempst,P. and Reinberg,D. (1997) Histone deacetylases and SAP18, a novel polypeptide, are components of a human Sin3 complex. *Cell*, **89**, 357–364.

Zhang,Y., Sun,Z.W., Iratni,R., Erdjument-Bromage,H., Tempst,P., Hampsey,M. and Reinberg,D. (1998) SAP30, a novel protein conserved between human and yeast, is a component of a histone deacetylase complex. *Mol. Cell*, **1**, 1021–1031.

Received November 4, 1998; revised and accepted April 29, 1999