immunoprecipitations were done as described[13]. Ethanol and methanol controls did not alter processing.

Co-precipitations. Notch-1 proteins were precipitated together with Flag–CSL^{RBP3} from transiently transfected 293T cells as described[7], with the following modifications: anti-Flag antibody M2 (Kodak) was used to precipitate Flag–CSL^{RBP3} and Myc-tagged Notch-1 proteins were detected with monoclonal antibody 9E10. Duplicate 60-mm plates were transfected with 1.5 μg Notch-1 plasmid and/or $pCS2^+$ Jagged-1 and $pCS2^+$ Flag–CSL^{RBP3} (3 μg), and $pCS2^+$ vector was added to a total of 6 μg. Cells were lysed and pooled in 1 ml buffer[7]. Notch-1 does not precipitate from cell extracts that were transfected separately with CSL^{RBP3} and Notch-1 and then mixed (data not shown).

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Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex

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Cytosine residues in the sequence 5′CpG (cytosine–guanine) are often postsynthetically methylated in animal genomes. CpG methylation is involved in long-term silencing of certain genes during mammalian development[1,2] and in repression of viral genomes[3,4]. The methyl-CpG-binding proteins MeCP1 (ref. 5) and MeCP2 (ref. 6) interact specifically with methylated DNA and mediate transcriptional repression[7–9]. Here we study the mechanism of repression by MeCP2, an abundant nuclear protein that is essential for mouse embryogenesis[10]. MeCP2 binds tightly to chromosomes in a methylation-dependent manner[11,12]. It contains a transcriptional-repression domain (TRD) that can

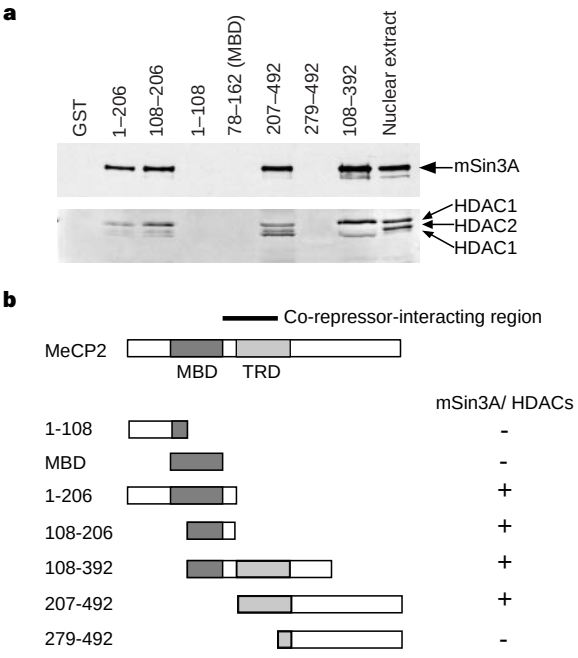

Figure 1 Interaction of MeCP2 with mSin3A and histone deacetylases. **a**, Western blot analysis of HeLa nuclear proteins pulled down by immobilized GST or by GST-fusion proteins that included different regions of MeCP2 (see **b**). Numbers correspond to amino-acid positions in the protein. Blots were probed with antibodies against mSin3A (antibody K-20), or against HDAC1 and HDAC2. The lower HDAC1 band probably corresponds to a degradation product of HDAC1. The HDAC2 band in lane 108–392 is displaced upwards because a large amount of fusion protein migrates with the HDAC2. **b**, Map of regions of MeCP2 that do (+) or do not (–) pull down mSin3A and HDACs. The thick black line corresponds to the corepressor-interacting region defined by these experiments. MBD, methyl-CpG-binding domain[11].

function at a distance *in vitro* and *in vivo*[9]. We show that a region of MeCP2 that localizes with the TRD associates with a corepressor complex containing the transcriptional repressor mSin3A and histone deacetylases[13–19]. Transcriptional repression *in vivo* is relieved by the deacetylase inhibitor trichostatin A[20], indicating that deacetylation of histones (and/or of other proteins) is an essential component of this repression mechanism. The data suggest that two global mechanisms of gene regulation, DNA methylation and histone deacetylation, can be linked by MeCP2.

DNA methylation is associated with altered chromatin structures[21–23]. As MeCP2 binds tightly to chromatin in a methylation-dependent manner, it seemed possible that transcriptional repression may be due to recruitment by MeCP2 of a chromatin-modifying corepressor. A corepressor complex containing mSin3 and histone deacetylases (HDACs) is thought to exert its effects by modulating chromatin structure[13–19]. We therefore asked whether regions of MeCP2 could 'pull down' the mSin3-containing corepressor complex from nuclear extracts. We used proteins containing glutathione *S*-transferase (GST) fused to different regions of MeCP2 in 'pulldown' reactions, and probed proteins bound to these complexes with antibodies against mSin3A, HDAC1 and HDAC2. A region in the centre of MeCP2 could bind all three proteins (Fig. 1a). GST alone and regions outside the interacting region of MeCP2 did not pull down any of the corepressor proteins (Fig. 1a). Control nuclear proteins (proliferating cell nuclear antigen and TATA-binding protein) were not bound by MeCP2 (data not shown). The region of MeCP2 that interacted with the corepressor substantially overlaps the TRD, as defined *in vivo* (Fig. 1b)[9].

To test for a possible *in vivo* interaction between native MeCP2 and the mSin3 corepressor complex, we used antibodies against MeCP2 and mSin3 to immunoprecipitate proteins from rat brain nuclear extracts (Fig. 2a). Both anti-MeCP2 antibodies precipitated mSin3A as well as MeCP2. Four different anti-mSin3 antibodies immunoprecipitated MeCP2 as well as mSin3 (the anti-Sin3 antibody AK11 precipitated less MeCP2 than the other antibodies). Immunoprecipitates of transiently expressed HDAC2 also contained MeCP2 (data not shown). These results led us to expect that MeCP2 would be associated with a catalytically active deacetylase complex. This was confirmed by the finding that both anti-MeCP2 antibodies precipitated deacetylation activity, which was abolished by the highly specific deacetylase inhibitor trichostatin A (TSA)[20] (Fig. 2b). Antibodies against components of the corepressor complex, mSin3A and HDAC1, also immunoprecipitated deacetylase activity, as expected, whereas control antibodies (anti-CDK7 antibody and preimmune serum) gave background activity (Fig. 2b).

Does MeCP2 interact with mSin3 itself or with another component of the complex? To answer this question, we assayed the ability

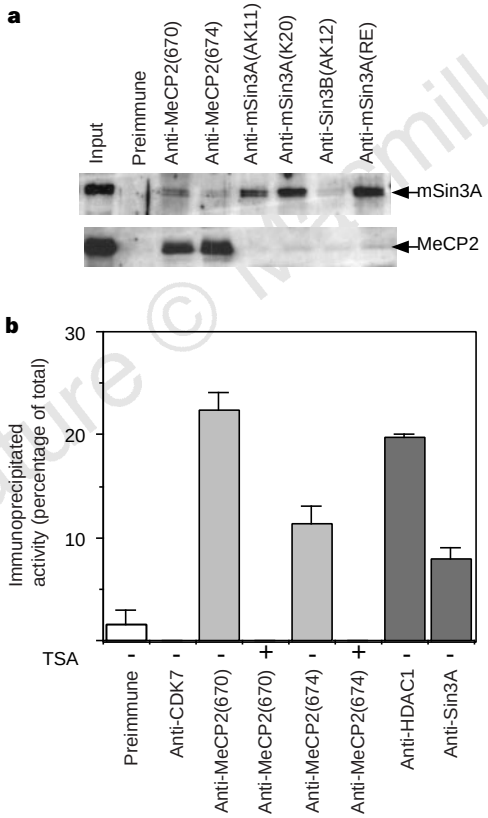

Figure 2 Co-immunoprecipitation of mSin3, MeCP2 and histone deacetylase activity from rat brain nuclear extracts. **a**, Western blot of immunoprecipitates prepared using antibodies against MeCP2 (antibodies 670 and 674) or mSin3 (antibodies AK11, AK12, K-20 and RE) or using preimmune serum. Probes were anti-mSin3A (RE) and anti-MeCP2 (670 and 674) antibodies. **b**, Immunoprecipitation of histone deacetylase activity from rat brain nuclear extracts with antibodies against MeCP2 (670 and 674), HDAC1, mSin3A or cyclin-dependent kinase 7 (CDK7) or with preimmune serum, in the presence or absence of 5 ng ml^{-1} TSA. Precipitated activities from duplicate parallel experiments are shown as percentage total activity added to each tube (19,200 d.p.m. per 2 h in the experiment shown).

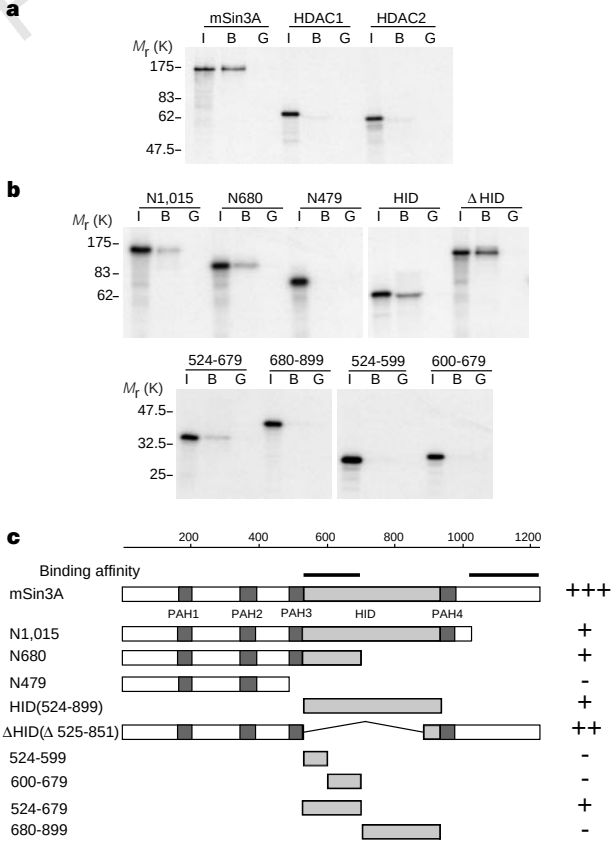

Figure 3 MeCP2 interacts with mSin3A, but not with HDACs. **a**, *In vitro*-translated ^{35}S-labelled mSin3A and HDACs 1 and 2 were incubated with an immobilized fusion protein containing GST and amino acids 108–392 of MeCP2 (Fig. 1b). Input protein (I) (20% of total), protein bound by the 108–392 fusion protein (B) and protein bound by GST alone (G) were analysed by SDS–PAGE. **b**, Deletion constructs of mSin3A were transcribed and translated *in vitro* to give ^{35}S-labelled products and processed as in **a**. Input (I) corresponds to 10% of total. **c**, Maps of the mSin3A deletion constructs[13], indicating truncated proteins that did (+) or did not (–) bind to MeCP2. Regions of strong binding to MeCP2 are indicated by the thick black lines (top).

of the corepressor-interacting region of MeCP2 (residues 108–392) to bind *in vitro*-translated mSin3A, HDAC1 and HDAC2. mSin3A is the preferred binding partner, as the HDACs have a much weaker affinity for MeCP2 (Fig. 3a). There is probably a direct interaction between MeCP2 and mSin3A, although we cannot exclude the possibility that an unknown component of the translation lysate mediates indirect binding. We mapped the sites of interaction between MeCP2 and mSin3A by translating *in vitro* a series of mSin3A proteins with deleted portions (ref. 13) and testing their ability to bind to amino acids 108–392 of MeCP2 (Fig. 3b, c). The amino-terminal half of the HDAC-interaction domain (HID)[13] of mSin3A was common to most fragments that were able to binding MeCP2, but a deletion lacking all but the carboxy-terminal 48 amino acids of HID (ΔHID) also bound efficiently, indicating a strong binding site within the C-terminal 200 amino acids of mSin3A (Fig. 3). We infer that the interaction between MeCP2 and mSin3A involves both the N-terminal half of the HID of mSin3A and the C-terminus of mSin3A. MeCP2 and deacetylases seem to bind to distinct sites within the 375-residue HID region, as both can bind simultaneously (Figs 1a and 2a, b).

To determine whether deacetylation is important for the effects of the TRD on transcription *in vivo*, we attempted to alleviate transcriptional repression with TSA[20]. We transiently transfected mouse L929 fibroblasts with a reporter gene, which either did (G_5) or did not (G_0) have GAL4-binding sites near its promoter, in the presence or absence of a GAL4–TRD fusion construct[9]. In the absence of repressor, the ratio of G_5 expression to control G_0 expression was unaffected by TSA, remaining close to unity (Fig. 4). In the presence of the GAL4–TRD repressor, the G_5:G_0 expression ratio was about 0.2, but was increased 2.8-fold by TSA. Incubations with TSA for 24 h (Fig. 4) or 9 h (data not shown) gave essentially the same result, eliminating the possibility that TSA exerted its effects by arresting the cells at a stage of the cell cycle where repression was minimal. Repression by GAL4–TRD was not completely alleviated by TSA in any of the experiments (Fig. 4, data not shown). The results indicate that a component of repression by the TRD may be deacetylase-independent, consistent with the observation that mSin3A retains some ability to repress transcription even in the absence of associated HDACs[13].

Our results indicate that repression by the TRD of MeCP2 relies, to a significant extent, on histone deacetylation. Evidence for a functional relationship between DNA methylation and chromatin has come from studies showing that methylated DNA packaged as chromatin is incapable of transcription, whereas methylated DNA alone or chromatin alone remains transcriptionally active[22,23]. Given the strong correlation between deacetylation of histone protein tails and transcriptional repression[24], the idea that methylated DNA provokes deacetylation is attractive[25], and is supported by evidence that TSA can substitute for the demethylating drug 5-azacytidine to derepress methylated ribosomal RNA genes in plants[26]. Our results indicate that MeCP2 may provide a mechanistic bridge between DNA methylation and histone deacetylation. Recruitment of the corepressor complex by chromatin-bound MeCP2 may lead to local deacetylation of core histones (and perhaps of other proteins involved in transcription[27]), with consequent elimination of transcription. □

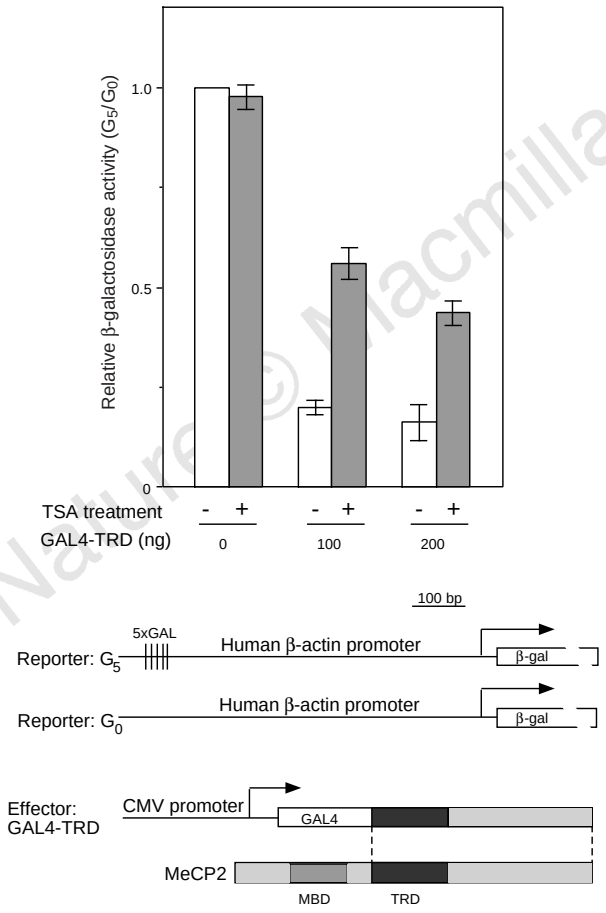

Figure 4 Relief of TRD-mediated repression of transcription by the deacetylase inhibitor TSA. Mouse fibroblasts (L cells) were transfected with reporter genes that did or did not contain GAL4 DNA-binding sites (G_5 or G_0, respectively), in the presence or absence of a fusion between the GAL4 DNA-binding domain and amino acids 207–492 of MeCP2. After 24 h, transfected cells were treated with TSA (shaded bars) or left untreated (white bars) and were incubated for a further 24 h. Columns represent the mean ratio between β-galactosidase activities of G_5 and G_0 in three independent experiments (± standard deviation). Relative β-galactosidase activity in the absence of TSA and GAL4–TRD was normalized to 1.0. CMV, cytomegalovirus; bp, base pairs.

Methods

GST pulldown assay. GST and GST fusion proteins[11] (6 μg) were first immobilized on glutathione–Sepharose beads. The coated beads were then incubated with 60 μg HeLa cell nuclear extract[28] in buffer A (20 mM HEPES pH 7.9, 150 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.1% Triton X-100) for 2 h at 4 °C. After five washes with buffer A, the proteins bound to the beads were eluted in Laemmli buffer and analysed by western blotting nearly as described[13]. The primary antibodies used were K20 (anti-mSin3A, Santa Cruz Biotech. Inc.), anti-HDAC1, and anti-HDAC2 (ref. 13). Nuclear extract (6 μg protein) was loaded as a positive control. Polyclonal antiserum against mammalian HDAC1 was raised against a synthetic peptide corresponding to the C-terminal domain of HDAC1 (EEKPEAKGVKEEVKLA) as described[29].

Immunoprecipitation. Antibodies (4 μl serum, or 2 μg purified IgG) were immobilized on protein A–Sepharose beads and washed with 20 mM HEPES pH 7.9, 0.1 M NaCl, 10% glycerol, 0.2 mM EDTA and 0.01% Triton X-100. The antisera were: anti-MeCP2 (670) raised against the amino acids 207–492 of MeCP2; anti-MeCP2 (674) raised against amino acids 1–392 of MeCP2; anti-mSin3A (RE)[13]; and anti-mSin3A/B antibodies AK-11, K-20 and AK12 (Santa Cruz Biotech. Inc.) Preimmune serum was from the rabbit that was subsequently immunized with MeCP2 amino acids 207–492. Bound antibodies were incubated with 20 μg brain nuclear extract[6,28] in 240 μl wash buffer plus 1 mg ml^{-1} BSA for 1.5 h. Beads were washed three times and protein was eluted in Laemmli buffer. Immunoprecipitated proteins were detected by western blotting.

Immunoprecipitation of histone deacetylase activity. Aliquots (100 μg protein) of rat brain nuclear extract were incubated for 16 h at 4 °C, with 5 μg affinity-purified antibodies against HDAC1 or mSin3A (Santa Cruz Biotech. Inc.) or with whole antiserum against MeCP2 (670 or 674) or CDK7 (a gift from J. Shuttleworth) or with preimmune serum. The final volume was 500 μl in 20 mM HEPES pH 7.9, 150 mM NaCl, 0.1% Nonidet P40, 0.25% gelatin, 1 mM EDTA, 0.5 mM EGTA, 0.2% NaN_3, 0.1 mM PMSF, and 1 μg ml^{-1} each of

aprotinin, leupeptin and pepstatin A. Antibody-bound material was pelleted with protein A–Sepharose (Pharmacia), washed and assayed for histone deacetylase activity as described[30]. The substrate was a synthetic peptide corresponding to the 18 N-terminal residues of histone H4, chemically acetylated using ^{3}H-labelled acetic anhydride (Amersham) and added at 3×10^5 d.p.m. per assay.

***In vitro* binding assay.** Intact mSin3A, HDAC1 (a gift from T. Kouzarides), HDAC2 and fragments of mSin3A (ref. 13) were labelled with [^{35}S]methionine using a coupled transcription-translation TNT system (Promega). GST–MeCP2 fusion proteins or GST (3 μg) were first bound to glutathione–Sepharose beads. The slurry was then incubated with the labelled products (1 μl) in 100 μl buffer B (50 mM HEPES pH 7.9, 250 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.1% Triton X-100, 0.5 mg ml^{-1} BSA) at 4 °C for 2 h. After washing the beads for three times with buffer B, the bound proteins were eluted in Laemmli loading buffer, fractionated by SDS–PAGE, and detected by autoradiography.

Transfection and β-galactosidase assay. Mouse fibroblasts (L cells) were transfected with reporters G_0 or G_5 in the presence or absence of GAL4–TRD(207–492). Transfection and β-galactosidase assays were performed nearly as described[9]. Fresh medium with or without 100 ng ml^{-1} TSA was added to the mouse L929 fibroblast cells 24 h after transfection and the cells were collected after a further 24 h. In some experiments, treatment with TSA was reduced to 9 h.

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Transcriptional activation independent of TFIIH kinase and the RNA polymerase II mediator *in vivo*

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The carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II becomes multiply phosphorylated by protein kinases during early steps in the gene transcription cycle both *in vivo*[1] and *in vitro*[2]. In yeast, the major CTD kinase is a subunit of the general transcription factor TFIIH, and is encoded by an essential gene, *KIN28* (ref. 3). Although the CTD and its phosphorylation are important for transcription[4–6], *in vitro* studies[7,8] have challenged whether CTD phosphorylation is an absolutely required step. The general importance of CTD phosphorylation by Kin28 for transcription in yeast has been suggested because, for all genes tested, transcription is inhibited at the non-permissive temperature in temperature-sensitive *kin28* mutants[9,10]. However, using such a mutant and a copper-inducible targeted destruction method, we show here that transcription of certain genes can be highly induced even when cells lack Kin28. We also show that transcription of these Kin28-independent genes is independent of Srb4 and Srb6, critical components of the CTD-associated transcriptional mediator complex[11]. These results indicate that there are at least two distinct pathways for transcriptional activation: one is dependent on Kin28 and the mediator complex, and the other is not.

In agreement with previous studies[9,10], constitutive transcription of the *MET19* and *ADH1* genes (Fig. 1a) and inducible transcription of the *GAL10* gene (Fig. 1b, left panel) were severely affected at the non-permissive temperature in a yeast (*Saccharomyces cerevisiae*) strain containing a conditional mutation in the TFIIH kinase subunit (*kin28-ts* mutant). Although some *ADH1* messenger RNA is still present at 1 h after the temperature shift because of its long half-life (>30 min), a run-on transcription experiment (Fig. 1a, right panel) shows that the level of polymerase found on the *ADH1* gene has decreased by >95%. We were surprised to find that some genes could be transcriptionally activated in cells that were severely depleted of this essential kinase and general transcription factor activity. After inactivating Kin28 by incubating the *kin28-ts* mutant at 37 °C for 1.5 h, the *CUP1* gene, a copper-inducible gene encoding metallothionein in yeast[12], remained highly inducible (85% of wild-type (WT) induction level) by copper sulphate (Fig. 1b, right panel). On the other hand, mutation in the largest subunit of RNA polymerase II (Pol II) (*rpb1-1*) severely decreased (to ~3% of WT levels) *CUP1* activation at the restrictive temperature (Fig. 1b, right panel). Thus, our data and those of others[9,10] show that many genes require Kin28 for transcription; however, here we show *CUP1* activation by copper can occur in cells in which Kin28 has been inactivated.

TFIIH contains several enzymatic activities, including ATPase