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Methyl-CpG-binding protein 2 is phosphorylated by homeodomain-interacting protein kinase 2 and contributes to apoptosis

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Mutations in the methyl-CpG-binding protein 2 (MeCP2) are associated with Rett syndrome and other neurological disorders. MeCP2 represses transcription mainly by recruiting various co-repressor complexes. Recently, MeCP2 phosphorylation at Ser 80, Ser 229 and Ser 421 was shown to occur in the brain and modulate MeCP2 silencing activities. However, the kinases directly responsible for this are largely unknown. Here, we identify the homeodomain-interacting protein kinase 2 (HIPK2) as a kinase that binds MeCP2 and phosphorylates it at Ser 80 *in vitro* and *in vivo*. HIPK2 modulates cell proliferation and apoptosis, and the neurological defects of *Hipk2*-null mice indicate its role in proper brain functions. We show that MeCP2 cooperates with HIPK2 in induction of apoptosis and that Ser 80 phosphorylation is required together with the DNA binding of MeCP2. These data are, to our knowledge, the first that describe a kinase associating with MeCP2, causing its specific phosphorylation *in vivo* and, furthermore, they reinforce the role of MeCP2 in regulating cell growth.

Keywords: HIPK2; MeCP2; phosphorylation; apoptosis

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INTRODUCTION

The methyl-CpG-binding protein 2 (MeCP2) is a ubiquitous protein, the mutations of which cause Rett syndrome, a severe neurodevelopmental disorder that mainly affects females. MeCP2 binds to methylated DNA and associates with various co-repressor complexes, thereby working as a methylation-dependent transcriptional repressor (Chahrour & Zoghbi, 2007), even though recent results indicated that it might also activate gene expression (Chahrour *et al*, 2008). In the nervous system, MeCP2 phosphorylation was shown to be influenced by extracellular stimuli and to dynamically regulate gene expression. In particular, MeCP2-mediated repression of the brain-derived neurotrophic factor (*Bdnf*) gene was found to be reversed by MeCP2 phosphorylation at Ser 421, causing a change in the binding affinity to the promoter (Chen *et al*, 2003). In addition, Ser 421 phosphorylation affects the ability of MeCP2 to regulate dendritic growth and spine maturation (Zhou *et al*, 2006). More recently, neuronal activity was found to trigger dephosphorylation at Ser 80, decreasing MeCP2 binding to some of its target promoters (Tao *et al*, 2009). Interestingly, *Mecp2^{S80A}*-knock-in and *Mecp2$^{S421A;S421A}$*-knock-in mice, carrying non-phosphorylatable MeCP2 mutations, showed altered locomotor activities (Tao *et al*, 2009), which highlights the relevance of these modifications in neurological functions. Besides Ser 80 and Ser 421, several other residues were found to be phosphorylated in the brain, supporting further the idea that phosphorylation might strongly influence MeCP2 activities. Whereas Ser 421 is phosphorylated by a CaMKII/IV-dependent mechanism, the upstream events causing the phosphorylation of MeCP2 at Ser 80 remain unknown.

We identified homeodomain-interacting protein kinase 2 (HIPK2) as a new MeCP2-associated kinase in a yeast two-hybrid screen. HIPK2 regulates cell growth and apoptosis in development and in response to genotoxic stress (Calzado *et al*, 2007; Rinaldo *et al*, 2007). Here, we identify and characterize HIPK2 as the first kinase that binds to MeCP2 and specifically phosphorylates it at Ser 80 *in vitro* and *in vivo*.

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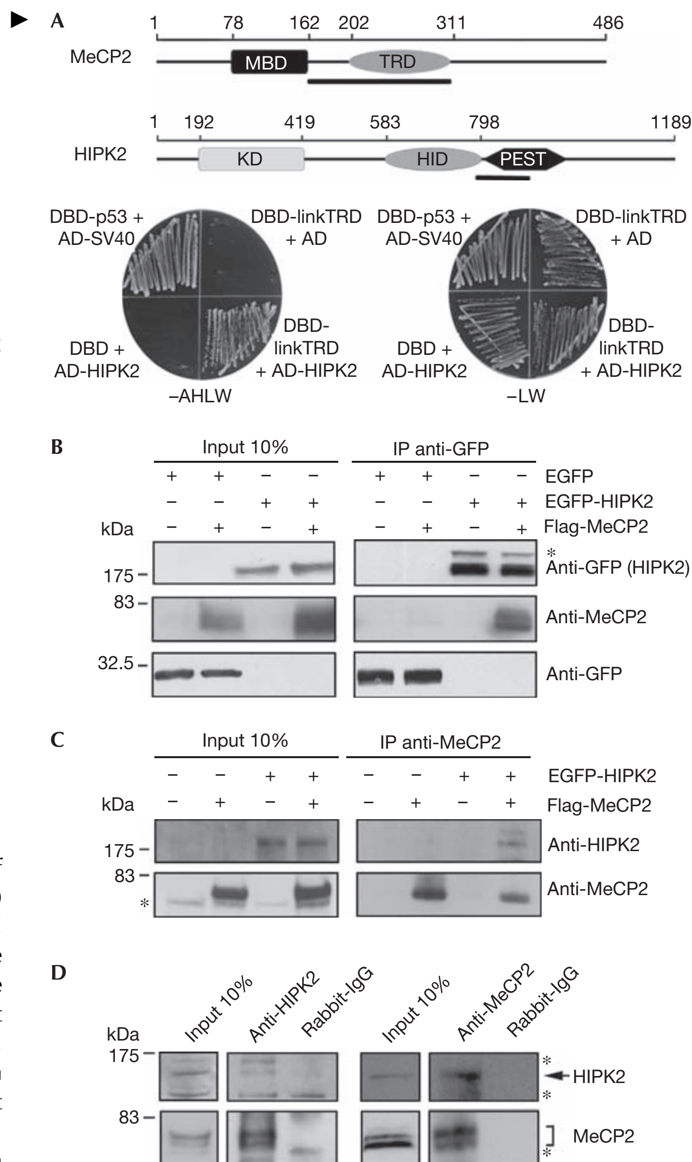

Fig 1 | HIPK2 interacts with MeCP2. (**A**) Schematic representation of human MeCP2 and mouse HIPK2. The shorter black bars indicate the linkTRD of MeCP2 (aa 162–311) and the MeCP2-interacting region of HIPK2 (aa 784–883) fused to GAL4 DBD and AD, respectively. AH109 yeast cells expressing DBD-linkTRD and AD-HIPK2 were tested for growth on selective media to verify bait and prey expression (right) and interaction (left). Cells expressing DBD-linkTRD + AD and DBD + AD-HIPK2 were used as negative controls and DBD-p53 + AD-SV40 as positive controls. (**B**) TCEs from HEK293T cells expressing EGFP or EGFP-HIPK2 with or without Flag-MeCP2 were immunoprecipitated (IP) with anti-GFP antibodies and analysed by wb as indicated. The asterisk indicates a nonspecific band. (**C**) TCEs from *Mecp2*-null MEFs expressing the indicated proteins were immunoprecipitated with MeCP2 antibody; inputs corresponding to 10% of TCEs and immunocomplexes were analysed by wb as indicated. (**D**) Endogenous HIPK2 and MeCP2 were immunoprecipitated with the indicated antibodies from TCEs of human fibroblasts; inputs corresponding to 10% of TCEs and immunocomplexes were analysed by wb with MeCP2 and HIPK2 antibodies. Rabbit IgGs were used as a negative control. The asterisks indicate non-specific bands. AD, activation domain; DBD, DNA-binding domain; EGFP, enhanced green fluorescent protein; HEK, human embryonic kidney; HIPK2, homeodomain-interacting protein kinase 2; KD, kinase-dead; MBD, methyl-CpG binding domain; MeCP2, methyl-CpG-binding protein 2; MEF, mouse embryonic fibroblast; TCE, total cell extract; TRD, transcriptional repression domain; wb, western blot.

RESULTS AND DISCUSSION

HIPK2 is a novel MeCP2-interacting protein

In a yeast two-hybrid screen that used the transcriptional repression domain (TRD) and the upstream linker region of MeCP2 fused to the GAL4 DNA-binding domain (DBD-linkTRD) as bait, we found the carboxy-terminus of HIPK2 to be a novel interactor of MeCP2. The ADE and HIS reporter genes were activated in cells coexpressing the HIPK2 C-terminus fused to the GAL4 activation domain (AD-HIPK2) and the DBD-linkTRD, but not in cells expressing only the bait or the prey (Fig 1A). Glutathione-*S*-transferase (GST) pulldown experiments with recombinant proteins confirmed the interaction, indicating that it might be direct (data not shown).

To evaluate whether the two proteins associate in mammalian cells, we expressed Flag-MeCP2 and enhanced green fluorescent protein (EGFP)-HIPK2 in human embryonic kidney 293T cells and immunoprecipitated total cell extracts (TCEs) with GFP antibodies. Flag-MeCP2 co-precipitates with EGFP-HIPK2 but not with EGFP alone (Fig 1B). The reciprocal experiment was performed on *Mecp2*-null mouse embryonic fibroblasts (MEFs; Fig 1C); EGFP-HIPK2 was present in anti-MeCP2 immunocomplexes obtained from MEFs expressing Flag-MeCP2, but not in cells transfected with the empty vectors. This interaction was confirmed with endogenous HIPK2 and MeCP2 in human fibroblasts (HFs) in which either MeCP2 or HIPK2 antibodies, but not an unrelated rabbit IgG, were able to co-precipitate both proteins (Fig 1D). Altogether, these data indicate that HIPK2–MeCP2 complexes can form both *in vitro* and *in vivo*.

MeCP2 is phosphorylated by HIPK2 *in vitro*

We next assessed whether HIPK2 phosphorylates MeCP2 in kinase assays *in vitro*. Immunopurified Flag-HIPK2, or a kinase-dead (KD) HIPK2 derivative (Flag-K221R), were incubated with recombinant MeCP2 in the presence of γ-[^{33}P]-ATP. Wild-type (wt) HIPK2—but not the KD derivative—phosphorylated both itself and MeCP2. Western blot (wb) confirmed that both wt and KD HIPK2 are efficiently immunoprecipitated, and that, as expected, the KD has faster mobility than wt HIPK2 (Fig 2A).

To identify the specific HIPK2 target residue(s) in MeCP2, we first mapped the region modified by the kinase. Considering that the linkTRD of MeCP2 contacts HIPK2, we performed the kinase assays using two deletion derivatives that retain the interaction surface. Only the C-terminal region (198–486) was significantly less phosphorylated compared with wt MeCP2, indicating that the amino-terminus of MeCP2 or the methyl-CpG binding domain (MBD) contains the main HIPK2 phosphorylation site(s) (Fig 2B).

MeCP2-Ser 80 is the specific target of HIPK2

The region in MeCP2 targeted by HIPK2 contains three of the total 11 serine/threonine-proline (S/T-P) sites constituting possible HIPK2 targets (Fig 3A). Thus, we first tested two MeCP2 derivatives in

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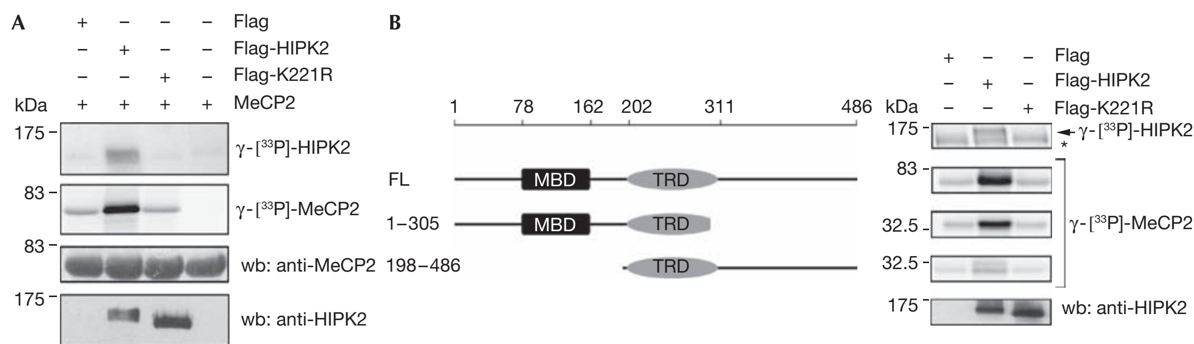

Fig 2 | HIPK2 phosphorylates the MeCP2 amino-terminus *in vitro*. (**A**) HEK293T cells were transiently transfected with the indicated Flag-tagged proteins or the empty vector (Flag alone). TCEs were immunoprecipitated with Flag antibody and processed as follows: 90% of the immunoprecipitated material was incubated with γ-[^{33}P]-ATP and recombinant MeCP2. The labelled proteins were separated by SDS–PAGE, transferred to nitrocellulose membranes, visualized by autoradiography and subsequently immunoblotted with MeCP2 antibody. Ten per cent of the immunoprecipitated material was immunoblotted with HIPK2 antibody (lower panel). The background signal, equally present in the empty vector (Flag) and the KD negative control samples, might be due to non-specifically precipitated cellular kinases. (**B**) *In vitro* kinase assay was performed as described in (**A**), with the indicated MeCP2 deletion derivatives. Upper four panels, autoradiogram; lower panel, wb. The asterisk indicates a non-specific band. HIPK2, homeodomain-interacting protein kinase 2; KD, kinase-dead; MBD, methyl-CpG binding domain; MeCP2, methyl-CpG-binding protein 2; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; TCE, total cell extract; TRD, transcriptional repression domain; wb, western blot.

which two or three N-terminal serine residues were substituted with alanine as HIPK2 substrates (Fig 3A). Two of the mutated residues (Ser 80 and Ser 164) are putative HIPK2 targets, whereas the third (Ser 229)—within the TRD—had already been excluded as substrate in the above experiments. Comparable levels of phosphorylation were obtained with wt MeCP2 and the double mutant (S164,229A), therefore we could exclude Ser 164 as an HIPK2 target. By contrast, a pronounced reduction in MeCP2 phosphorylation was seen with the triple mutant (S80,164,229A). This differs from the first by having eliminated the Ser 80 phosphorylation site as well, indicating that this residue is an HIPK2 target. As the substitution of Ser 80 with alanine also significantly reduces MeCP2 phosphorylation, this residue seems, at least *in vitro*, to be the principal HIPK2 target. This was further confirmed on *in vitro* phosphorylated MeCP2 by immunoblotting with an MeCP2 Ser 80 phospho-site-specific antibody (S80^{P}), the specificity of which was confirmed on either endogenous MeCP2 or exogenous wt or the S80A non-phosphorylatable MeCP2 derivative in cortical neurons and in *Mecp2*-null MEFs (supplementary Fig S1 online).

To test whether Ser 80 is an HIPK2 target *in vivo,* we immunoprecipitated endogenous MeCP2 from HFs transiently transfected with wt or KD EGFP–HIPK2 or EGFP alone. Immunocomplexes were resolved on SDS–polyacrylamide gel electrophoresis (SDS–PAGE), blotted and immunoreacted with MeCP2 or S80^{P} antibodies. Aliquots of the same TCEs (input) were used for wb analyses to demonstrate that equal amounts of EGFP chimeras were present in the input. As shown in Fig 3B, wt HIPK2, but not the KD, strongly increased the reactivity for the S80^{P} antibodies, indicating that HIPK2 can phosphorylate endogenous MeCP2 at Ser 80 *in vivo*. As indicated by the empty vector control, MeCP2 is weakly phosphorylated at Ser 80 in HFs. By contrast, neuronal cells show higher MeCP2 phosphorylation at Ser 80 (Tao *et al*, 2009; Fig 3D) and, under our experimental conditions, further phosphorylation upon ectopic HIPK2 expression could not be detected by our antibody (Fig 4B; data not shown). Thus, to confirm the causal role of HIPK2 in MeCP2-Ser 80 phosphorylation *in vivo*, we used neuron-differentiated P19 cells (Latella *et al*, 2001) and primary mouse cortical neurons in which endogenous HIPK2 was depleted by RNA interference. A strong reduction in reactivity for the S80^{P} antibody was observed in both cell types upon HIPK2 depletion, although, as previously described for other cells (Iacovelli *et al*, 2009), HIPK2 expression could be only partly depleted (Fig 3C,D).

Altogether, these data depict HIPK2 as a kinase capable of phosphorylating MeCP2 *in vivo* through direct complex formation. Two other kinases have been reported to be involved in MeCP2 phosphorylation. Of these, CaMKII and/or IV probably mediate the activity-dependent phosphorylation of Ser 421 in neurons, but as no interaction has been established between the two proteins, it is still unknown whether the CaM kinases target MeCP2 directly or whether other kinases are involved (Zhou *et al*, 2006; Tao *et al*, 2009). Cyclin-dependent kinase-like 5 (CDKL5), the second kinase, is able to associate with MeCP2 and promote its phosphorylation *in vitro* (Mari *et al*, 2005). In this case, however, the' specific target residue has not been mapped and it is unclear whether MeCP2 is a target of CDKL5 *in vivo*.

MeCP2 and HIPK2 cooperate in inducing apoptosis

To begin investigating the functional role of the interaction between HIPK2 and MeCP2, we expressed the two proteins either alone or together in various cell lines—HFs, NIH 3T3 fibroblasts, MEFs and neuron-differentiated P19 cells—and assessed cell survival. We found increased cell death in each of the tested cell lines not only, as expected, when HIPK2 was overexpressed but also with MeCP2 alone (Fig 4A,B). When both proteins were expressed together, the number of dead cells increased in an additive manner. This additive effect depends on the catalytic activity of HIPK2 as coexpression of MeCP2 and the KD mutant induces a level of cell death similar to that of MeCP2 alone. Comparable results were obtained with each cell line tested by using either Trypan blue exclusion or TdT-mediated dUTP

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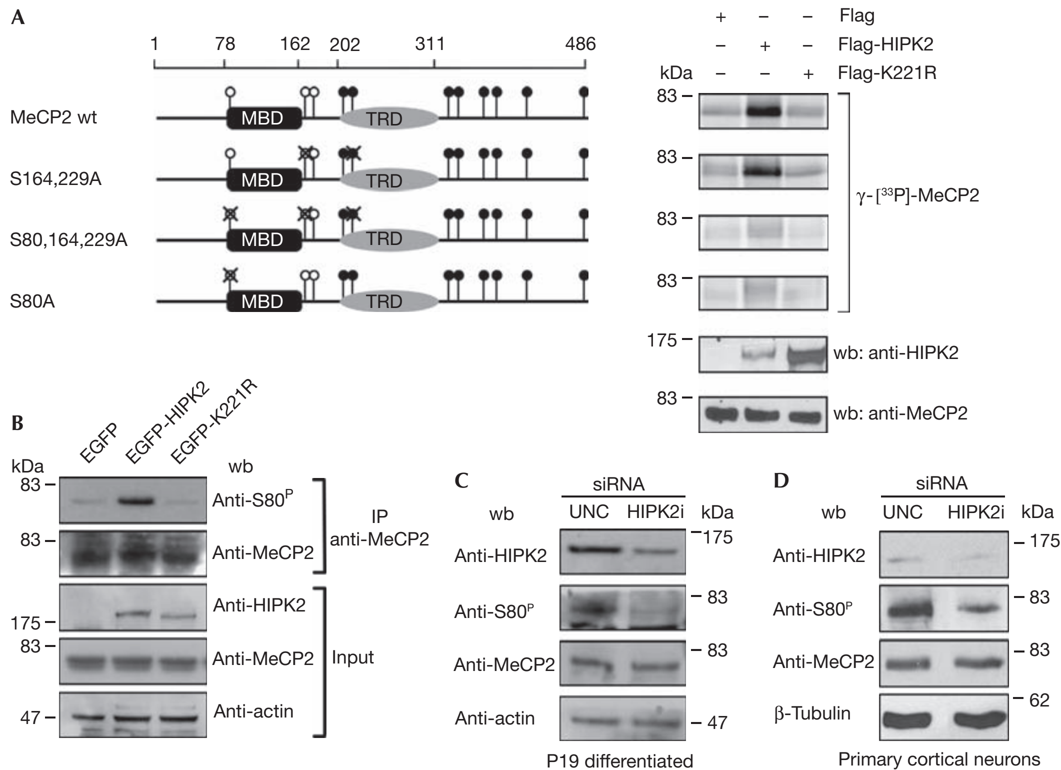

Fig 3 | MeCP2 is specifically phosphorylated at Ser 80 by HIPK2. (**A**) *In vitro* kinase assay (as in Fig 2) with wt MeCP2 and mutated derivatives carrying the indicated serine (S) to alanine (A) substitutions. Autoradiograms (γ-[^{33}P]-MeCP2) and wb with the indicated antibodies are reported. S/T-P sites are represented by lollipops; white lollipops fall in the region modified by HIPK2, whereas black lollipops lie outside; crossed lollipops are serine-to-alanine substitutions. (**B**) Human fibroblasts were transfected with the indicated EGFP-tagged proteins or the empty vector (EGFP alone). TCEs were immunoprecipitated with MeCP2 antibody and analysed by wb with the indicated antibodies. TCEs (inputs) were also analysed by direct wb with the indicated antibodies using actin as a loading control. (**C**) P19 cells were differentiated by retinoic acid treatment and assessed for neuronal marker as reported (Latella *et al*, 2001). At day 14 of differentiation, cells were transfected with a stealth siRNA duplex specific for HIPK2 (HIPK2i) or with the universal negative control (UNC). Wb analyses of the indicated proteins were performed on TCEs from the indicated cells, 48 h after transfection. (**D**) Primary cortical neurons were transfected with a stealth siRNA duplex at day 7 and analysed as shown in (**C**) at day 11. β-Tubulin was used as a loading control. EGFP, enhanced green fluorescent protein; HIPK2, homeodomain-interacting protein kinase 2; MBD, methyl-CpG binding domain; MeCP2, methyl-CpG-binding protein 2; siRNA, short interfering RNA; TCE, total cell extract; TRD, transcriptional repression domain; wb, western blot; wt, wild type.

nick end labelling assays (data not shown), indicating that MeCP2-induced cell death, similarly to that induced by HIPK2, is apoptotic.

As HIPK2 phosphorylates MeCP2, we tested whether MeCP2 works downstream from HIPK2 in the induction of cell death and, therefore, assessed cell viability of *Mecp2*-wt and *Mecp2*-null MEFs upon expression of wt or KD HIPK2. HIPK2-mediated cell death was reduced in the *Mecp2*-null MEFs as compared with that in the *Mecp2*-wt MEFs (Fig 4C), indicating that MeCP2 is involved in HIPK2-mediated apoptosis. However, the level of cell death in the *Mecp2*-null cells expressing HIPK2 was above the background levels at each time tested, indicating, as expected, that the kinase has MeCP2-independent functions in promoting cell death. The reduced cell death induced by HIPK2 in the *Mecp2*-null MEFs was rescued by the concomitant expression of wt MeCP2 (Fig 4D), further confirming that MeCP2 contributes to HIPK2-mediated apoptosis.

As MeCP2 performs its best-characterized functions by binding methylated DNA, we also tested whether the apoptotic functions require the DNA-binding properties. Indeed, expression of the Rett syndrome-associated MeCP2-R106W mutant, which is unable to bind to methylated DNA (Yusufzai & Wolffe, 2000), was unable to restore cell death levels (Fig 4D). Additionally, when HIPK2 was expressed with the non-DNA-binding mutant, not only was the additive effect in inducing apoptosis lost, but also a reduction in the proapoptotic activity of HIPK2 was observed in a small but reproducible manner. This apparent dominant-negative effect of the R106W mutant on the apoptotic function of HIPK2 indicates that MeCP2 binding to methylated DNA might modulate the interaction of HIPK2 with the proper transcription factors and cofactors involved in the apoptotic response (Calzado *et al*, 2007; Rinaldo *et al*, 2007).

MeCP2 has recently been reported to be required for the growth of prostate cancer cells (Bernard *et al*, 2006). In these cells, MeCP2 depletion results in inhibition of proliferation, whereas its overexpression confers a growth advantage that allows these androgen-dependent tumour cells to proliferate in the absence of the hormone. Interestingly, we could confirm the reduced

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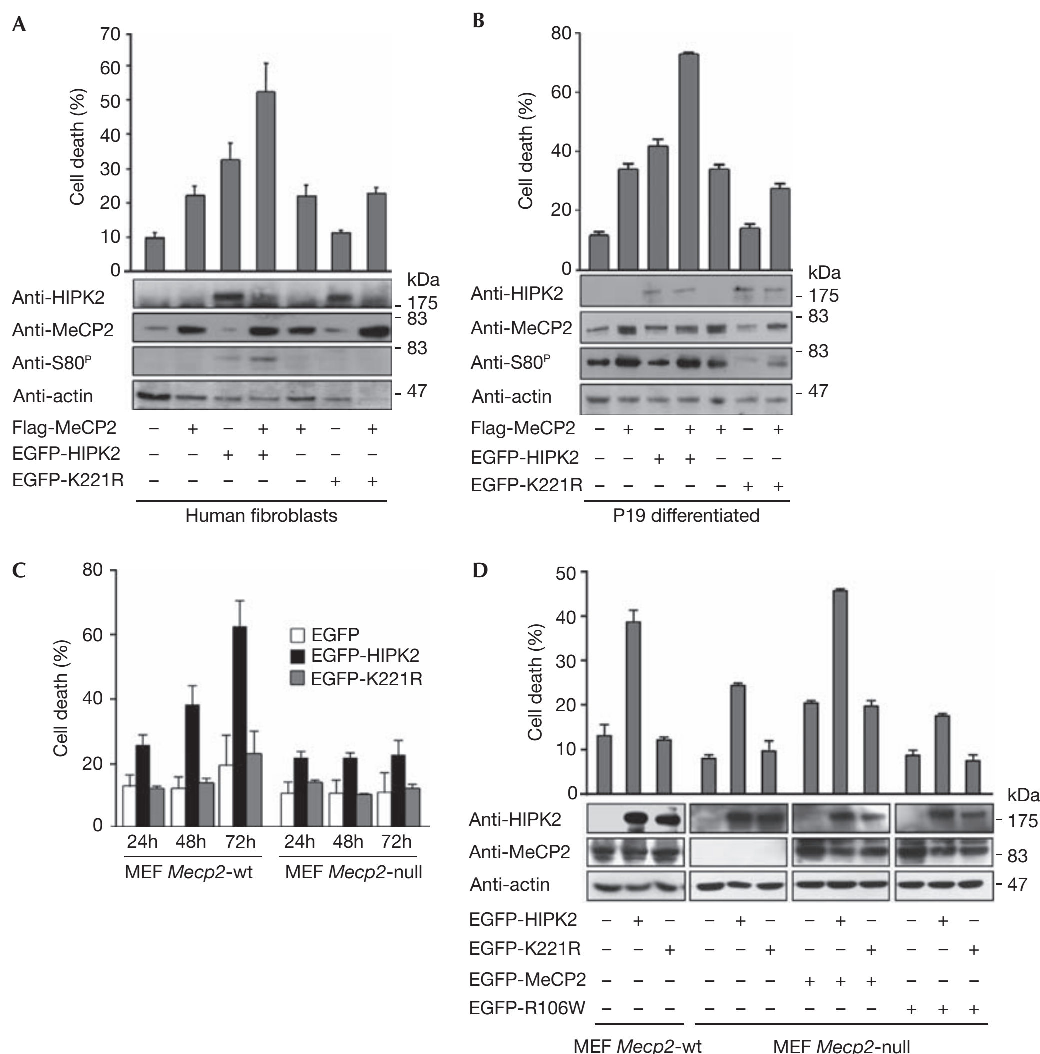

Fig 4 | MeCP2 contributes to HIPK2-induced apoptosis. (**A**) Human fibroblasts expressing Flag-MeCP2 with EGFP-HIPK2 or EGFP-K221R were analysed 24 h after transfection for cell death by Trypan blue exclusion. Mean ± s.d. of three independent experiments is reported. wbs on TCE (HIPK2, actin) or immunoprecipitates (MeCP2, S80^P) were performed. (**B**) P19 cells were induced to differentiate as in Fig 3D, transfected and TCEs were analysed by wb as indicated. (**C**) *Mecp2*-wt and *Mecp2*-null MEFs were transfected with EGFP-HIPK2, EGFP-K221R or the EGFP control vector. Cell death was evaluated as described in part (**A**) at the indicated time points. Mean ± s.d. of three experiments is reported. (**D**) *Mecp2*-wt and *Mecp2*-null MEFs were transfected with the indicated proteins and analysed 40 h after transfection for cell death and protein expression. EGFP, enhanced green fluorescent protein; HIPK2, homeodomain-interacting protein kinase 2; MeCP2, methyl-CpG-binding protein 2; MEF, mouse embryonic fibroblast; TCE, total cell extract; wb, western blot; wt, wild type.

proliferation rate in our *Mecp2*-null MEFs that have a mean doubling time of 39 h compared with 24 h of the wt MEFs (data not shown). However, the apoptotic effect induced by MeCP2—even without coexpression of HIPK2—that we report here is in apparent contrast with the data obtained by using prostate cancer cells. One of the main differences between our experiments and those of Bernard and co-workers is that we used only non-transformed or terminally differentiated cells, whereas they used only tumour cells. We, therefore, tested whether tumour transformation might explain our divergent results. Surprisingly, in tumour cells—HeLa, RKO, U2OS or spontaneously transformed NIH 3T3 cells—we found neither induction of cell death by MeCP2 overexpression nor the additive effect caused by its expression with HIPK2 (data not shown); this indicates that the divergent response to MeCP2 overexpression might depend on tumour transformation. However, the molecular basis of this divergence is unknown at present.

Ser 80 phosphorylation contributes to apoptosis

We next addressed whether phosphorylation at Ser 80 is involved in the apoptotic function of the two proteins and assessed the capacity of some MeCP2 non-phosphorylatable mutants to induce cell death in *Mecp2*-null MEFs alone or in combination with HIPK2. As shown in Fig 5A, the alanine substitutions at Ser 164 and Ser 178 did not significantly alter the capacity of MeCP2 to induce cell death either alone or with HIPK2. By contrast, the

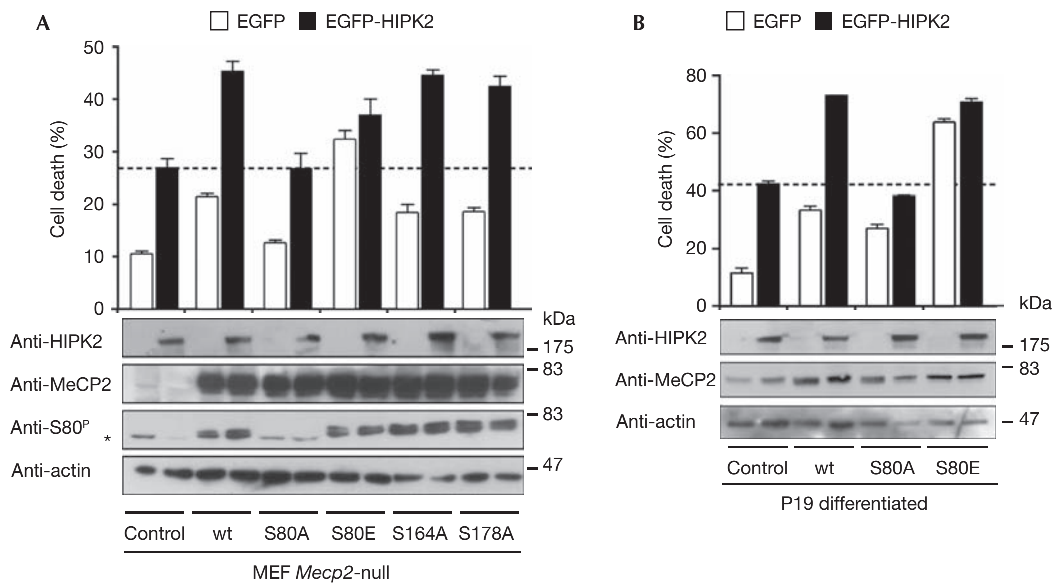

Fig 5 | MeCP2 phosphorylation at Ser 80 contributes to cell death. (**A**) *Mecp2*-null MEFs were cotransfected with EGFP or EGFP-HIPK2 and Flag-tagged wt MeCP2 or its indicated derivatives and analysed 40 h after transfection for cell death. Mean ± s.d. of three experiments is reported. TCEs were analysed by wb as indicated using actin as a loading control. Asterisk indicates a non-specific band. (**B**) P19 cells were induced to differentiate as in Fig 3D and cotransfected with EGFP or EGFP-HIPK2 and MeCP2 or its indicated derivatives. Cell death and protein expression were analysed. The dashed lines indicate cell death levels induced by HIPK2 alone. EGFP, enhanced green fluorescent protein; HIPK2, homeodomain-interacting protein kinase 2; MeCP2, methyl-CpG-binding protein 2; MEF, mouse embryonic fibroblast; TCE, total cell extract; wb, western blot; wt, wild type.

S80A mutant that cannot be phosphorylated (Fig 5A) was not able to induce a level of cell death significantly above that of the control cells and, when coexpressed with HIPK2, did not show any additive effect in MEFs (Fig 5A) or in neuron-differentiated P19 cells (Fig 5B). Thus, we tested whether substitution of Ser 80 with glutamate (S80E), mimicking phosphorylation, might render MeCP2 independent from HIPK2 activation in the cell viability assay. When expressed in *Mecp2*-null MEFs (Fig 5A) or in neuron-differentiated P19 cells (Fig 5B), MeCP2-S80E induced a level of cell death similar to that obtained by coexpressing wt MeCP2 and HIPK2. Coexpression with wt HIPK2 did not significantly increase this level, therefore supporting the idea that, under these conditions, MeCP2-Ser 80 phosphorylation is mediated by HIPK2 and contributes to induction of cell death.

To conclude, here we provide strong evidence of HIPK2 as a kinase associating with MeCP2 and causing its phosphorylation at Ser 80 *in vitro* and *in vivo*; furthermore, our studies show that the two proteins cooperate in the HIPK2-mediated apoptotic pathway in an Ser 80-dependent manner. As described, Ser 80 has previously been identified as one of several serine residues within MeCP2 that are phosphorylated in the brain (Zhou *et al*, 2006; Tao *et al*, 2009). Accordingly, our $S80^P$ antibody detects this specific phosphorylation on endogenous MeCP2 in brain extracts as well as in neuron cultures. Thus, from one viewpoint, it will be interesting to evaluate whether and which types of HIPK2 function are related to MeCP2 phosphorylation in non-neuronal cells (e.g. DNA-damage response). From another viewpoint, given the role of MeCP2 in the brain, which is best illustrated by the onset of Rett syndrome in females with *MECP2* mutations, it will be interesting to analyse the function of the HIPK2–MeCP2 interaction in the nervous system. Importantly, HIPK2 is highly expressed in both the central and peripheral nervous system, and *Hipk2*-null mice show an array of psychomotor abnormalities, underscoring an important role of this kinase in the nervous system (Wiggins *et al*, 2004; Isono *et al*, 2006; Zhang *et al*, 2007). Interestingly, locomotor defects were also observed in the recently developed $Mecp2^{S80A}$-knock-in mice in which MeCP2 cannot be phosphorylated at Ser 80 (Tao *et al*, 2009). Our unpublished results indicate a strong overlap between MeCP2 and HIPK2 expression in the brain, underscoring the possibility that they belong to the same molecular pathway also in this organ. Therefore, it will be challenging to analyse whether HIPK2 contributes to MeCP2-Ser 80 phosphorylation in the brain, and whether it might be involved in the pathogenesis of the Rett syndrome.

METHODS

Plasmids and reagents. For the yeast two-hybrid screen, the linkTRD region (amino acids (aa) 163–311) of human MeCP2 fused with the GAL4 DBD (pGBKT7) was expressed in the yeast strain AH109 with a mouse embryo day 11 cDNA library (Clontech, Milan, Italy). The preparation and source of other plasmids, antibodies and reagents are described in the supplementary information online.
***In vitro* kinase assay.** Recombinant proteins were expressed and purified as previously described (Bertani *et al*, 2006). Kinase assays were performed by incubating immunoprecipitated kinases with recombinant substrates in the presence of γ-[^{33}P]-ATP as described (Bertani *et al*, 2006). Labelled proteins were separated by SDS–PAGE, transferred to membranes, visualized by autoradiography or by PhosphorImager analysis (GE Healthcare, Milan, Italy) and immunoblotted.
siRNA interference. HIPK2-specific (HIPK2i) and universal negative control (UNC) short interfering RNA (siRNA) were HIPK2i

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stealth RNAi sequences (a mix of three different sequences) and stealth RNAi Negative, Medium GC Duplexes, respectively (all from Invitrogen, Milan, Italy). Cells were transfected using RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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