Methyl-CpG binding protein 2 (MeCP2) is phosphorylated by HIPK2 and contributes to apoptosis

***Giorgia Bracaglia,1\* Barbara Conca,2\* Anna Bergo,2 Laura Rusconi,2 Zhaolan Zhou,3 Michael E. Greenberg,3 Nicoletta Landsberger,2,4 Silvia Soddu,1† and Charlotte Kilstrup-Nielsen2†***

1Department of Experimental Oncology, Molecular Oncogenesis Laboratory, Regina Elena Cancer Institute, 00158 Rome, Italy, 2Department of Structural and Functional Biology, Laboratory of Genetic and Epigenetic Control of Gene Expression, University of Insubria, 21052 Busto Arsizio (VA), Italy, 3Department of Neurobiology, Harvard Medical School, Boston, MA 02115, 4Division of Neuroscience, San Raffaele Hospital, 20132 Milan, Italy.

\* These authors contributed equally to the work

† Cocorresponding last authors

Running title:

MeCP2-S80 phosphorylation by HIPK2

Total character count: 27.475

Subject Category: Proteins

ABSTRACT

**Mutations in the methyl CpG-binding protein 2 (MeCP2) are associated with Rett syndrome and other neurological disorders. MeCP2 represses transcription mainly by recruiting different corepressor complexes. Recently, MeCP2 phosphorylation at S80, S229, and S421 was shown to occur in brain and modulate MeCP2 silencing activities. However, the kinases directly responsible are largely unknown. Here, we identify the homeodomain interacting protein kinase 2 (HIPK2) as a kinase binding MeCP2 and phosphorylating it at S80 *in vitro* and *in vivo*. HIPK2 modulates cell proliferation and apoptosis and the neurologic defects of *Hipk2*-null mice underscores its role for proper brain functions. We show that MeCP2 cooperates with HIPK2 in induction of apoptosis and that S80-phosphorylation is required together with the DNA-binding of MeCP2. These data are thus the first describing a kinase associating with MeCP2 causing its specific phosphorylation *in vivo* and, moreover, they reinforce the role of MeCP2 in regulating cell growth.**

Keywords: HIPK2/MeCP2/Phosphorylation/Apoptosis

INTRODUCTION

The methyl CpG-binding protein MeCP2 is a ubiquitous protein whose mutations cause Rett syndrome, a devastating neurodevelopmental disorder affecting mainly females. MeCP2 binds methylated DNA and associates with different corepressor complexes thereby working as a methylation-dependent transcriptional repressor (Chahrour & Zoghbi, 2007) even if recent results suggested 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 dynamically regulate gene expression. In particular, MeCP2 mediated repression of the *Bdnf* gene was found to be reversed by MeCP2 phosphorylation at S421 causing a change in the binding affinity to the promoter (Chen *et al*, 2003). In addition, S421 phosphorylation affects MeCP2’s ability to regulate dendritic growth and spine maturation (Zhou *et al*, 2006). More recently, neuronal activity was found to trigger dephosphorylation at S80 decreasing MeCP2 binding to some of its target promoters (Tao *et al*, 2009). Interestingly, *Mecp2S80A* and *Mecp2S421A;S421A* knock-in mice, carrying non-phosphorylatable MeCP2 mutations, showed altered locomotor activities (Tao *et al*, 2009), highlighting the relevance of these modifications in neurological functions. Besides S80 and S421, a number of additional residues were found phosphorylated in brain, further supporting the idea that phosphorylation might strongly influence MeCP2 activities. Whereas S421 is phosphorylated by a CaMKII/IV-dependent mechanism, the upstream events causing the phosphorylation of MeCP2 at S80 remain unknown.

We identified HIPK2 as a novel 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 MeCP2 and specifically phosphorylates it at S80 *in vitro* and *in vivo*.

RESULTS AND DISCUSSION

**HIPK2 is a novel MeCP2-interacting protein**

In a yeast two-hybrid screen using as bait the transcriptional repression domain (TRD) and the upstream linker region of MeCP2 fused to the GAL4 DNA-binding domain (DBD-linkTRD) we found the C-terminus of HIPK2 as 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). GST pull-down experiments with recombinant proteins confirmed the interaction, suggesting that it might be direct (data not shown).

To evaluate if the two proteins associate in mammalian cells, we expressed Flag-MeCP2 and EGFP-HIPK2 in HEK293T cells and immunoprecipitated total cell extracts (TCEs) with anti-green fluorescent protein (GFP) antibodies (Abs). Flag-MeCP2 coprecipitates with EGFP-HIPK2 but not with EGFP alone (Fig 1B). The reciprocal experiment was performed in *Mecp2-*null mouse embryo 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) where either anti-MeCP2 or anti-HIPK2 Abs, but not an unrelated rabbit-IgG, were able to coprecipitate 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 if 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-[33P]-ATP. Wild-type (WT) HIPK2 but not the KD derivative phosphorylated both itself and MeCP2 while Western blot (WB) confirmed that both WT and KD HIPK2 are efficiently immunoprecipitated and that, as expected, the KD has a faster mobility than WT HIPK2 (Fig 2A).

To identify the specific HIPK2 target residue/s within MeCP2, we first mapped the region modified by the kinase. Considering that the linkerTRD of MeCP2 contacts HIPK2, we performed the kinase assays employing two deletion derivatives that retain the interaction surface. Only the C-terminal region (198-486) was significantly less phosphorylated compared to WT MeCP2, indicating that the N-terminus of MeCP2 or the MBD contains the main HIPK2 phosphorylation site(s) (Fig 2B).

**MeCP2-S80 is the specific target of HIPK2**

The region within MeCP2 targeted by HIPK2 contains three of the total 11 S/T-P sites constituting possible HIPK2 targets (Fig 3A). Thus, we first tested two MeCP2 derivatives in which two or three N-terminal serine residues were substituted with alanine as HIPK2 substrates (Fig 3A). Two of the mutated residues (S80, S164) are putative HIPK2 targets whereas the third (S229), within the TRD, had already been excluded as substrate in the above experiments. Since comparable levels of phosphorylation were obtained with WT MeCP2 and the double mutant (S164,229A), we could exclude S164 as an HIPK2 target. In contrast, a dramatic reduction in MeCP2 phosphorylation was observed with the triple mutant (S80,164,229A). This differs from the first by having eliminated also the S80 phosphorylation site suggesting that this residue is an HIPK2 target. Since the substitution of only S80 with alanine also reduces significantly MeCP2 phosphorylation this residue appears, at least *in vitro*, to be the major HIPK2 target. This was further confirmed on *in vitro* phosphorylated MeCP2 by immunoblotting with an anti-MeCP2 S80 phospho-site-specific Ab (anti-S80P) whose specificity 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 if S80 is an HIPK2 target also *in vivo,* we immunoprecipitated endogenous MeCP2 from HFs transiently transfected with WT or KD EGFP-HIPK2 or EGFP alone. Immunocomplexes were resolved on SDS-PAGE, blotted, and immunoreacted with anti-MeCP2 or anti-S80P Abs. 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 anti-S80P Abs indicating that HIPK2 can phosphorylate endogenous MeCP2 at S80 *in vivo*. As indicated by the empty vector control, MeCP2 is weakly phosphorylated at S80 in HFs. In contrast, neuronal cells show higher MeCP2 phosphorylation at S80 (Tao et al, 2009 and Fig 3D) and, in our experimental conditions, further phosphorylation upon ectopic HIPK2 expression could not be detected by our Ab (Fig. 4B and data not shown). Thus, to confirm the causal role of HIPK2 in MeCP2-S80 phosphorylation *in vivo*, we employed 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 anti-S80P Ab was observed in both cell types upon HIPK2 depletion although, as previously described for other cells (Iacovelli *et al*, 2009), HIPK2 expression could only be partially depleted (Fig 3C and 3D).

Altogether, these data depict HIPK2 as a kinase capable of phosphorylating MeCP2 *in vivo* through direct complex formation. Other two kinases have been reported to be involved in MeCP2 phosphorylation. Of these, CaMKII and/or IV likely mediate the activity-dependent phosphorylation of S421 in neurons but since 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). CDKL5, the other kinase, is capable of associating with MeCP2 and promoting its phosphorylation *in vitro* (Mari *et al*, 2005). In this case, however, the specific target residue has not been mapped and it is still not clear 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 different cell lines (i.e., HFs, NIH3T3 fibroblasts, MEFs, and neuron differentiated P19 cells) and assessed cell survival. Interestingly, 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 and 4B). When both proteins were expressed together, the number of dead cells increased in an additive manner and this additive effect depends on the catalytic activity of HIPK2 since coexpression of MeCP2 and the KD mutant induced a level of cell death similar to that of MeCP2 alone. Comparable results were obtained with each tested cell line by either Trypan blue exclusion or TUNEL assays (data not shown) suggesting that the MeCP2-induced cell death is apoptotic like that induced by HIPK2.

Since HIPK2 phosphorylates MeCP2, we tested the possibility that MeCP2 works downstream HIPK2 in the induction of cell death and therefore assessed cell viability of *Mecp2*-WT and-null MEFs upon expression of WT or KD HIPK2. HIPK2-mediated cell death was reduced in the *Mecp2*-null MEFs compared to 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.

Since 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, unable to bind methylated DNA (Yusufzai *et al*, 2000), was unable to restore cell death levels (Fig 4D). Additionally, when HIPK2 was expressed with the non DNA-binding mutant, not only the additive effect in inducing apoptosis was lost but also a reduction in the pro-apoptotic 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 suggests 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 growth of prostate cancer cells (Bernard *et al*, 2006). In these cells, MeCP2 depletion results in inhibition of proliferation while MeCP2 overexpression confers a growth advantage allowing these androgen-dependent tumor cells to proliferate in the absence of the hormone. Interestingly, we could confirm the reduced proliferation rate in our *Mecp2*-null MEFs that have a mean doubling time of 39 hrs compared to 24 hrs of the WT MEFs (data not shown). However, the apoptotic effect induced by MeCP2, even without coexpression of HIPK2, we report here is in apparent contrast with the data obtained with prostate cancer cells. One of the major differences between our experiments and those of Bernard *et al*. is that we used only non-transformed or terminally differentiated cells, while they employed only tumor cells. We therefore tested whether tumor transformation might explain our divergent results. Surprisingly, in tumor cells (i.e., HeLa, RKO, U2OS, or spontaneously transformed NIH-3T3 cells), we found neither induction of cell death by MeCP2 overexpression nor the additive effect by its expression with HIPK2 (data not shown); this suggests that the divergent response to MeCP2 overexpression might depend on tumor transformation. However, the molecular basis of this divergence is currently unknown.

**S80 phosphorylation contributes to apoptosis**

We next addressed if phosphorylation at S80 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 substitution at S164 and S178 did not significantly alter the capacity of MeCP2 to induce cell death either alone or with HIPK2. By contrast, the 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, it did not show any additive effect either in MEFs (Fig 5A) or in neuron differentiated P19 cells (Fig 5B). Thus, we tested whether the substitution of S80 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 an amount of cell death similar to that obtained by coexpressing WT MeCP2 and HIPK2. In addition, this amount was not further significantly increased by coexpression with WT HIPK2, supporting the idea that, in these conditions, MeCP2-S80 phosphorylation is mediated by HIPK2 and contributes to induction of cell death.

To conclude, we here provide strong evidence of HIPK2 as a kinase associating with MeCP2 causing its phosphorylation at S80 *in vitro* and *in vivo*; moreover, our functional studies show that the two proteins cooperate in the HIPK2-mediated apoptotic pathway in a S80-dependent manner. As described, S80 has previously been identified as one of several serine residues within MeCP2 that are phosphorylated in brain (Zhou *et al*, 2006; Tao *et al*, 2009). Accordingly, our anti-S80P antibody detect this specific phosphorylation on endogenous MeCP2 in brain extracts as well as in neuron cultures. Thus, from one side, it will be interesting to evaluate whether and which types of HIPK2 functions are related to MeCP2 phosphorylation in non-neuronal cells (e.g., DNA-damage response). From another side, given the role of MeCP2 in brain, which is best illustrated by the onset of Rett syndrome in girls with *MECP2* mutations, it will be interesting to analyze 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 *Mecp2S80A* knock-in mice in which MeCP2 cannot be phoshorylated at S80 (Tao *et al*, 2009). Eventually, our unpublished results indicate a strong overlap between MeCP2 and HIPK2 expression in brain, underscoring the possibility that they belong to the same molecular pathway also in this organ. Therefore it will be challenging to analyze whether HIPK2 contributes to MeCP2-S80 phosphorylation in brain and whether it might be involved in the pathogenesis of the Rett syndrome.

MATERIALS AND METHODS

**Plasmids and reagents.** For the yeast two-hybrid screen the linkerTRD region (aa 163-311) of hMeCP2 fused with the GAL4-DBD (pGBKT7) was expressed in the yeast strain AH109 with a mouse embryo d11 cDNA library (Clontech). The preparation and source of other plasmids, Abs 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-[33P]-ATP essentially as described (Bertani *et al*, 2006). Labeled proteins were separated by SDS-PAGE, transferred to membranes, visualized by autoradiography or by PhosphorImager analysis (GE Healthcare) and immunoblotted.

**siRNA interference.** HIPK2-specific (HIPK2i) and universal negative control (UNC) siRNA were: HIPK2i stealth RNAi sequences (a mix of 3 different sequences) and stealth RNAi Negative, Medium GC Duplexes, respectively (all from Invitrogen). 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>).

ACKNOWLEDGEMENTS

The financial support of National Institute of Health NS048276 (MEG), NS058391 (ZZ), Italy-USA Program for Rare Diseases (SS), Associazione Italiana per la Ricerca sul Cancro (AIRC; NL), Rett Syndrome Research Foundation (RSRF; CKN), Telethon (NL), Fondazione Cariplo (NL), the E-Rare EuroRETT network (NL, CKN, SS) are gratefully acknowledged. We also thank all the people cited for providing us with cells, reagents, expression vectors and, finally, we thank ProRETT Ricerca for their continuous and encouraging trust in the laboratory.

**The authors declare that they have no conflict of interest.**

REFERENCES

Bernard D, Gil J, Dumont P, Rizzo S, Monté D, Quatannens B, Hudson D, Visakorpi T, Fuks F, de Launoit Y (2006) [The methyl-CpG-binding protein MECP2 is required for prostate cancer cell growth.](http://www.ncbi.nlm.nih.gov/pubmed/16331274?ordinalpos=2&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DefaultReportPanel.Pubmed_RVDocSum) *Oncogene* **25**:1358-1366

Bertani I, Rusconi L, Bolognese F, Forlani G, Conca B, De Monte L, Badaracco G, Landsberger N, Kilstrup-Nielsen C (2006) [Functional consequences of mutations in CDKL5, an X-linked gene involved in infantile spasms and mental retardation.](http://www.ncbi.nlm.nih.gov/pubmed/16935860?ordinalpos=2&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DefaultReportPanel.Pubmed_RVDocSum) *J Biol Chem* **281**:32048-32056

Calzado MA, Renner F, Roscic A, Schmitz ML (2007) [HIPK2: a versatile switchboard regulating the transcription machinery and cell death.](http://www.ncbi.nlm.nih.gov/pubmed/17245128?ordinalpos=2&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DefaultReportPanel.Pubmed_RVDocSum) *Cell Cycle* **6**:139-143

Chahrour M, Zoghbi HY (2007) [The story of Rett syndrome: from clinic to neurobiology.](http://www.ncbi.nlm.nih.gov/pubmed/17988628?ordinalpos=2&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DefaultReportPanel.Pubmed_RVDocSum) *Neuron* **56**:422-437

Chahrour M, Jung SY, Shaw C, Zhou X, Wong ST, Qin J, Zoghbi HY (2008) [MeCP2, a key contributor to neurological disease, activates and represses transcription.](http://www.ncbi.nlm.nih.gov/pubmed/18511691?ordinalpos=1&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DefaultReportPanel.Pubmed_RVDocSum) *Science* **320**:1224-1229

Chen WG, Chang Q, Lin Y, Meissner A, West AE, Griffith EC, Jaenisch R, Greenberg ME (2003) [Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2.](http://www.ncbi.nlm.nih.gov/pubmed/14593183?ordinalpos=13&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DefaultReportPanel.Pubmed_RVDocSum) *Science* **302**:885-889

Iacovelli S, Ciuffini L, Lazzari C, Bracaglia G, Rinaldo C, Prodosmo A, Bartolazzi A, Sacchi A, Soddu S (2009) HIPK2 is involved in cell cycle regulation and its down-regulation promotes growth arrest independently from DNA damage. *Cell proliferation* **42**:737-84

Isono K, Nemoto K, Li Y, Takada Y, Suzuki R, Katsuki M, Nakagawara A, Koseki H (2006) [Overlapping roles for homeodomain-interacting protein kinases hipk1 and hipk2 in the mediation of cell growth in response to morphogenetic and genotoxic signals.](http://www.ncbi.nlm.nih.gov/pubmed/16537918?ordinalpos=2&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DefaultReportPanel.Pubmed_RVDocSum) *Mol Cell Biol* **26**:2758-2771

Latella L, Sacco A, Pajalunga D, Tiainen M, Macera D, D'Angelo M, Felici A, Sacchi A, Crescenzi M (2001) Reconstitution of cyclin D1-associated kinase activity drives terminally differentiated cells into the cell cycle. *Mol Cell Biol* **21**:5631-5643

Mari F, Azimonti S, Bertani I, Bolognese F, Colombo E, Caselli R, Scala E, Longo I, Grosso S, Pescucci C, Ariani F, Hayek G, Balestri P, Bergo A, Badaracco G, Zappella M, Broccoli V, Renieri A, Kilstrup-Nielsen C, Landsberger N (2005) [CDKL5 belongs to the same molecular pathway of MeCP2 and it is responsible for the early-onset seizure variant of Rett syndrome.](http://www.ncbi.nlm.nih.gov/pubmed/15917271?ordinalpos=2&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DefaultReportPanel.Pubmed_RVDocSum) *Hum Mol Genet* **14**:1935-1946

Rinaldo C, Prodosmo A, Siepi F, Soddu S (2007) [HIPK2: a multitalented partner for transcription factors in DNA damage response and development.](http://www.ncbi.nlm.nih.gov/pubmed/17713576?ordinalpos=1&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DefaultReportPanel.Pubmed_RVDocSum) *Biochem Cell Biol* **85**:411-418

Tao J, Hu K, Chang Q, Wu H, Sherman NE, Martinowich K, Klose RJ, Schanen C, Jaenisch R, Wang W, Sun YE (2009) Phosphorylation of MeCP2 at Serine 80 regulates its chromatin association and neurological function. *PNAS* **106**:4882-4887.

[Wiggins AK](http://www.ncbi.nlm.nih.gov/sites/entrez?Db=pubmed&Cmd=Search&Term=%22Wiggins%20AK%22%5BAuthor%5D&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DiscoveryPanel.Pubmed_RVAbstractPlus), [Wei G](http://www.ncbi.nlm.nih.gov/sites/entrez?Db=pubmed&Cmd=Search&Term=%22Wei%20G%22%5BAuthor%5D&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DiscoveryPanel.Pubmed_RVAbstractPlus), [Doxakis E](http://www.ncbi.nlm.nih.gov/sites/entrez?Db=pubmed&Cmd=Search&Term=%22Doxakis%20E%22%5BAuthor%5D&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DiscoveryPanel.Pubmed_RVAbstractPlus), [Wong C](http://www.ncbi.nlm.nih.gov/sites/entrez?Db=pubmed&Cmd=Search&Term=%22Wong%20C%22%5BAuthor%5D&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DiscoveryPanel.Pubmed_RVAbstractPlus), [Tang AA](http://www.ncbi.nlm.nih.gov/sites/entrez?Db=pubmed&Cmd=Search&Term=%22Tang%20AA%22%5BAuthor%5D&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DiscoveryPanel.Pubmed_RVAbstractPlus), [Zang K](http://www.ncbi.nlm.nih.gov/sites/entrez?Db=pubmed&Cmd=Search&Term=%22Zang%20K%22%5BAuthor%5D&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DiscoveryPanel.Pubmed_RVAbstractPlus), [Luo EJ](http://www.ncbi.nlm.nih.gov/sites/entrez?Db=pubmed&Cmd=Search&Term=%22Luo%20EJ%22%5BAuthor%5D&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DiscoveryPanel.Pubmed_RVAbstractPlus), [Neve RL](http://www.ncbi.nlm.nih.gov/sites/entrez?Db=pubmed&Cmd=Search&Term=%22Neve%20RL%22%5BAuthor%5D&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DiscoveryPanel.Pubmed_RVAbstractPlus), [Reichardt LF](http://www.ncbi.nlm.nih.gov/sites/entrez?Db=pubmed&Cmd=Search&Term=%22Reichardt%20LF%22%5BAuthor%5D&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DiscoveryPanel.Pubmed_RVAbstractPlus), [Huang EJ](http://www.ncbi.nlm.nih.gov/sites/entrez?Db=pubmed&Cmd=Search&Term=%22Huang%20EJ%22%5BAuthor%5D&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DiscoveryPanel.Pubmed_RVAbstractPlus) (2004) Interaction of Brn3a and HIPK2 mediates transcriptional repression of sensory neuron survival. [*J Cell Biol*](javascript:AL_get(this,%20'jour',%20'J%20Cell%20Biol.');) **167**:257-267

Yusufzai TM, Wolffe AP (2000) [Functional consequences of Rett syndrome mutations on human MeCP2.](http://www.ncbi.nlm.nih.gov/pubmed/11058114?ordinalpos=1&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DefaultReportPanel.Pubmed_RVDocSum) *Nucleic Acids Res* **28**:4172-4179

Zhang J, Pho V, Bonasera SJ, Holtzman J, Tang AT, Hellmuth J, Tang S, Janak PH, Tecott LH, Huang EJ (2007) [Essential function of HIPK2 in TGFbeta-dependent survival of midbrain dopamine neurons.](http://www.ncbi.nlm.nih.gov/pubmed/17159989?ordinalpos=3&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DefaultReportPanel.Pubmed_RVDocSum) *Nat Neurosci* **10**:77-86

Zhou Z, Hong EJ, Cohen S, Zhao WN, Ho HY, Schmidt L, Chen WG, Lin Y, Savner E, Griffith EC, Hu L, Steen JA, Weitz CJ, Greenberg ME (2006) [Brain-specific phosphorylation of MeCP2 regulates activity-dependent Bdnf transcription, dendritic growth, and spine maturation.](http://www.ncbi.nlm.nih.gov/pubmed/17046689?ordinalpos=4&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DefaultReportPanel.Pubmed_RVDocSum) *Neuron* **52**:255-269

FIGURE LEGENDS

**Fig 1** HIPK2 interacts with MeCP2. (**A**) Schematic representation of hMeCP2 and mHIPK2. Black bars indicate the linkTRD of hMeCP2 (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 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 with anti-GFP Ab and analyzed by WB as indicated. The asterisk \* indicates a non-specific band (**C**) TCEs from *Mecp2-*null MEFs expressing the indicated proteins were immunoprecipitated with anti-MeCP2 Ab; inputs corresponding to 10% of TCEs and immunocomplexes were analyzed by WB as indicated. (**D**) Endogenous HIPK2 and MeCP2 were immunoprecipitated with the indicated Abs from TCEs of HFs; inputs corresponding to 10% of TCEs and immunocomplexes were analyzed by WB with anti-MeCP2 and -HIPK2 Abs. Rabbit-IgGs were used as negative control. The asterisks \* indicate non-specific bands.

**Fig 2** HIPK2 phosphorylates the MeCP2 N-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 anti-Flag Ab and processed as follows: 90% of the immunoprecipated material was incubated with γ-[33P]-ATP and recombinant MeCP2. The labelled proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, visualized by autoradiography and subsequently immunoblotted with anti-MeCP2 Ab. 10% of the immunoprecipitated material was immunoblotted with anti-HIPK2 Ab (lower panel). The background signal equally present in the empty vector (Flag) or the KD negative control samples might be due to non-specifically precipitated cellular kinases. (**B**) *In vitro* kinase assay was performed as in (A) with the indicated MeCP2 deletion derivatives. Upper four panels: autoradiogram; lower panel: WB. The asterisk \* indicates a non-specific band.

**Fig 3** MeCP2 is specifically phosphorylated at S80 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 (-[33P]-MeCP2) and WB with the indicated Abs are reported. S/T–P sites are represented by lollipops; white lollipops fall within the region modified by HIPK2 whereas black lollipops lie outside; crossed lollipops are S to A substitutions. (**B**) HFs were transfected with the indicated EGFP-tagged proteins or the empty vector (EGFP alone). TCEs were immunoprecipitated with anti-MeCP2 Ab and analyzed by WB with the indicated Abs. TCEs (Inputs) were also analyzed by direct WB with the indicated Abs using actin as 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 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 hrs post-transfection. (**D**) Primary cortical neurons were transfected with stealth siRNA duplex at day 7 and analyzed as in C at day 11. -tubulin was used as loading control.

**Fig 4** MeCP2 contributes to HIPK2-induced apoptosis. (**A**) HFs expressing Flag-MeCP2 with EGFP-HIPK2 or EGFP-K221R were analyzed 24h post-transfection for cell death by Trypan blue exclusion. Mean ± standard deviation (± SD) of three independent experiments is reported. WBs on TCE (HIPK2, actin) or IPs (MeCP2, S80P) were performed. (**B**) P19 cells were induced to differentiate as in Fig 3D, transfected, and TCEs analyzed by WB as indicated. (**C**) *Mecp2*-WT and-null MEFs were transfected with EGFP-HIPK2, EGFP-K221R, or the EGFP control vector. Cell death was evaluated as in (A) at the indicated time points. Mean ± SD of three experiments is reported. (**D**) *Mecp2*-WT and -null MEFs were transfected with the indicated proteins and analyzed 40h post-transfection for cell death and protein expression.

**Fig 5** MeCP2 phosphorylation at S80 contributes to cell death. (**A**) *Mecp2*-null MEFs were co-transfected with EGFP or EGFP-HIPK2 and Flag-tagged WT MeCP2 or its indicated derivatives and analyzed 40h post-transfection for cell death. Mean ± SD of three experiments is reported. TCEs were analyzed by WB as indicated using actin as loading control. (**B**) P19 cells were induced to differentiate as in Fig 3Dand co-transfected with EGFP or EGFP-HIPK2 and MeCP2 or its indicated derivatives. Cell death and protein expression were analyzed. The dashed lines indicate cell death levels induced by HIPK2 alone.