Collapsin response mediator protein 3 deacetylates histone H4 to mediate nuclear condensation and neuronal death

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CRMP proteins play critical regulatory roles during semaphorin-mediated neurite outgrowth, neuronal differentiation and death. Albeit having a high degree of structure and sequence resemblance to that of liver dihydropyrimidinase, purified rodent brain CRMPs do not hydrolyze dihydropyrimidinase substrates. Here we found that mouse CRMP3 has robust histone H4 deacetylase activity. During excitotoxicity-induced mouse neuronal death, calpain-cleaved, N-terminally truncated CRMP3 undergoes nuclear translocation to cause nuclear condensation through deacetylation of histone H4. CRMP3-mediated deacetylation of H4 leads to de-repression of the E2F1 gene transcription and E2F1-dependent neuronal death. These studies revealed a novel mechanism of CRMP3 in neuronal death. Together with previous well established bodies of literature that inhibition of histone deacetylase activity provides neuroprotection, we envisage that inhibition of CRMP3 may represent a novel therapeutic approach towards excitotoxicity-induced neuronal death.

Collapsin response mediator proteins (CRMPs), consisting of five highly related members (CRMP-1 to 5), are homologues of Unc33 whose mutation in Caenorhabditis elegans causes an ubiquitous impairment in the formation of neural circuits and severely uncoordinated locomotion1,2. During rodent development, CRMPs are involved in neurite path-finding in response to semaphorin-mediated growth cone collapse through neuropilin receptors and neuropilin co-receptors3–6. Activated by the upstream serine/threonine protein kinases, CRMPs directly modulate the integrity of cytoskeletons to affect growth cone morphology and neurite outgrowth7,8.

CRMP2 is the most intensely studied amongst all CRMPs. Expressed abundantly in growth cones and distal parts of growing axons, CRMP2 modulates neurite length through direct binding to tubulin and promoting microtubule polymerization9,10. Over-expression of CRMP2 promotes neurite genesis in cultured hippocampal neurons8. In addition to regulating microtubule assembly, CRMP2 is also involved in modifying actin filaments, microtubule cytoplasmic flow and polarized Numb-mediated endocytosis of proteins such as L111. CRMP4 has also been shown to promote F-actin bundling in vitro12. The in vivo functions of the CRMP family members remain less clear. Recent genetic studies showed that CRMP1 regulates neuronal migration by mediating reelin signaling. Deletion of CRMP1 during mouse development caused severe retardation of radial migration13, impaired long-term potentiation and impaired spatial learning and memory14. Similarly, deletion of CRMP3 also negatively affects hippocampal CA1 dendritic organization and plasticity15, suggesting that CRMPs are important in maintaining cognitive functions.

More recent works, including those from our laboratory, showed that CRMP proteins participate in adult brain injury response leading to neuronal death16,17. Calpain cleavage of CRMP3 produces a truncated fragment with a molecular weight of 54 kD (hence p54) which translocates into the nucleus to modulate neuronal death both during glutamate excitotoxicity in vitro and cerebral ischemic in vivo16–18. However, how CRMP proteins participate in these processes remains unclear.

Ever since their discovery, CRMP proteins were believed to have no enzymatic activities and function as regulatory proteins. Albeit having a high degree of structure and sequence resemblance to that of liver dihydropyrimidinase, purified rodent brain CRMPs do not hydrolyze dihydropyrimidinase substrates. Here we found that mouse CRMP3 has robust histone H4 deacetylase activity. During excitotoxicity-induced mouse neuronal death, calpain-cleaved, N-terminally truncated CRMP3 undergoes nuclear translocation to cause nuclear condensation through deacetylation of histone H4. CRMP3-mediated deacetylation of H4 leads to de-repression of the E2F1 gene transcription and E2F1-dependent neuronal death. These studies revealed a novel mechanism of CRMP3 in neuronal death. Together with previous well established bodies of literature that inhibition of histone deacetylase activity provides neuroprotection, we envisage that inhibition of CRMP3 may represent a novel therapeutic approach towards excitotoxicity-induced neuronal death.
dihydropyrimidinase (DHPase), purified brain CRMPs do not hydrolyze DHPase substrates\(^1\)\(^9\)\(^,\)\(^2\), leading to the widely held belief that CRMP proteins have acquired a regulatory function instead\(^3\)\(^,\)\(^9\)\(^,\)\(^2\). Surprisingly, we found that the CRMP3 protein has robust histone H4 deacetylase (HDAC) activities. During excitotoxicity-induced mouse neuronal death, calpain-cleaved, N-terminally truncated CRMP3 undergoes nuclear translocation to cause deacetylation of histone H4, nuclear condensation and death. Interestingly, we found nuclear CRMP3 modulates the expression of the transcription factor E2F1, serving as a potential mechanism for CRMP3-induced neuronal death.

**Results**

CRMP3 has histone deacetylase (HDAC) activity. To understand how CRMP proteins function, we compared the peptide sequences of CRMP1-5 using SBASE (http://hydra.icgeb.trieste.it/sbase/), and found that all CRMPs shared a highly conserved domain with predicted structural homology to the amidohydrolase family (80–100% amongst CRMPs, Fig. 1a)\(^2\). The cyclic amidohydrolase superfamily of enzymes, including hydantoinase, DHPase, allantoinase, dihydroorotase and histone deacetylase (HDAC), play a crucial role in the metabolism of purine and pyrimidine in prokaryotic and eukaryotic cells\(^2\). We also reported that during excitotoxicity-induced neuronal death in vitro and in vivo, the full-length CRMP3 was N-terminally cleaved by calcium-activated calpain to produce a truncated molecule, p54 (54 kD). Truncated CRMP3 (p54) underwent nuclear translocation through nuclear pores to interact with chromatin-bound proteins causing nuclear condensation and neuronal death\(^1\)^\(^7\)^\(^,\)\(^1\)^\(^8\).

**Figure 1** | CRMP3 has histone deacetylase activity. (a) A schematic representation of cDNA constructs of the full-length CRMP3, p54, D domain and the EGFP vector. Crosshatch-filled bar areas represent structural homology to amidohydrolase family member DHPase (D domain). Solid black-filled areas represent homology to ankyrin repeat. (b) cDNAs encoding these domains were subcloned into the pEGFP-C1 expression vector and expressed as EGFP fusion proteins. Determination of CRMP3 HDAC activity using a cell-free fluorimetric activity assay. The CRMP3 expression plasmid was transfected into HEK293 cells in the presence or absence of TSA (1 \(\mu\)M) for 24 h before collection of total cell lysates or the nuclear fractions (see Method section). CRMP3 was affinity-purified using an EGFP antibody. A cell-free fluorimetric activity assay with histone-derived fluorogenic substrate was used to determine HDAC activity. (c) Purified full-length CRMP3, p54 and D domain proteins exhibited significantly increased HDAC activity in comparison with EGFP, and which was completely inhibited by the addition of TSA (1 \(\mu\)M). (d) Nuclear fractions from p54 and D domain transfected HEK293 cells showed significantly increased HDAC activities compared to EGFP transfected nuclei. As the full-length CRMP3 was excluded from entering the nucleus, therefore no HDAC activity was present in the nuclear fraction. Two-way ANOVA was used with Tukey’s post hoc analysis in (c) and (d) to determine statistical significance with ** indicating \(p < 0.01\) compared to the EGFP group.
To investigate the properties of CRMP3 and its cleaved products, recombinant cDNAs encompassing the full-length CRMP3, p54 and the conserved DH-Pase domain (D domain) were made (Fig. 1a). The expressed proteins were separated on a SDS-PAGE and probed with an antibody to EGFP (Fig. 1b). Since HEK293 cells do not have endogenous CRMP3 \( ^3 \), the expression vector was transfected into HEK293 cells in the presence or absence of a histone deacetylase inhibitor, Trichostatin A (TSA). The HDAC activities of affinity-purified CRMP3, p54 and D domain from HEK293 cells were quantified using a cell-free fluorescent assay with a substrate derived from histones. As shown in Fig. 1(c and d), purified full-length CRMP3 had the strongest HDAC activity, followed by that from the D domain and p54. EGFP protein served as a negative control. TSA (1 \( \mu \text{M} \)) completely inhibited CRMP3 HDAC activities. The C-terminal fragment of CRMP3 covering the Ankrin repeat domain (see Fig. 1a) did not show HDAC activity (not shown). Because our previous studies showed that the full-length CRMP3 was only localized in the cytoplasm, while p54 and the D domain were mostly localized in the nucleus (cf. Figs. 5 and 6), we therefore isolated the nuclei of CRMP3 cDNA transfected in HEK293 cell. HDAC activities were assessed using the same cell-free fluorescent assay. Indeed, only p54 and D domain transfected nuclei appeared to have significant HDAC activities compared to the full-length CRMP3 and EGFP-transfected nuclei (Fig. 2b), supporting the hypothesis that nuclear p54 has HDAC activity.

Interestingly, CRMP3, p54 and D domain expression in HEK293 cells resulted in a selective and reversible deacetylation of histone H4 (Fig. 2a), while the addition of TSA restored the level of acetylation of histone H4 due to CRMP3 expression. Furthermore, CRMP3 only deacetylated histone H4, but not H2A, H2B and H3 (Fig. 2), which was further supported by the fact that affinity-purified CRMP3 was also effective in deacetylating H4 in an in vitro test tube reaction (Fig. 2b), while HDAC6 was effective in deacetylating all histones (Fig. 2b).

In order to eliminate the possibility that contaminating proteins from the host HEK293 cells contributed to the CRMP3 HDAC activity seen in the cell-free fluorescent assay, we determined HDAC activity derived from CRMP3 proteins expressed using a heterologous expression system, i.e. Sf9 insect cells. Recombinant cDNA was inserted into the pIEx-1 expression vector with an N-terminal His-tag (\( \times 6 \) Histidine) and a S-tag (Fig. 3a). Expressed proteins were confirmed as shown in Figure 3b. An antibody to CRMP3 detected the full-length CRMP3 and p54, while an antibody to S-tag protein detected all three constructs, except the empty vector serving as a negative control. His-tag affinity-purified proteins from Sf9 cells were used in the cell-free fluorescent assay (Fig. 3c, d). CRMP3 full-length protein, p54 and D domain all showed significantly increased HDAC activities in a dose-dependent manner, while addition of TSA (1 \( \mu \text{M} \)) to the reaction completely inhibited CRMP3 HDAC activities (Fig. 3c, d).

CRMP3 protein dose-dependently deacetylates histone H4, but not tubulin. CRMP3 HDAC activity was further confirmed in an in vitro reaction and the immunoblotting (Fig. 4). Affinity-purified, Sf9 cell expressed CRMP3 proteins were incubated with HEK293 cell extract in the presence or absence of TSA. HDAC 6 protein served as a positive control (Fig. 4a). The full-length CRMP3, p54 and D domain proteins dose-dependently reduced the level of acetylated histone H4 (Fig. 4a–c), and such deacetylation of histone H4 was completely reversed by TSA (1 \( \mu \text{M} \)). In contrast, CRMP4, another member of the CRMP family, did not deacetylate histone H4 (Fig. 4b, c). Since the CRMP proteins modulate microtubules \( ^{33} \), we also examined whether CRMP3 deacetylates tubulin. As shown in Fig. 4(a and b), although HDAC6 reversibly deacetylated tubulin, serving as a positive control, the full-length CRMP3, p54, D domain and CRMP4 proteins did not have any effect on tubulin deacetylation, suggesting that CRMP modulation of microtubule changes may be independent of reversible acetylation of tubulins. This is further supported by the fact that adding TSA, a general inhibitor of HDACs, to cultured mouse cortical explants did not prevent neurite outgrowth as measured by immunostaining of tubulins (ref to Fig. 8 f and g).

Direct association of p54 with histone H4 in the nucleus. When CRMP3 and p54 were coexpressed in HEK293 cells, the full-length CRMP3 was localized exclusively in the cytoplasm (Fig. 5a–d), while p54 was predominantly associated with condensed nuclei \( ^{17,21} \) (Fig. 5c–h). EGFP was found to be expressed everywhere in the cell (Fig. 5i–l). Histone H4 was also exclusively expressed in the nuclei (not shown). Furthermore, p54 expressing cells appeared positive to propidium iodide (PI) staining indicating p54-mediated cell death (arrowhead in Fig. 5f). Adding TSA (1 \( \mu \text{M} \)) prevented p54-induced nuclear condensation (Fig. 5h) and reduced nuclear accumulation of p54 proteins (Fig. 5h), lending further support for a function of p54 in mediating nuclear condensation through HDAC activities. The number of condensed and PI positive nuclei were counted and plotted in Fig. 5(m and n) to indicate p54-induced cell death.

Immunoprecipitation pull-down with EGFP antibody, protein G beads of total cell lysate transfected with CRMP3 cDNA constructs and Western blotting against histone H4 showed that p54 protein was associated with histone H4 (Fig. 5o). Sub-cellular fractionation of p54 cDNA transfected HEK293 cells was performed to generate cytoplasm and nuclear fractions which were further separated into soluble and non-soluble nuclear fractions (solubilized using sonication) \( ^31 \). EGFP antibody pull-down and Western blotting against histone H4 showed that p54 was directly associated with histone H4 in the non-soluble nuclear fraction (Fig. 5p). Nuclear association of p54 with histone H4 was further confirmed using mass spectrometry analysis. The non–soluble nuclear fraction was subjected to EGFP pull-down, separation on a 10% native polyacrylamide gel and silver staining. Differentially–expressed protein bands from p54 transfected cells, when compared with those derived from the non-soluble...
nuclear fraction of HEK293 cells transfected with the full-length CRMP3, EGFP vector and the non-treated HEK293 cells, were cut out and subjected to mass spectrometry analysis (nanoHPLC-MS/MS Q-TOF) to identify p54 interacting proteins. We identified several putative p54 interacting nuclear proteins such as histone H4 (data not shown), confirming direct interaction of p54 with histone H4. Collectively, these studies strongly suggest that p54, the N-terminal cleaved product of CRMP3, is structurally amenable for nuclear translocation to deacetylate histone H4 through direct association.

To determine whether CRMP3 HDAC activity is involved in neuronal death, CRMP3 expression was examined in glutamate-treated cultured cortical neurons (Fig. 6a–j) and in mouse brain samples subjected to 1 h middle cerebral artery occlusion (MCAO) and 24 h reperfusion (Fig. 6k and l). Increased CRMP3 expression was clearly associated with condensed nuclei in damaged neurons (arrows in Fig. 6b, l), which were also TUNEL positive (Fig. 6e), confirming our previous reports. The significant increase in nuclear localization of CRMP3 under glutamate toxicity or MCAO was quantified using Image J (Fig. 6m). The presence of nuclear p54 was determined using sub-cellular fractionation and Western blotting, which showed that only p54 was present in the nuclear fraction following glutamate toxicity and MCAO (Fig. 6n–p), confirming our previous reports.

CRMP3 modulates E2F1 expression. Our laboratory has previously showed that glutamate-induced neuronal death requires the expression of E2F1. Other studies showed the HDAC activity constitutively represses the E2F1 gene in mature neurons in order

Figure 3 | CRMP3 proteins expressed in Sf9 insect cells have HDAC activity. (a) cDNAs encoding the full-length CRMP3, p54 and D domain were subcloned into an insect expression plasmid vector, which has a His tag (×6 Histidine) and a S-tag to the N-terminus. Crosshatch-filled bar areas represent structural homology to the amidohydrolase family member DHPase (D domain). Solid black-filled areas represent homology to the ankyrin repeat. (b) Expressed fusion proteins were detected using Western blotting against CRMP3 (left panel, lanes 1–4) and an antibody to the S-tag (right panel, lanes 5–8). The CRMP3 antibody does not recognize the D domain fragment, so no band was visible on lane 3. Cells transfected with the empty vector showed no expression of CRMP3 (lanes 4 and 8). (c) Ni affinity column-purified full-length CRMP3, p54 and the D domain proteins showed a significant increase in HDAC activities which were completely inhibited by the addition of TSA (1 μM) in the cell-free fluorimetric assay. Hela cell nuclear extract was used as a positive control. (d) Dose-dependent increase in HDAC activities from affinity-purified full-length CRMP3, p54 and D domain proteins as determined using the cell-free fluorimetric assay. Two-way ANOVA was used with Tukey’s post hoc analysis in (c) and (d) to determine statistical significance with ** indicating p < 0.01 and * indicating p < 0.05.
transfected cells with glutamate (50 μM) for 2 and 4 h elicited a clear activation of E2F1 promoter activity (Luciferase assay) at 4 h treatment in comparison with those cells transfected with the empty control plasmid vector (pGL3 plasmid).

To determine if CRMP3 expression affects E2F1 expression, cortical neurons were transfected with the CRMP3 domain specific plasmids (Fig. 7c). Over-expression of p54 and D domain clearly increased neuronal expression of E2F1 (Fig. 7c and d’), while adding TSA (1 μM) completely inhibited E2F1 expression. These data strongly support the hypothesis that CRMP3 may directly or indirectly modulate E2F1 promoter and E2F1 protein expression during neuronal death. To see whether CRMP3 directly binds to the E2F1 promoter during neuronal death, cortical neurons, treated with glutamate for 1 and 4 h, were collected for a chromatin immunoprecipitation (ChIP) assay. As shown in Fig. 7d and d’, 4 h glutamate treatment elicited a clear increase in association of CRMP3 with the E2F1 promoter sequence, while, adding TSA inhibited CRMP3 HDAC activity and also reduced CRMP3 binding to the E2F1 promoter sequence (Fig. 7d and d’). Taken together, these experiments showed that CRMP3 translocates into the nucleus during neuronal death to augment E2F1 expression.

Figure 4 | CRMP3 protein dose-dependently deacetylates histone H4, but not tubulin. S9 insect cell-expressed, affinity-purified full-length CRMP3, p54 and the D domain proteins were co-incubated with nuclear extracts from HEK293 cells. HDAC6 protein and CRMP4 protein were also used as controls. Western blotting was used to assess the level of acetylation of histone H4 and tubulin. (a) and (b), the full-length CRMP3, p54 and D domain proteins dose-dependently deacetylated histone H4. TSA (1 μM) completely inhibited deacetylation caused by the highest tested dose of CRMP3 (16 μg). However, CRMP3 proteins did not deacetylate tubulin. Interestingly, CRMP4 exhibited no HDAC activity to histone H4 and tubulin. As a positive control, HDAC6 (5 μg/per reaction) showed complete deacetylation of histone H4 and partial (50%) deacetylation of tubulin under the same condition. Western blot band intensities, averaged from at least three independent experiments, were quantified and the percentage of intensity to the non-treated control was presented in (c).

Discussion
Here we report an unexpected finding that CRMP3 has histone H4 deacetylase enzymatic activity. During excitotoxicity-induced neuronal death, an influx of intracellular of calcium triggers calpain cleavage of the full-length CRMP3 into a N-terminally truncated p54. Nuclear translocation of p54 and its direct association with histone H4 caused reversible deacetylation of histone H4, nuclear condensation, increased E2F1 promoter activity, E2F1 expression and neuronal death (Fig. 9).

Down-regulation of CRMP3 expression increases histone H4 acetylation and protects neurons from glutamate-induced neuronal death. Down-regulation of CRMP3 expression using siRNA against CRMP3 (Fig. 8a and b) not only effectively reduced CRMP3 levels, but also resulted in increased levels of acetylated histone H4, which, again, supports the fact that CRMP3 has HDAC activities. Treating cortical neurons with glutamate (50 μM) led to increased nuclear condensation, TUNEL positivity and shortening of neurites (Fig. 8c, d, e). Neurons transfected with CRMP3 siRNA were protected against this insult (Fig. 8c, d, e). Interestingly, CRMP3 HDAC activity appears to be not essential in microtubule-mediated neurite outgrowth, as TSA treatment of cultured cortical explants did not show significant inhibition of neurite outgrowth in cultured cortical explants (Fig. 8f and g).

Collectively, we demonstrated that during excitotoxicity-induced neuronal death, intracellular influx of calcium triggers calpain cleavage of the full-length CRMP3 into a N-terminally truncated p54. Nuclear translocation of p54 and its direct association with histone H4 caused reversible deacetylation of histone H4, nuclear condensation, increased E2F1 promoter activity, E2F1 expression and neuronal death (Fig. 9).
Figure 5 | p54 directly interacts with histone H4 in the nucleus. Since HEK293 cells do not express endogenous CRMP3, we used these cells to determine p54 interaction with histone H4. EGFP-tagged full-length CRMP3 (a–d), p54 (e–h) and EGFP (i–l) were transfected into HEK293 cells (EGFP in green color) with (d, h, l) or without additional treatment using TSA. After 24 h transfection, cells were live-stained with PI (red color) to show cell death (b, f, j). Arrows indicate cells positive for CRMP protein expression. Arrowhead indicates PI positive dead cells (f). (d, h, l) TSA (1 μM) was added to transfected HEK293 cells, which clearly inhibited nuclear condensation caused by p54 expression (h). The percentage of condensed nuclei (m) and PI positive cells (n) in the treated cells were counted and plotted to indicate increased cell death caused by p54. (o) Nuclear fractions were immunoprecipitated (IP) with an antibody to EGFP and probed with antibodies to histone H4 by immunoblotting (IB). Only p54 showed co-immunoprecipitation with nuclear histone H4 in the treated cells were counted and plotted to indicate increased cell death caused by p54. (p) The nuclear fraction from p54-transfected HEK293 cells was further divided into soluble fractions and non-soluble fractions. IP was performed using an EGFP antibody and probed with antibodies to histone H4 and GAPDH. Association of p54 with histone H4 occurred in the non-soluble nuclear fraction. Scale bars = 60 μm. One-way ANOVA was used with Tukey’s post hoc analysis in (m) and (n) to determine statistical significance with ** indicating p < 0.01 compared to the p54 group.

selective HDAC activities to histone H4. Histone H4 is one of the five main histone proteins involved in the structure of chromatin in eukaryotic cells. H4 is a structural component of the nucleosome, and is subject to covalent modification, such as acetylation. Acetylation of histone H4 may alter the expression of genes located on DNA associated with its parent histone octamer. It is very interesting that the present study showed a clear selectivity in CRMP3 deacetylating histone H4. Although it is not clear of the structural basis for this selectivity, it is known that H2A, H2B H3, and histone H4 are core histones with relatively similar structures and a highly conserved feature of long ‘tails’ on one end of the amino acid structure where the location of post-translational modification, such as acetylation, is located. Future studies will be aimed at delineating the specific location where CRMP3 deacylates histone H4.

Our previous studies showed that the full-length CRMP3 undergoes cleavage at the N-terminus to produce a large fragment with a molecular weight of 54 kD. Now we show that both the full-length and cleaved product of CRMP3 have HDAC activities and that the HDAC activity comes from the D domain region. The biological functions of CRMP3 HDAC activities appear to be regulated through spatial partitioning of the full-length CRMP3 in the cytoplasm and through p54 in the nucleus. Indeed, p54 translocation into the nucleus and its association with nuclear condensation and histone H4 deacetylation clearly support this idea. Cell biology data from this study showed that nuclear p54 participates in nuclear condensation and cell death during glutamate-induced toxicity. It is very interesting to see that nuclear CRMP3 binds to the E2F1 promoter sequence. This interaction appears to be direct as the ChIP assay showed. However, it may be possible that CRMP3 interacts with the E2F1 promoter through another protein, for example, histone H4, to modulate the de-repression of the E2F1 promoter. Nevertheless, based on several previous studies, including those early reports from our own laboratory that E2F1 plays a key role in neuronal death and the fact that over-expression of CRMP3 in neurons induces E2F1 expression, it is highly possible that E2F1 is a target of nuclear CRMP3 during neuronal death. CRMP3 de-acetylates histone H4 to allow de-repression of the E2F1 gene and ultimately leads to E2F1-dependent neuronal death.

The biological function of the full-length CRMP3 in the cytoplasm remains unclear. An obvious target for the cytoplasmic localized full-length CRMP3 is the microtubule. Our in vitro experiments showed that the full-length CRMP3 does not target tubulin in acetylation. In
fact inhibition of HDAC using TSA does not affect neurite outgrowth (Fig. 8), lending further support to the argument that cytoplasmic CRMP3 does not modulate microtubules through its HDAC activity. So the full-length CRMP3 in the cytoplasm may regulate microtubules through other means such as phosphorylation as previously reported. Nevertheless, the biological role of CRMP3 HDAC activity in the cytoplasm warrants further investigation.

In summary, this is the first report that CRMP3 protein has HDAC activity. The biological role of nuclear CRMP3 is to mediate nuclear condensation during glutamate-induced excitotoxicity, possibly through up-regulation of E2F1 expression. These findings not only revealed a previously unknown function of CRMP3, but also indicated a potential therapeutic value of targeting CRMP3 in neuroprotection.
**Methods**

**Materials.** All chemicals were purchased from Sigma Aldrich Canada (Oakville, ON, Canada) unless stated otherwise. Insect Direct pEx-1 vector (Cat. Number M00046806) was purchased from EMD Chemicals (Gibbstown, NY). Taq DNA polymerase (Cat. Number 1164477), BamHI (Cat. Number 2704027), KpnI (Cat. Number 250328B), Dnase Competent Cells (Cat. Number 407878), ChargeSwitch Pro Filter Plasmid Miniprep Kit (Cat. Number 42546863), Sf9 insect cell line (Cat. Number 574237), Supplemented Grace's Insect Medium (Cat. Number 608497), Lipofectamine 2000 (Cat. Number 569156), ProBond Purification System (Cat. Number 479871), and plasmid Midi Kit (Cat. Number K210014) were purchased from Invitrogen (Carlsbad, CA). GeneClean Spin Kit (Cat. Number 1101-2001-20149) was purchased from MP Biomedicals, LLC (Solon, OH). T4 DNA ligase (Cat. Number 0001827) was purchased from Fermentas (Burlington, ON). PCR and sequencing primers were purchased from Alpha DNA (Montreal, QC, Canada). Amersham ECL Western Blotting Detection Reagents (Cat. Number 163) were purchased from GE Healthcare (Mississauga, ON, Canada). HDAC Fluorimetric Cellular Activity Assay – AK-503 (Cat. Number 3-T6505) was purchased from Enzo Life Sciences (Plymouth Meeting, PA). DPYSL4 Recombinant Protein (CRMP3) (Cat. Number 0980513) was purchased from Abnova (Taipei City, Taiwan).

**Mouse neuronal culture and treatment.** Primary cortical neurons were prepared from embryonic E13-16 female mice (strain CD1) and cultured in B27 and N2 supplemented neurobasal media for 7 days as described previously. Cells were plated onto 24-well plates containing poly-L-lysine coated coverslips at a density of 5 × 10^5 per well. Glutamate (50 μM) was added to the cultures either at 7 or 8 days in vitro (DIV) in the presence or absence of TSA. PCR was performed using a primer pair to the E2F1 promoter sequence. Total genomic DNA was used as an input control. As shown in the lower left panel, glutamate treatment increased CRMP3 binding to the E2F1 promoter. The level of E2F1 expression was quantified against the input DNA and normalized to the non-treated control (c). The ChIP assay was performed on cortical neurons treated with glutamate (50 μM) for 1 h and 4 h in the presence or absence of TSA. PCR was performed using a primer pair to the E2F1 promoter sequence. Total genomic DNA was used as an input control. As shown in the lower left panel, glutamate treatment increased CRMP3 binding to the E2F1 promoter. The level of E2F1 PCR was quantified against the input DNA and normalized to the non-treated control (d').

**Recombinant mammalian expression plasmid construction, purification and transfection.** The full-length CRMP3, p54 and D domain constructs were amplified by PCR from the plasmid CRMP3-pBKCMV as a template. These primer pairs contain sequences encoding the restriction sites KpnI and BamHI at the end. The amplified fragments were then digested with KpnI and BamHI and subcloned into the pEGFP-C1 (Clontech).
Figure 8 | Knock-down CRMP3 expression prevented glutamate-induced nuclear condensation and neuronal death. (a) CRMP3 specific stealth siRNA and a sequence-mutated negative control siRNA (Ctl siRNA) were transfected into cultured cortical neurons. CRMP3 siRNA down regulated CRMP3 expression after 48 and 60 h of transfection. Acetylated histone H4 level increased in CRMP3 siRNA transfected neurons (lower panels of a). GAPDH was used to indicate equal protein loading. (b) Untreated neurons and neurons transfected with siRNAs showed cytoplasmic expression of CRMP3 (green color) and normal nuclei (arrowheads). (c) Glutamate caused shortening of neurites and increased co-localization of CRMP3 with condensed nuclei (arrows in c). Control siRNA to CRMP3 neither reduced nuclear accumulation of CRMP3, nor prevented nuclear condensation (arrows in c). In contrast, CRMP3 siRNA prevented neurite shortening (c) and nuclear condensation. (d) Quantification of neuronal protection conferred by the treatment with CRMP3 siRNA using the TUNEL assay and a neurite length measurement (e). (f and g) cortical explants were cultured for 7 days in the presence of TSA. These explants were immunostained with an antibody to tubulin (red color) and phalloidin (green color) to show neurites and growth cones, respectively. TSA treatment inhibited HDAC, but did not affect the length of neurites when compared with the non-treated control. Neurite length was measured and plotted in panel (g). Two-way ANOVA was used with Tukey’s post hoc analysis to determine statistical significance with ** indicating p < 0.01. Scale bars = 60 μm.
Miniprep Kit according to the manufacturer’s instructions. Centrifugation steps were performed using a Biofuge fresco microcentrifuge from Heraeus Instruments. The fidelity of plasmid sequences was confirmed by restriction digestion using BamH1 and Kpn1 and sequencing using the following primers:

- Full-length/D/p54 forward: 5’-CGTGTTCCGCTATGGGCGAATA-3’
- Full-length/D/p54 reverse: 5’-GCTTCAGCGTTATAACGCTG-3’

**Growth of S9** insect cell line and transfection. S9 insect cells were initiated from frozen stock and grown to confluency at 27°C according to the manufacturer’s instructions. Cells were grown in complete TNM-FH media (Grace’s Insect Medium, supplemented with 10% fetal bovine serum and 10 μg/ml gentamycin). Confluent cells were passaged at a 1:5 dilution by gentle scraping. S9 insect cells were passaged and grown to confluency in 10 cm dishes. Transfection was performed using 15 μg DNA with 15 μl Lipofectamine 2000 in 10 ml complete TNM-FH media per dish according to the manufacturer’s instructions.

**Purification of total protein from S9 insect cells.** Insect cells were harvested on days 2 through 4 following transfection. Briefly, cells were washed and suspended in 1 ml PBS. Cells were then pelleted by centrifugation at 3000 g for 5 minutes using a Labofuge 400 centrifuge from Heraeus Instruments. Total protein was isolated under native conditions using a ProBond Purification System according to the manufacturer’s instructions. During the steps subsequent to the harvesting of insect cells, centrifugation steps were performed using a Biofuge fresco microcentrifuge from Heraeus Instruments.

**Double immunofluorescent staining,** TUNEL labelling and confocal imaging. The procedures for fluorescent immunocytochemistry were exactly as described previously36,39–41. The primary antibodies against CRMP3 were a gift from Dr J Kappler (Bern, Germany) and used at a dilution of 1 : 1000. The appropriate Cy3- or Alexa488-conjugated secondary antibodies were used at 1 : 5000. Staining was examined under a fluorescent microscope. Neuronal death was determined using cortical neurons fixed with freshly prepared paraformaldehyde (4%) and followed by a reaction for in situ DNA end labeling (TUNEL) using a kit purchased from Roche as previously described36. Cells were counter stained using DAPI (blue color). Images were collected on a confocal laser scanning microscope with a 60 × oil-immersion objective (Olympus Optical, Tokyo, Japan).

**Western blotting.** Western blotting was performed with the indicated amount of protein as described previously42. Proteins were electrophoresed in an 8% sodium dodecyl sulfate mini gel and then electroblotted onto a nitrocellulose membrane in transfer buffer. Full-length, p54, and D domain CRMP3 samples were probed with a polyclonal antibody specific to CRMP3 at a 1 : 1000 dilution at 4°C overnight or at room temperature for 1 hour. After washing with TBST (10 mM Tris, 150 mM NaCl and 0.1% Tween 20), a horseradish peroxidase-conjugated secondary antibody at a dilution of 1 : 5000 dilution was applied to the membrane at room temperature for 1 hour. Alternatively, full-length, p54, and D domain CRMP3 proteins were probed with a polyclonal antibody specific to tetra His-tag at a dilution of 1 : 2000. After washing with TBST (10 mM Tris-HCl, pH 7.8, and 150 mM NaCl), a horseradish peroxidase-conjugated secondary antibody at a dilution of 1 : 5000 was applied to the membrane for 1 hour at room temperature. Enhanced chemiluminescence detection of the target protein was performed using Amersham ECL Western Blotting Detection Reagents and X-ray film.

**Cell-free HDAC fluorescent assay.** The cell-free HDAC fluorescent assay was performed following the manufacturer’s instructions (Enzo Life Sciences, Plymouth Meeting, PA). CRMP3 proteins were diluted in HDAC assay buffer and Fluor de Lys substrate to a final volume of 50 μl. HeLa nuclear extract (4 μg) was used as a positive control. As negative controls, Tischostatin (TSA) was added at a final concentration of 1 μM. Ten minutes following addition of the Fluor de Lys substrate, the reactions were stopped with an addition of 50 μl 1X Fluor de Lys Developer. The fluorescence of the reactions was immediately read using a CytoFluor 2350 Fluorescence Measurement System from Millipore with an excitation wavelength of 360 nm. Fluorescence was detected at a wavelength of 460 nm.

**Immunoprecipitation (IP) and Western blotting.** IP was performed using the Protein A beads method (Dynal Inc, Lake Success, NY) as exactly described by the manufacturer43. Briefly, 200 μg of total protein was incubated for 2 h at 4°C with an antibody to EGFP followed by an incubation with protein A beads. After washing away unbound proteins, the immunoprecipitated protein complex was subjected to Western blotting for the proteins of interests, including histone H4 and tubulin. The procedures for Western blotting were exactly as described previously43. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a protein loading control. The intensities of the bands were quantified using NIH Image J software.

**In Vitro histone and tubulin deacetylation assay.** A histone deacetylation assay was performed in a 100 μl volume of HDAC deacetylation buffer (10 mM Tris-HCl, pH 8.0, and 10 mM NaCl) containing 0.1 mg/ml BSA and 50 μg of total cellular lysate (as substrate). Non-treated HEK293 cell lysate was harvested in HDAC deacetylation buffer (containing 1% NP-40) and underwent brief sonications. For the tubulin deacetylation assay, total HEK293 cell lysates were either left as monomers or polymerized in PEM buffer (80 mM PIPES, pH 6.8, 1 mM MgCl2, 1 mM EGTA) in the presence of 20 μM Taxol (Sigma) and 1 mM GTP (Sigma) for 30 min at 37°C as described previously39,40.
previously described34,43. Reactions that included HDAC inhibitor, TSA (1 μM), were performed using InvestigatorTM Progest (Genomic Solutions, Ann Arbor, MI). Briefly, destaining protein gel bands of interest were excised and automatically destained and digested overnight. The average intensity and standard deviations were calculated using Prism least four measurements were taken from non-overlapping areas for each region of interest. The supernatant contained a soluble nucleic acid fraction. The pellet was sonicated for 15 s in Buffer II and spun at 55,000 rpm for 10 min at 4°C. This supernatant contained proteins from the insoluble nucleic fraction.

Chromatin immunoprecipitation (ChIP) assay. The ChIP assay was performed using a magnetic bead-based ChIP-IT Express kit exactly following the manufacturer’s instructions (Active Motif, Carlsbad, CA). Briefly, mouse cortical neurons grown on a 100 mm size tissue culture dishes were treated with 50 μM of glutamate for 1 and 4 h with or without the pre-treatment of TSA (1 μM) which was added acetylation of histone H4 (1:100, Cell signalling) and an anti-acetylated tubulin (1:1000, Sigma)

E2F1 Promoter construct and activity assay. The E2F1 promoter-luciferase lightSwitch promoter reporter GoClone plasmid construct (product ID 5719961) was purchased from SwitchGear Genomics (Menlo Park, CA). The purified plasmid (1 μg) was transfected into neurons at a density of 5 × 104 in a 24 well plate using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The plasmid pGL3 construct (Promega) was used as a negative control. After 24 h of transfection, with either the E2F1 Promoter or pGL3 construct, transfected cortical neurons were further treated with glutamate (50 μM) with or without prior treatment with TSA (1 μM) for 15 min. Neurons were treated with glutamate for 2 or 4 h before collection for the luciferase assay.

The LightSwitch Luciferase Assay kit was purchased from SwitchGear Genomics and the assay was performed according to the manufacturer’s protocol. Briefly, the luciferase substrate was reconstituted with substrate solvent to a final concentration of 100×. The assay solution was prepared in 1× substrate solvent with assay buffer in a 1:10 ratio. Neuronal samples were placed into a 96 well plate in the amount of 100 μl cells + media. Assay solution (100 μl) was also added to the samples plate. The plate was incubated for 30 min at room temperature protected from light and then read immediately for two seconds with an appropriate luciferase counter with a luminescence protocol. The neuronal sample protein concentrations were determined in order to obtain an OD/μg protein concentration. Also a background subtraction was done using the readings from conditioned media with only assay solution added.

Image analysis and data analysis. To quantify the expression levels of CRMP3 both in the nucleus and the cytoplasm, images of immunostaining were taken under a fluorescent microscope using identical fluorescent intensity settings. Identical areas were selected on digitized images with resolutions of 1300 × 1300 pixels. Fluorescent intensities were measured using NIH Image J software (http://rsb.info.nih.gov/ij/). At least four measurements were taken from non-overlapping areas for each region of interest. The average intensity and standard deviations were calculated using Prism 5.0 software. All experiments were repeated at least three times.

Mass spectrometry analysis of CRMP3 interaction with nuclear proteins. The protein bands of interest were excised and automatically digested and digested using Progesterone (Genomic Solutions, Ann Arbor, MI). Briefly, destaining was achieved using a 1 : 1 ratio of 30 mM potassium ferricyanide and 100 mM sodium thiourea (Gharahdaghi et al., 1999). The gel bands were then washed with deionized water, shrunk with acetonitrile and reswollen with 50 mM ammonium bicarbonate. Gel bands were placed in 100 μl of 5% m/18 C18 micro-pre-column cartridge (Dionex/LC-Packings) which was then brought online with a 75-μm × 500-μm C18 reversed phase media. The peptides were separated using a gradient supplied by a 0.05% formic acid in 5 min at 4°C. The cell pellet was transferred into a 1 ml eppendorf tube and washed with deionized water, shrunk with acetonitrile and reswollen with 50 mM ammonium bicarbonate (Gharahdaghi et al., 1999). The gel bands were then washed with 300 nL/min flow rate). The mass spectrometer was set to automatically acquire MS/2. Li, W., Herman, R. K. & Shaw, J. E. Analysis of the Caenorhabditis elegans axonal guidance and outgrowth gene unc-33. Genetics 133, 675–689 (1992).

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