Regulation of neuron survival and death by p130 and associated chromatin modifiers

David X. Liu,³ Niharika Nath,¹ Srikumar P. Chellappan,² and Lloyd A. Greene

Department of Pathology, Columbia University Medical Center, New York, New York 10032, USA

E2F-mediated gene repression plays a key role in regulation of neuron survival and death. However, the key molecules involved in such regulation and the mechanisms by which they respond to apoptotic stimuli are largely unknown. Here we show that p130 is the predominant Rb family member associated with E2F in neurons, that its major partner for repression of pro-apoptotic genes is E2F4, and that the p130–E2F4 complex recruits the chromatin modifiers HDAC1 and Suv39H1 to promote gene silencing and neuron survival. Apoptotic stimuli induce neuron death by sequentially causing p130 hyperphosphorylation, dissociation of p130–E2F4–Suv39H1–HDAC complexes, altered modification of H3 histone and gene derepression. Experimental suppression of such events blocks neuron death while interference with the synthesis of E2F4 or p130, or with the interaction of E2F4–p130 with chromatin modifiers, induces neuron death. Thus, neuron survival and death are dependent on the integrity of E2F4–p130–HDAC/Suv39H1 complexes.

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Although neurons are post-mitotic, the cell cycle machinery plays an important role in their sustenance. On one hand, this machinery must be suppressed to maintain a post-mitotic state and survival; on the other, a growing body of evidence indicates that inappropriate activation of this machinery mediates neuron apoptotic death during both normal developmental and pathological conditions (Liu and Greene 2001a; Becker and Bonni 2004; Greene et al. 2004). However, many questions remain about the identities of the molecules involved in such events and the mechanisms by which they regulate neuron survival and death.

The E2F and Rb families are pivotal regulators of gene expression and of cell cycle. In actively dividing cells, E2F transactivates genes required for S-phase progression; in post-mitotic or quiescent cells, E2Fs bind Rb family members and form complexes that both suppress the transactivational activity of E2Fs and that actively repress transcription of genes with E2F-binding sites (Stevaux and Dyson 2002). The association of Rb family members with E2Fs is regulated through their phosphorylation by cyclin-dependent kinases such as Cdks 2, 4, and 6. Hypophosphorylated Rb family members bind

E2Fs while Cdk-dependent phosphorylation of Rb family members leads to dissociation of E2F–Rb complexes and to loss of E2F-dependent gene repression (Dyson 1998).

Multiple studies show that a variety of apoptotic stimuli including DNA damage and trophic factor deprivation promote Cdk 4/6 activity and hyperphosphorylation of Rb family members in neuronal cells and derepression of apoptotic E2F-responsive genes (Park et al. 1997a,b, 2000; Padmanabhan et al. 1999; Osuga et al. 2000; Liu and Greene 2001b). B-myb and C-myb in particular have been identified as pro-apoptotic genes that undergo such derepression, and recent work reveals that they play an essential role in neuron death (Liu and Greene 2001b; Liu et al. 2004).

The Rb family has three members—Rb, p107, and p130—with both redundant and unique properties (Dyson 1998). The importance of the Rb family in maintaining survival of post-mitotic neurons was underscored by observations that targeting SV40 T-antigen to Purkinje cells induces their death and that this requires the T-antigen Rb-family-binding domain (Feddersen et al. 1995). However, the roles of specific family members in maintaining the neuronal post-mitotic state and in neuron survival have been less clear. Although early reports with knockout mice indicated that Rb was required for neuron survival, more recent work indicates that Rb loss causes defects in cell cycle, but not neuron death (Ferguson et al. 2002; MacPherson et al. 2003). Targeted deletion of p107 had no observable effect on neuron survival (Cobrinik et al. 1996; LeCouter et al. 1998a), while massive neuron loss was reported in one strain of p130-

Present addresses: ¹Department of Physiology and Pharmacology, City University of New York Medical School, New York, NY 10031, USA;

²Drug Discovery Program, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL 33612, USA.

³Corresponding author.

E-MAIL dl345@columbia.edu; FAX (212) 305-5498.

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null mice, but not in another (Cobrinik et al. 1996; LeCouter et al. 1998b).

Rb family members form complexes with a variety of partners that participate in suppression of gene expression (Stevaux and Dyson 2002). Among such partners detected in nonneuronal cells are chromatin-modifying enzymes such as histone deacetylases (HDACs) (Luo et al. 1998) and histone methyltransferases (HMTs) (Nielsen et al. 2001; Vandel et al. 2001). Thus, transcriptional repression of some E2F target genes can be relieved by HDAC inhibitors (Luo et al. 1998) or by overexpression of an enzymatically inactive mutant of the HMT Suv39H1 (Vandel et al. 2001). This supports a model in which complexes of promoter-bound E2F–Rb family members recruit enzymes that modify nearby chromatin and thereby cause transcriptional repression.

The present work addresses the molecular mechanisms by which cell cycle molecules regulate neuron survival and death. Our studies reveal that complexes of p130 with E2F4 and chromatin modifiers including HDAC and Suv39H1 play an essential role in maintaining neuron survival and do so by repressing pro-apoptotic genes such as B-myb. Apoptotic stimuli trigger dissociation of such complexes, resulting in gene derepression and neuron death.

Results

p130 is the predominant Rb family member in neuronal E2F complexes

We first determined the nature of Rb–E2F complexes in viable neurons. Extracts of cultured cortical neurons and neuronal (nerve growth factor [NGF]-treated) PC12 cells were subjected to electrophoretic mobility shift assays (EMSAs) using a variety of E2F-site-containing DNA fragments including those from the adenovirus E2 promoter, mouse and rat B-myb promoters, human cdc25A promoter, and a commercial E2F consensus site, with or without antibodies/antisera specific to individual Rb family members. Initial tests with R386 or CHO cells overexpressing Rb, p107, or p130 verified that each antibody recognized and shifted its appropriate target (data not shown). For the neuronal material, irrespective of binding-site fragment used, there was no evident effect of Rb- or p107-specific antibodies (Fig. 1A; data not shown). In contrast, anti-p130 almost entirely supershifted or abolished the E2F complexes (Fig. 1A).

We next used chromatin immunoprecipitation (ChIP) assays to identify Rb family members that occupy E2F-site-containing promoters in neurons. Chromatin from neuronal PC12 cells stably transfected with constructs containing cdc25 or B-myb promoters was subjected to saturating IP (immunoprecipitation) with Rb, p107, or p130 antibodies, and the IPs were analyzed by quantitative PCR for relative level of promoter DNA. We consistently recovered 8–20 times more cdc25A (Fig. 1B, pellet) or B-myb (Fig. 1C, pellet) promoter DNA from p130 IPs than from Rb or p107 IPs. A second round of IPs with the same antibodies did not yield significant signals over

background (Fig. 1B,C, supernatant), indicating that the first IP was complete. ChIP/PCR assays also confirmed occupancy of E2F-responsive promoters by p130 in neuronal PC12 cells transiently transfected with reporters B-myb-luc or cdc25-luc (Fig. 1D). In contrast, there was no detectable association between p130 and E2F-site mutants of B-myb (Fig. 1D) or cdc25 promoter sequences (data not shown). Finally, ChIP/PCR assays revealed association of the endogenous B-myb promoter with p130 in cultured rat cortical neurons (Fig. 1E, upper panel). As control, no signal was recovered in ChIPs generated with anti-p27 antiserum (Fig. 1E) or when the p130 IPs were analyzed for the actin promoter (Fig. 1E, lower panel). Taken together, these data indicate that p130 is the predominant Rb family member associated with E2F complexes in cultured post-mitotic cortical neurons and neuronal PC12 cells.

Apoptotic stimuli cause loss of chromatin-associated p130–E2F complexes in neuronal cells

We next compared interaction of p130 with E2F and with E2F-binding sites under control and apoptotic conditions. EMSAs revealed that in both cortical neurons and neuronal PC12 cells, levels of p130–E2F complexes fell within 3–5 h of exposure to camptothecin (Cpt), a topoisomerase I inhibitor that evokes apoptotic neuron death by 1 d (Park et al. 1997b), and were nearly undetectable by 8–9 h of treatment (Fig. 1F). Similarly, levels of p130–E2F complexes in cultured sympathetic neurons dropped significantly within 8 h (data not shown) and were nearly absent by 18 h of NGF deprivation (Fig. 1G). Loss of these complexes was prevented by flavopiridol (Flavo), a Cdk inhibitor that blocks neuron death and derepression of E2F-regulated genes evoked by Cpt and NGF withdrawal (Fig. 1F,G; Park et al. 1996, 1997b; Liu and Greene 2001b). To study this effect further, we performed ChIP/PCR assays on neuronal PC12 cells stably or transiently expressing B-myb or cdc25A promoters. Promoter occupancy by p130 significantly fell by 2 h with Cpt (Fig. 1C; data not shown) and was nearly absent by 9 h (Fig. 1B–E). As with the EMSAs, Flavo substantially blocked Cpt- and NGF-deprivation-promoted loss of promoter occupancy (Fig. 1F,G). Finally, Cpt compromised association of p130 with the endogenous B-myb promoter in cortical neurons (Fig. 1E). Taken together, these observations indicate that neuronal chromatin-associated E2F–p130 complexes are lost in response to DNA damage and NGF deprivation.

Hypophosphorylated p130 is lost from nuclei of dying neurons

Because p130 associates with E2F only when hypophosphorylated, we examined neuronal p130 protein by Western blot in whole-cell extracts of cortical neurons treated $\pm$ Cpt or of neuronal PC12 cells $\pm$ NGF withdrawal. At least three or four p130 isoforms were resolved in each case (Fig. 2A,B). Pretreatment of PC12 cell

Figure 1. p130 is the major Rb-family member complexed with E2F in neurons, and such complexes are lost in response to apoptotic stimuli. (A) EMSAs on extracts of cortical neurons and the E2F-binding sequence from the adenovirus E2 promoter (Wang et al. 1999) (lanes 1–4) or rat B-myb promoter sequence (lanes 5–8). Samples in lanes 1–4 were incubated without (control) or with anti-p130 (Transduction Laboratories), p107 (Santa Cruz C-18), or Rb (Oncogene Science), and in lanes 5–8 with Santa Cruz antisera C-20 (p130), C-18 (p107), and C-15 (Rb) and control antibody p27 (C-19). The solid arrow marks the E2F–p130 complex that is selectively supershifted by anti-p130. The open arrow indicates free E2F. Irrelevant lanes were removed from the original image. (B) ChIP assays on neuronal PC12 cells stably transfected with the cdc25A-luc reporter and treated for 9 h ± Cpt. Chromatin from the cells was subjected to IP with the indicated antibodies, and the relative amount of promoter in the IPs (pellet) was quantified by PCR and reported as relative promoter occupancy (the value for the p130 pellet at 0 h was arbitrarily set at 100%). Supernatants from the IPs were subjected to a second round of IP/PCR to verify that the first round of IP was complete. “Mock” indicates values for ChIP assays on samples in which antibodies were omitted. The data here and all other quantified experiments below are means of values (±SEM) from three to six independent experiments. (C) ChIP assays carried out as in B except that cells were stably transfected with the B-myb-luc reporter. Times of exposure to Cpt and Flavo are as indicated. Data were quantified and expressed as in B. (D) ChIP assays were carried out as in C using p130 antibody

and chromatin from neuronal PC12 cells transfected for 2 d with the indicated promoters and exposed as indicated to Cpt and Flavo. The gel shows PCR on recovered DNA using primers for mouse B-myb (lanes 1–6) and human cdc25A (lanes 7–10) promoters. (Lanes 5,6) PCR results for controls containing 5% of the pre-IP input for wild-type and mutant mB-myb-luc. (Lane 10) Ten percent of the pre-IP input for cdc25A-luc (hscdc25A). (E) As in D except that chromatin was prepared from cortical neurons treated ± Cpt as indicated. IPs were carried out with a control antibody (p27) in lane 1 and with anti-p130 in lanes 2–5. For lane 6, the input was 10% of the pre-IP DNA. PCR was carried out using either primers for the rat B-myb (upper panel) or actin (lower panel) promoters. (F) EMSA on extracts of cortical neurons (lanes 1–4) or neuronal PC12 cells (lanes 5–9) treated with Cpt for the indicated times in the absence or presence of Flavo (+F). The commercial E2F consensus DNA sequence was used. (G) As in F except that extracts of SCG neurons undergoing NGF withdrawal (W/D) for the indicated times ± Flavo (F) were used.

extracts with λ phosphatase (Ptase) converted the isoforms into a single, fast-migrating band (Fig. 1C), consistent with the interpretation that the isoforms correspond to variably phosphorylated p130 (Laplantine et al. 2002). In whole-cell extracts of viable cultured cortical neurons, the most rapidly migrating p130 band, which is hypophosphorylated, was the most prominent. Selective loss of this isoform was detectable within 2–4 h of Cpt treatment (data not shown) and a substantial loss after 9 h (Fig. 2A). Studies with whole-cell extracts of PC12 cells also indicated selective loss of hypophosphorylated

p130 in response to Cpt (data not shown) or NGF deprivation (Fig. 2B).

To analyze the cellular localization of the affected p130 isoforms, nuclear and cytosolic fractions from PC12 cells were resolved by Western immunoblotting. The hypophosphorylated p130 selectively fractionated with nuclei, while the more hyperphosphorylated forms selectively fractionated with cytosol (Fig. 2D). Similar findings were made with cortical neurons (data not shown). Examination after various times of Cpt exposure revealed that the nuclear-localized hypophosphorylated

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Figure 2. Apoptotic stimuli evoke loss of hypophosphorylated p130 and exodus of E2F4 from the nuclei of neuronal cells. (A) Western immunoblot analysis of p130 in whole-cell extracts of cultured cortical neurons treated $\pm$ Cpt. Blots were probed with p130 antibody C-20. Equal loadings were confirmed by ERK staining (data not shown) and the presence of nonspecific bands on the *top* and *bottom* of the blot. (B) Western immunoblot analysis of p130 isoforms in whole-cell extracts of neuronal PC12 cells at 0, 3, and 18 h after NGF withdrawal. (C) Conversion of hyperphosphorylated p130 (pp130) into hypophosphorylated p130 (p130) by λ -phosphatase (Ptase). (D) Western immunoblot analysis of p130 isoforms (p130, hypophosphorylated; pp130, hyperphosphorylated) in nuclear (N) and cytosolic (C) fractions of neuronal PC12 cells. Actin and PCNA are cytosolic and nuclear markers, respectively. (E,F) Western immunoblot analysis of p130 levels in nuclear (D) and cytosolic (E) fractions of neuronal PC12 cells treated with Cpt for the indicated times. The presence of Flavo is indicated by +F. (G) E2F4 bound to the endogenous B-myb promoter specifically associates with p130, but not Rb or p107 in neuronal PC12 cells. Neuronal PC12 cells were subjected to ChIP using 30 μ g of cross-linked chromatin and 30 μ g of Agarose-conjugated anti-E2F4 antibody as described in Materials and Methods. Bound material was eluted and equal aliquots were subjected to IP with antibodies against either Rb, p107, or p130. (Top panel) One-tenth of each pellet was de-cross-linked in sample buffer, and the samples were resolved by SDS-PAGE on a 4%–12% gel and analyzed by Western immunoblotting with anti-p130. (Bottom panel) Half of each pellet was used to extract DNA that was subjected to PCR to detect B-myb promoter sequence. The supernatants of the first IPs were subjected to a second round (2nd IP) with the same antibodies to ensure that the first round had reached completion. As positive controls, a fraction (1.7%) of the eluate from the ChIP with anti-E2F4 was loaded into the far-left lane (E2F4), while the same fraction from the chromatin before IP was loaded into the far-right lane (input). (H) Change in distribution of E2F4 from nuclear (N) to the cytosolic (C) fraction in cortical neurons after Cpt treatment.

form was gradually lost, while the cytoplasmic hyperphosphorylated forms remained nearly unchanged (Fig. 2E,F). Flavo blocked the Cpt-evoked loss of nuclear p130 (Fig. 2D).

E2F4 associates with p130 on the B-myb promoter and changes distribution in dying neurons

Among E2F family members, E2F4 is a major and preferred p130 partner (Takahashi et al. 2000; Rayman et al. 2002) and is highly and differentially expressed in neuronal PC12 cells and neurons (Persengiev et al. 1999). In consonance with this, ChIP assays indicated that of all E2F family members, only E2F4 detectably occupies the

endogenous B-myb promoter (Supplementary Fig. S1). We therefore next determined whether E2F4 bound to the endogenous B-myb promoter associates with p130 in neuronal cells. E2F4-associated chromatin from neuronal PC12 cells was isolated with an agarose-conjugated anti-E2F4 antibody, and equal aliquots were used for a subsequent round of ChIP with antibodies against Rb, p107, or p130. The pellets were de-cross-linked, and equal aliquots were subjected to PCR to detect B-myb promoter sequence. This revealed that endogenous B-myb promoter sequence was brought down only by anti-p130, but not anti-Rb or anti-p107 (Fig. 2G, 1st IP, lower panel). A second round of IPs indicated that the first round was complete (Fig. 2G, 2nd IP). Western im-

munoblotting confirmed the presence of p130 in the original extract (input), E2F4 ChIP, and in the p130 ChIP (Fig. 2G, upper panel).

Given the association of E2F4 with p130, we next compared the localization of E2F4 in viable and dying cortical neurons. Although overall E2F4 levels were not changed, there was a shift from a predominantly nuclear distribution to a predominantly cytoplasmic localization after Cpt exposure (Fig. 2H). These findings are consistent with EMSA data showing loss of p130–E2F complexes in dying neurons (Fig. 1F,G) and support the model that apoptotic stimuli promote loss of nuclear p130–E2F4 complexes by Flavo-sensitive hyperphosphorylation of nuclear p130.

p130 and E2F4 are required for neuron survival

The selective presence of p130–E2F4 complexes in neurons and their loss with apoptotic stimuli led us to query their role in maintaining neuron survival. We prepared siRNA constructs against rat Rb, p107, and p130 and confirmed that they effectively down-regulate the corresponding endogenous protein in rat cortical neurons (Fig. 3A,B). We also showed that rat si-p130 and si-Rb spare expression of wild-type human p130 and Rb, but block expression of human p130 or Rb transcripts mutated to contain the corresponding targeted rat sequences (data not shown). We next assessed the constructs for their effects on neuron survival. While neither si-Rb nor si-p107 significantly affected survival, si-p130 induced death of cortical neurons (Fig. 3C), with a threefold increase in apoptotic nuclei after 2 d (Fig. 3A,D). Death was blocked by BAF and by overexpression of human p130 (Fig. 3C; Supplementary Fig. S2), supporting the conclusion that apoptosis was due to depletion of endogenous p130.

We further tested the p130 role in neuron survival using a battery of constructs that included wild-type, full-length antisense (AS) and various p130 mutants, which gave comparable levels of expression in U2OS cells (Hansen et al. 2001) and in PC12 cells (data not shown). Both the p130 AS construct and p130(C894F), a point mutant defective for interaction with corepressors such as CtIP and in repressor activity (Meloni et al. 1999), induced death of cortical neurons (Fig. 3E) and neuronal PC12 cells (data not shown). In contrast, there was no significant death after transfection with empty vector, wild-type p130, or p130 mutants defective for phosphorylation by cdk4 (Δ CDK4) or missing a large proportion of potential phosphorylation sites (PM19A).

To determine if p130 perturbation and death are accompanied by E2F derepression, we cotransfected cortical neurons with various p130 constructs and an E2F reporter (B-myb-luc). Each construct that induced death (p130-siRNA, AS and C894F) led to reporter derepression, while wild-type p130 did not (Fig. 3F). Cotransfection of rat p130-siRNA with human p130 fully blocked derepression of the reporter (Fig. 3F).

We also queried whether the p130 survival requirement reflects the post-mitotic state of neurons. Such ex-

periments revealed that in contrast to its death-inducing effect on neuronal (NGF-treated, nonmitotic) PC12 cells, p130 siRNA had no effect on survival of cycling, NGF-untreated PC12 cells (Supplementary Fig. S2).

Finally, we prepared an siRNA for rat E2F4 and found by immunostaining that it reduced endogenous E2F4 staining in cortical neurons, but spared coexpressed human E2F4 that is different from rat E2F4 by 4 nt in the targeted sequence (data not shown). Similarly to si-p130, overexpressed si-E2F4 induced death of cortical neurons (Fig. 3C,D), and elevated B-myb promoter activity by threefold (Fig. 3F).

Taken together, these findings indicate that loss of either p130 or E2F4 leads to derepression of E2F sites and neuron apoptosis. In contrast, Rb and p107 play no apparent roles in neuron survival.

Overexpression of p130 protects neurons from death evoked by apoptotic stimuli

Our observations that Flavo blocks p130 hyperphosphorylation and loss of E2F–p130 complexes (and neuron death) suggests that this drug acts upstream of p130 as a Cdk inhibitor. Consistent with this model, Flavo failed to protect from loss of p130 induced by p130-siRNA (Fig. 3C).

If neuron death results from p130 hyperphosphorylation and loss of E2F4–p130 complexes, then overexpression of p130 or of p130 mutants resistant to phosphorylation should be protective. To assess this, we transfected neuronal PC12 cells with wild-type p130 as well as with constructs encoding the phosphorylation-resistant p130 mutants Δ CDK4 and PM19A and determined their response to NGF withdrawal (Fig. 3G). Each construct significantly protected the cells from death compared with control vector. We also observed comparable protection of neuronal PC12 cells and cortical neurons from death evoked by Cpt (data not shown). As anticipated by its susceptibility to phosphorylation, wild-type p130 was somewhat less effective in protection than the phosphorylation-resistant mutants (Fig. 3G).

p130 forms complexes with corepressors HDAC1 and Suv39H1, and these complexes are lost with apoptotic stimuli

Our findings indicate that neuron survival requires p130-mediated repression of E2F-responsive genes. To determine how this occurs, we examined interaction of p130 with HDAC1 and Suv39H1, two chromatin modifiers that cooperate with Rb family members to promote E2F-dependent gene repression in nonneuronal cells (Luo et al. 1998; Nielsen et al. 2001; Vandel et al. 2001; Vaute et al. 2002; Macaluso et al. 2003). Using cotransfection and co-IPs followed by Western blotting, we first showed that Flag-HDAC and Myc-Suv39H1 reciprocally associate with p130 and p130PM19A in CHO cells (Supplementary Fig. S3). We also demonstrated association of endogenous p130 with Suv39H1 and HDAC1 by co-IPs

Figure 3. p130 and E2F4 are required for neuronal survival. (A) siRNAs against individual Rb family members down-regulate their specific targets in cortical neurons, and only p130 siRNA induces apoptosis. Cortical neurons were cotransfected with eGFP and siRNA constructs against Rb (panels *a–c*), p107 (panels *d–f*), or p130 (panels *g–i*). (Panels *b,e,h*) Two days later, cultures were fixed and immunostained as indicated for expression of Rb family members. (Panels *c,f,i*) Nuclei were stained with Hoechst 33342. (Panels *a,d,g*) Arrows show transfected cells (green). Bar, 20 μ m. (B) Efficiency of indicated siRNA constructs in down-regulating Rb family members in cortical neurons. Transfection was as in A. The proportions of transfected (eGFP⁺) cells (35–136/culture in triplicate) expressing each antigen were assessed. (C,D) Down-regulation of p130 or E2F4, but not of Rb or p107, induces apoptosis of cortical neurons. In C, cells were cotransfected with indicated siRNA and human (Hs) constructs and eGFP and 4 h later, the transfection medium was replaced with culture medium $\pm$ BAF or Flavo as indicated. The number of eGFP⁺ cells was counted 24 h after transfection (day 0) and on each of the next 2 d and percent surviving cells expressed relative to that at day 0 (set arbitrarily to 100%). In D, transfection was as in C, and 2 d later cells were stained with Hoechst dye. Transfected (eGFP⁺) cells were scored for the presence of apoptotic nuclei. (E) Loss of p130 or displacement with mutant p130(C894F) compromises survival of cortical neurons. Cells were cotransfected with eGFP and the indicated constructs. The number of GFP⁺ cells in the cultures was counted as in C. (F) Loss of p130, E2F4, or expression of p130(C894F) causes derepression of a B-myb-luc reporter. Neuronal PC12 cells were cotransfected with pcDNA-lacZ, B-myb-luc, and indicated constructs. Cells were harvested 48 h later and subjected to luciferase and β -gal assays. p130(h) is a human p130 construct. (G) p130 and p130 mutated at phosphorylation sites protect neuronal PC12 cells from NGF withdrawal (W/D). Experiments were carried out and data were expressed as in E except cells were washed and anti-NGF was added 24 h after transfection.

and WB using extracts of PC12 cells and cortical neurons (Fig. 4A,B; data not shown).

We next examined whether association between p130 and HDAC1 or Suv39H1 changes in response to apoptotic stimuli. While endogenous p130 co-IPed with HDAC1 in extracts of viable cortical neurons, association was undetectable at 9 h of Cpt exposure (Fig. 4B). Similarly, association between p130 and Suv39H1 was lost in PC12 cells after 8 or 18 h of NGF deprivation (Fig. 4C). We further measured p130-associated HMTase (his-

tone H3-methyltransferase) activity in extracts of cortical neurons treated $\pm$ Cpt (Fig. 4D,E) and in PC12 cells undergoing NGF deprivation (Fig. 4F). Cpt caused a substantial drop of p130-associated H3-HMT activity while the total cellular levels of HDAC1 and Suv39H1 showed relatively little change (Fig. 4B,C). Similar loss of p130-associated H3-HMT activity was observed in PC12 cells after 16 h of NGF withdrawal (Fig. 4F). These data thus indicate that in viable neuronal cells, p130 complexes with corepressors HDAC1 and Suv39H1 and that such

Figure 4. p130 associates with HDAC1 and Suv39H1, and this association is lost in response to apoptotic stimuli. (A) Cellular association between endogenous p130 and Suv39H1. IPs were prepared from whole-cell extracts of neuronal PC12 cells using the indicated antibodies. Irr is an irrelevant (control) antibody (p53). IPs were subjected to Western blotting with antibodies against p130 and Suv39H1 as indicated. (B,C) Apoptotic stimuli lead to loss of cellular association between endogenous p130 and HDAC1 (B) or Suv39H1 (C). IPs were prepared from extracts of cortical neurons treated $\pm$ Cpt for 9 h using anti-HDAC1 (B) or of neuronal PC12 cells undergoing NGF withdrawal for 0, 8, and 18 h using anti-Suv39H1 (C). The IPs were subjected to Western immunoblotting using anti-p130 and anti-HDAC1 (B) or anti-Suv39H1 (C). (D,E) Cpt treatment leads to loss of association between p130 and H3-

HMTase activity. IPs were prepared as in B using antibodies against either p53 (Irr, irrelevant) or p130. Half of each IP was used for fluorographic analysis (D) and the other half for H3-HMTase assay (E). In D, reaction products were separated by PAGE and subjected to fluorography. Loading of H3 histone is shown by Ponceau Red staining. In E, reaction products were spotted on filters and processed as described in Materials and Methods. (F) As in E except that IPs were prepared from extracts of neuronal PC12 cells undergoing NGF withdrawal for 0 and 16 h.

complexes are lost in response to DNA damage or NGF deprivation.

p130-associated HDAC1 and Suv39H1 are required for neuron survival

To assess whether association of p130 with HDAC and Suv39H1 is required for neuron survival, we again used p130(C894F). We found that although this mutant retains interaction with various E2Fs (Supplementary Fig. S4), it fails to associate with HDAC1 and Suv39H1 (Supplementary Fig. S3). Expression of p130(C894F) led to derepression of the E2F reporter B-myb-luc in PC12 cells (Fig. 3F) and evoked death of cortical neurons and neuronal PC12 cells (Fig. 3E; data not shown). To extend these findings, we transfected neuronal PC12 cells and cortical neurons with si-Suv39H1 and Suv39H1(1–332), a truncation mutant lacking HMTase activity that appears to act as a dominant-negative (Vandel et al. 2001). Expression of si-Suv39H1 in cortical neurons down-regulated endogenous rat Suv39H1, but not a coexpressed mouse Suv39H1 that differs by 2 nt in the targeted region (data not shown). Expression of either si-Suv39H1 or Suv39H1(1–332), but not of wild-type Suv39H1, led to derepression of the B-myb-luc reporter and death of neuronal PC12 cells (Fig. 5A). Importantly, coexpression of mouse Suv39H1 reversed the derepression and apoptotic effects of si-Suv39H1 in these cells (Fig. 5A). si-Suv39H1 also provoked death of cortical neurons (data not shown). Finally, treatment of PC12 cells with the HDAC inhibitor trichostatin A (TSA), which causes death of cultured cerebellar granule neurons (Boutillier et al. 2003) greatly elevated expression of the B-myb-luc reporter and pro-

moted death of cortical neurons (Fig. 5B). Taken together, these findings support the model that association of HDAC and Suv39H1 with p130 is required for p130-dependent gene repression and neuron survival.

Apoptotic stimulation increases histone H3 acetylation and phosphorylation in nucleosomes associated with E2F-derepressed genes

Histone deacetylation by HDACs is often associated with gene repression, and phosphorylation and acetylation of histone H3 at Ser 10 and Lys 14, respectively, with gene activation (Lo et al. 2000; Nicolas et al. 2003). To assess whether these changes occur in neurons responding to apoptotic stimuli, we performed ChIP assays on PC12 cell lines stably expressing cdc25A-luc or B-myb-luc with an antibody recognizing P-ser10/Ac-lys14 histone H3 (Ac-p-H3), and the relative levels of IPed promoter were determined by quantitative PCR. As shown in Figure 5C, the levels of Ac-p-H3 in viable cells were indistinguishable from background. However, there was a sevenfold increase within 5 h of Cpt exposure and an 18-fold increase by 9 h. Results were comparable for cells expressing B-myb-luc (data not shown). In contrast, a control E2F-binding site mutant of cdc25A and the endogenous actin promoter (Fig. 5C), both of which lack E2F-dependent repression, showed unrepressed levels of associated Ac-p-H3 that were little changed with Cpt.

We next examined the endogenous B-myb promoter and associated chromatin in neuronal PC12 cells expressing either HA-tagged wild-type p130 or mutant p130 (C894F) defective in association with chromatin

Figure 5. Relationship between chromatin modification and neuron survival and death. (A) Expression of truncated Suv39H1 or si-Suv39H1 leads to gene derepression of an E2F-regulated construct and evokes death. Neuronal PC12 cells were cotransfected with pcDNA-lacZ, B-myb-luc (*right*), or with eGFP (*left*) and the indicated Suv39H1 constructs. (*Left*) Cells were harvested 2 d later and assessed for relative luciferase activity as in Figure 3F. (*Right*) The number of eGFP-positive cells was assessed as in Figure 3C. Significance comparison to the vector on same day: (*) $p < 0.03$. (B) Inhibition of HDAC activity leads to derepression of an E2F-regulated construct and evokes neuron cell death. (*Left*) Neuronal PC12 cells were cotransfected with pcDNA-lacZ and B-myb-luc constructs. Two days later, cells were exposed to 20 nM TSA for 24 h. Luciferase activity was measured as in Figure 3F. (*Right*) Cortical neurons were treated with or without 20 nM TSA, and survival was assessed. Cell counts before the addition of TSA on 3 DIV (Day 0) were set to 100%, and survival was assessed as in Figure 3C. Significance comparisons on same day: (*) $p < 0.01$. (**) $p < 0.001$. (C) An apoptotic stimulus leads to selective modification of chromatin associated with E2F-binding sites. ChIP assays were carried out using material from PC12 cells stably transfected with wild-type or mutant cdc25A-luc (mutant for E2F binding) (*left*) or wild-type PC12 cells (*right*). Cells were treated for indicated times with Cpt, and chromatin samples were subjected to IP with saturating amounts (6 μ g/assay) of anti-Ac-p-H3 antibody. Real-time PCR (including Mock setup) was carried out as in Figure 1B. Values are normalized so that the level of promoter detected for the Mut cdc25A or Actin after 9 h of Cpt treatment equals 100. (D) p130-associated corepressors regulate histone modification at the endogenous B-myb promoter. ChIP assays were carried out as in Figure 1E except that neuronal PC12 cells were transfected with the indicated constructs or treated $\pm$ 20 nM TSA. Cross-linked chromatin was prepared as in Materials and Methods 24 h after transfection or TSA treatment. Forty micrograms per transfected sample was subjected to ChIP with 8 μ g of agarose-conjugated anti-HA (*left*) or anti-Myc (*middle*). The IPs were eluted by three rounds of treatment with the Pro-Found Co-IP Kit (Pierce) according to the manufacturer's instructions and each was divided into four equal aliquots, subjected to a second ChIP using antibodies as indicated. (*Right*) In the case of cells $\pm$ TSA, a single ChIP was carried out using anti-MeH3 or anti-Ac-p-H3. DNA was recovered from the IPs as in Materials and Methods, and an equal amount (25%) was used for PCR detection using primers specific to the rat B-myb promoter. As positive controls for PCR, 0.5 μ g of chromatin from each sample before HA-Flag-IP was included in lanes 1 and 5, respectively.

modifiers. The transfected cells were subjected to ChIP with anti-HA, and the HA-associated chromatin was subjected to a second round of IP with antibodies against p130, E2F4, methylated histone H3 (Me-H3), or Ac-p-H3. Quantitative PCR was then used to detect endogenous B-myb promoter DNA in the immunocomplexes (Fig. 5D). This revealed association of the promoter with both wild-type and mutant p130 as anticipated, as well as with E2F4. Significantly, while the endogenous myb promoter occupied by wild-type p130 was associated with Me-H3 (indicative of gene repression), in contrast, that occupied by functionally defective p130C894F was associated with Ac-p-H3 (indicative of gene derepression).

We similarly examined the endogenous B-myb promoter and associated chromatin in neuronal PC12 cells transfected with Myc-Suv39H1 or Myc-Suv39H1(1–332) or before or after TSA treatment (Fig. 5D). The promoter in control cells or complexed with Myc-Suv39H1 exclusively associated with Me-H3-containing chromatin. In contrast, chromatin associated with the endogenous B-myb promoter in cells expressing Myc-Suv39H1(1–332) or treated with TSA contained Ac-p-H3. Taken together, these findings indicate that in viable neuronal cells, promoters of E2F-silenced genes such as B-myb associate with Me-H3-containing chromatin and that apoptotic stimuli or manipulations that compromise the presence or activity of promoter-associated E2F–p130–HDAC–Suv39H1 complexes lead to reduced Me-H3 and elevated Ac-p-H3.

E2F4 and p130 tether HDAC and Suv39H1 to the B-myb promoter in viable, but not apoptotic neuronal cells

The presence of E2F4–p130 complexes on the endogenous B-myb promoter in viable neurons raises several questions regarding their association with HDAC and Suv39H1. Are these the complexes that associate with HDAC and Suv39H1, is this association regulated by p130 phosphorylation, and what happens to these complexes under apoptotic conditions? To address these, we created a battery of Flag-tagged constructs (Supplementary Fig. S5) that encode various E2F4–p130 fusion proteins including several mutated to affect their capacity for Cdk-dependent phosphorylation (E2F4–p130 Δ CDK and E2F4–p130PM19A) or interaction with corepressors (E2F4–p130 Δ C). The constructs were transfected into neuronal PC12 cells, and ChIP assays were performed with anti-Flag before and after 4 h of Cpt treatment. The IP brought down wild-type E2F4–p130 fusion protein as anticipated, as well as endogenous HDAC1 and Suv39H1 and B-myb promoter sequence (Fig. 6A, column 3, 1st IP: FLAG). In response to Cpt, the wild-type E2F4–p130 fusion protein remained associated with the B-myb promoter, but no longer bound HDAC1 or Suv39H1 (Fig. 6A). This loss of binding appeared to be due to phosphorylation of the p130 portion of the fusion protein, which showed a retarded electrophoretic mobility (Fig. 6A). In agreement with this interpretation, transfected phosphorylation-site mutant fusion proteins E2F4–

p130 Δ CDK and E2F4–p130PM19A neither shifted nor lost binding to HDAC1 or Suv39H1 in response to Cpt (Fig. 6A). In contrast, although E2F4–p130 Δ C associated with the B-myb promoter, it did not tether either HDAC1 or Suv39H1 to the complex irrespective of Cpt exposure (Fig. 6A).

To further investigate changes in the complexes during apoptosis, we subjected aliquots of the solubilized Flag IPs to a second IP with anti-HDAC1 and assessed the pellets for endogenous HDAC1, B-myb promoter, and H3 HMTase activity (Fig. 6A, 2nd IP: HDAC1). In this case, HDAC1 was associated with the wild-type E2F4–p130 fusion protein, the B-myb promoter, and HMTase activity in viable cells, but not in Cpt-treated cells. Moreover, these associations occurred for E2F4–p130 Δ CDK and E2F4–p130PM19A irrespective of Cpt treatment, but were not detected for E2F4–p130 Δ C. Taken together, these data indicate that E2F4–p130 anchors HDAC1 and Suv39H1 to the B-myb promoter and that an apoptotic stimulus abrogates this association by a Cdk-dependent mechanism.

Active E2F-dependent gene repression by p130-associated HDAC1 and Suv39H1 is required and sufficient for neuron survival

We next asked whether E2F4–p130–HDAC–Suv39H1 repressor complexes at E2F-responsive promoters are specifically required for neuron survival. First, we tested the ability of E2F4–p130 fusion constructs to modulate B-myb-luc expression in PC12 cells. Constructs encoding the wild-type and phosphorylation-resistant fusion proteins had little effect on reporter activity (Fig. 6B). This is as anticipated because B-myb is already repressed in viable cells (Liu and Greene 2001b). In contrast, E2F4, E2F4–p130 Δ C, and E2F4–p130C894F, all of which lack the capacity to tether HDAC1 and Suv39H1, raised reporter activity four- to sevenfold (Fig. 6B). These data thus suggest that association of HDAC and Suv39H1 with E2F4–p130 complexes is required for gene repression.

We next evaluated the effects of the fusion proteins on survival of cortical neurons. While E2F4–p130, E2F4–p130 Δ CDK, and E2F4–p130PM19A did not affect survival, significant death was induced by E2F4, E2F4–p130 Δ C, and E2F4–p130C894F (Fig. 6C). Thus, association of HDAC and Suv39H1 with E2F4–p130 complexes at E2F sites is required for neuron survival.

We also evaluated the effects of the various E2F4–p130 fusion constructs on survival of cortical neurons exposed to Cpt. While E2F4 and E2F4–p130 Δ C showed little or no protection, E2F4–p130 Δ CDK and E2F4–p130PM19A provided full protection from Cpt-induced death (Fig. 6D). E2F4–p130, which is subject to phosphorylation in response to apoptotic stimuli and loss of interaction with HDAC1 and Suv39H1, retarded death but failed to provide sustained protection like the phosphorylation-resistant proteins. Similar protection was obtained with NGF-deprived PC12 cells (data not shown). Taken together, these results demonstrate the key role of p130 in

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Figure 6. Neuronal survival is regulated by chromatin modifiers tethered to DNA by E2F4–p130. (A) Association of HDAC1 and Suv39H1 with the B-myb promoter is regulated by an apoptotic stimulus and requires anchored interaction with E2F4–(hypophosphorylated) p130. Neuronal PC12 cells were transfected with the indicated Flag-tagged constructs and 24 h later treated for 4 h $\pm$ Cpt as indicated. Twenty micrograms of cross-linked chromatin per sample was subjected to ChIP with 4 μ g of anti-Flag (M2) antibody. The IPs were each divided into two equal aliquots. One (1st IP: FLAG; upper panel) was de-cross-linked, and 1/10 of this was analyzed by 4%–12% gradient SDS-PAGE and immunoblotted with the indicated antisera. Half of each of the same aliquots was used to extract DNA that was analyzed by PCR for detection of B-myb promoter. The second aliquot was subjected to a second IP with 2 μ g of anti-HDAC1 (2nd IP, HDAC1; lower panel). After de-cross-linking, 1/10 of the IP was analyzed as above for HDAC1, 50% was used for PCR detection of B-myb promoter as above, and 40% to measure HMT activity as in Materials and Methods. (B) Derepression of the B-myb promoter by mutant E2F4–p130 fusion proteins compromised for interaction with corepressors. Neuronal PC12 cells were transfected with indicated constructs and processed as in Figure 3F. (C) Induction of neuron death by mutant E2F4–p130 fusion proteins compromised for interaction with corepressors. Cortical neurons were cotransfected with the indicated constructs, and eGFP and the number of transfected (eGFP⁺) cells were counted as in Figure 3E. (D) Phosphorylation-resistant E2F4–p130 fusion proteins protect neurons from apoptotic death. Cortical neurons were cotransfected with the indicated constructs and eGFP. One day later, the number of eGFP-positive cells was assessed (Day 0) and Cpt was added. eGFP-positive cells were counted on each of the following 3 d as in Figure 3E.

regulating neuron survival by tethering the corepressors HDAC1 and Suv39H1 to E2F4-binding promoters.

Discussion

A critical role for p130–E2F4 complexes in survival of post-mitotic neurons

Among key observations here are that p130 is the predominant Rb family member in neuronal E2F-containing complexes and that loss of p130 or its displacement from such complexes led to E2F derepression and apoptosis. In agreement with their paucity in neuronal E2F complexes, down-regulation of Rb or p107 evoked little neuron death. The selective survival function of p130 is not due to absence of Rb or p107 in neurons. Prior work detected both in neurons as well as their hyperphosphorylation and loss with apoptotic stimuli (Park et al. 2000).

Our findings are consistent with conditional and germ-cell knock-out studies on the role of Rb family members in brain development. Although loss of Rb leads to enhanced and ectopic neuroblast proliferation, its presence is not required for survival or differentiation

of post-mitotic neurons (Ferguson et al. 2002; MacPherson et al. 2003). Targeted deletion of p107 also had no observable effect on neuron survival (LeCouter et al. 1998a). In contrast, deletion of p130 led to neuron death in Balb/cJ mice by embryonic day 11–13 (E11–E13) (LeCouter et al. 1998b). Moreover, while post-mitotic Purkinje cells were normal in mice null for both pRb and p107 (Marino et al. 2003), they died when expressing a mutant SV40 T-antigen that binds p130 as well as pRb and p107 (Feddersen et al. 1995). In apparent contrast to findings on p130-null Balb/cJ mice, p130-null C57BL/6J mice had no apparent phenotype (LeCouter et al. 1998b). This led to the suggestion of a “second site modifier gene” (LeCouter et al. 1998b). In a related study, Cobrinik et al. (1996) reported no major phenotype in C57BL/6 mice null for either p130 or p107, but a major phenotype and lethality in mice null for both. This suggested that p107 and p130 have overlapping functions and that each fills in for the other in singly null animals. Thus, it appears likely that strain-dependent compensatory responses may account for lack of a consistent neuronal phenotype in p130-null mice.

Our observations are also consistent with prior developmental studies. p130 is up-regulated during brain de-

velopment and neuronal differentiation of P19 cells (Kusek et al. 2001), and p130 binding to the B-myb promoter rises during neuroblastoma differentiation (Raschella et al. 1997). Similarly, differentiation of PC12 cells and cortical and cerebellar neuroblasts is associated with elevated E2F4 levels and formation of p130–E2F4 complexes (Persengiev et al. 1999). The absence/low level of E2F–p107 complexes found here is in line with reports that p107 is highly expressed in proliferating neuroprogenitor cells but undetectable (Jiang et al. 1997) or at low levels (Baldi et al. 1997) in post-mitotic neurons.

Additional key findings here are that the chief partner for p130, at least on the critical B-myb promoter, is E2F4 and that the presence of E2F4 is essential for neuron survival. These are consistent with the roles of E2F4 as a major partner for p130 and in gene repression (Stevaux and Dyson 2002).

Loss of p130–E2F complexes occurs in response to apoptotic stimuli and mediate neuron death

Another key observation here is that apoptotic stimuli promote loss of nuclear hypophosphorylated p130 and of chromatin-associated p130–E2F/p130–E2F4 complexes in neuronal cells. Given its role in neuron death, it is particularly relevant that such complexes are lost from the endogenous B-myb promoter. The effects of apoptotic stimuli on p130 may not be limited to neurons; Fusaro et al. (2002) reported that Cpt leads to p130 phosphorylation and release of free E2F in Ramos B cells.

Our findings also indicate that loss of p130–E2F4 complexes is a required component of the mechanism by which apoptotic stimuli induce neuron death. Flavo, a neuroprotective agent with Cdk-inhibitory activity, prevented loss of hypophosphorylated p130 and of p130–E2F complexes. Moreover, phosphorylation-resistant p130 and E2F4–p130 fusion protein mutants effectively protected neuronal cells from apoptotic stimuli.

p130 gene repression/derepression and promotion of neuron survival and death: the role of corepressors and of chromatin modification

Active repression of E2F-regulated genes by Rb family members is achieved by recruitment of chromatin-modifying proteins to complexes with E2F (Stevaux and Dyson 2002). Thus, another key finding here is that the mechanism by which p130–E2F4 complexes promote neuron survival is via gene repression that requires their interaction with the chromatin-modifying corepressors HDAC and Suv39H1. Moreover, we found that apoptotic stimuli induce death by causing loss of such interactions. In support of this, p130 and p130–E2F4 fusion proteins mutated to abolish interaction with HDAC and Suv39H1 promoted neuron death, while phosphorylation-resistant E2F4–p130 fusion proteins that do not lose association with HDAC or Suv39H1 under apoptotic conditions were protective. Although our studies identified HDAC1 as a partner for p130 in neuronal cells, pre-

liminary findings indicate that other HDAC family members may also be involved in p130-mediated gene repression and neuron survival.

How might p130-tethered HDAC1 and Suv39H1 promote gene repression and neuron survival? One target for these enzymes is the N-terminal tail of histone H3. In the absence of corepressors, H3 is phosphorylated on Ser 10, and this facilitates acetylation of Lys 14. These modifications promote transcription and are essential for cell cycle progression in mitotically competent cells (Stevaux and Dyson 2002). When tethered to chromatin by Rb–E2F complexes, Suv39H1 methylates H3 residue Lys 9 (Rea et al. 2000; Nielsen et al. 2001). This, in turn, inhibits phosphorylation of Ser 10 and, in concert with HDACs, favors deacetylation of Lys 14. Such changes lead to condensation of local chromatin and gene silencing (Iizuka and Smith 2003). Consistent with this mechanism, we observed that levels of Ac-p-H3 associated with the endogenous B-myb promoter are low in viable neuronal cells and greatly increase in response to an apoptotic stimulus. In support of the involvement of HDAC and Suv39H1, we found that a death stimulus abolished the association between p130 and HDAC and substantially diminished levels of p130-associated HMT activity.

Myb as a death-associated target of E2F-mediated gene repression

An issue raised by our studies is the target of E2F-mediated gene repression that regulates neuron survival and death. A variety of observations support the closely related B-myb and C-myb genes in such a role. The promoters for these genes contain E2F-binding sites, and their expression is subject to E2F-dependent repression (Lam and Watson 1993; Catchpole et al. 2002). We found here and elsewhere (Liu and Greene 2001b) that apoptotic stimuli, including p130 down-regulation, induce Myb reporter activity in neuronal cells, and the findings here show that apoptotic stimuli lead to loss of repressive complexes containing E2F4–p130–HDAC–Suv39H1 from the endogenous B-myb promoter as well as changes in associated chromatin consistent with gene derepression. Moreover, B-myb and C-myb transcripts and proteins are significantly induced by apoptotic stimuli, and overexpression of B-myb and C-myb promotes neuron death (Freeman et al. 1994; Liu and Greene 2001b). Finally, down-regulation of B-myb and C-myb with antisense and siRNA constructs protects neurons from apoptotic stimuli (Liu et al. 2004).

A model for regulation of neuron survival and death

Our findings strongly support a repression/derepression model for regulation of neuron survival/death by E2F4–p130 and associated chromatin modifiers. They identify both the molecules and mechanisms by which p130 promotes silencing of E2F-responsive genes in viable neurons and by which apoptotic stimuli lead to derepression

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Figure 7. A model for the mechanism by which neuron survival and death are regulated by p130 and associated chromatin modifiers.

of E2F-responsive genes and death. Key aspects of this mechanism are depicted in Figure 7.

p130 has been implicated as a promoter of quiescence in nonneuronal cells and may well contribute to the post-mitotic state of neurons. Thus, stimuli that lead to dissolution of p130 complexes and that thereby trigger the derepression of pro-apoptotic genes such as *Mybs*, might concomitantly stimulate neurons to attempt cell cycle re-entry. Such a situation may explain not only why a variety of cell cycle markers are observed in neurons affected by injury and neurodegenerative disorders, but also why treatments targeted at suppressing derepression of E2F-regulated genes in neurons may have a therapeutic benefit in preventing neuron degeneration (Raina et al. 2000; El-Khodori et al. 2003; Greene et al. 2004). In this regard, the present findings provide several additional molecular targets for such an approach.

Materials and methods

Culture, transfection, and survival assays of PC12 cells and cortical neurons

These were previously described (Greene and Tischler 1976; Liu and Greene 2001b). Where used, BAF (100 μ M, Enzyme Systems Products) or Flavo (1 μ M) were added 4–6 h after transfection.

Subcellular fractionation, immunoprecipitation, Western blot analyses, luciferase reporter assay, quantitative PCR, and gel-shift assay

See Supplemental Material.

ChIP

ChIP was performed as previously detailed (Guan et al. 2002) except that the final concentration of formaldehyde used for cross-linking was 0.5%. Immunological reagents used were mouse anti-Rb (Pharmingen), mouse anti-p107 (NeoMarkers), rabbit anti-p130 (Transduction Laboratories), agarose-conjugated anti-E2F4 (Santa Cruz), anti-HA, Me-(K9), and p-(S10)-Ac-(K14) His H3 rabbit antiserum (Upstate).

HMTase assay

This assay was performed as previously described (Mizzen et al. 1999). All experiments were performed three to six times and are reported as mean $\pm$ SEM.

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