

The N-terminal Set- β Protein Isoform Induces Neuronal Death*

Received for publication, December 17, 2014, and in revised form, March 31, 2015. Published, JBC Papers in Press, April 1, 2015, DOI 10.1074/jbc.M114.633883

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Background: Set- β protein can suppress or promote neuronal recovery. We investigated Set- β isoforms to understand its different roles in neurons.

Results: Neurons express several Set- β transcripts, Set- β proteins localize to different compartments, and the N-terminal Set- β induces neuronal death.

Conclusion: N-terminal Set- β induces neuronal death. Other isoforms could play different roles.

Significance: Different Set- β isoforms could underlie its diverse roles in neurons.

Set- β protein plays different roles in neurons, but the diversity of Set- β neuronal isoforms and their functions have not been characterized. The expression and subcellular localization of Set- β are altered in Alzheimer disease, cleavage of Set- β leads to neuronal death after stroke, and the full-length Set- β regulates retinal ganglion cell (RGC) and hippocampal neuron axon growth and regeneration in a subcellular localization-dependent manner. Here we used various biochemical approaches to investigate Set- β isoforms and their role in the CNS, using the same type of neurons, RGCs, across studies. We found multiple alternatively spliced isoforms expressed from the Set locus in purified RGCs. Set transcripts containing the Set- β -specific exon were the most highly expressed isoforms. We also identified a novel, alternatively spliced Set- β transcript lacking the nuclear localization signal and demonstrated that the full-length (~39-kDa) Set- β is localized predominantly in the nucleus, whereas a shorter (~25-kDa) Set- β isoform is localized predominantly in the cytoplasm. Finally, we show that an N-terminal Set- β cleavage product can induce neuronal death.

Set- β , described as an oncogene (1, 2) and an epigenetic regulator of transcription (3–6), has different roles depending on subcellular localization, isoform, posttranslational modification, and cell type (7). The diversity of Set- β functions is represented in its naming; it is also known as template-activating factor (TAF)-I β (8), putative human leukocyte antigen class II-associated protein (9), inhibitor of GzmA-activated DNase (GAAD) (10), and inhibitor 2 of protein phosphatase 2A (I₂PP2A) (11–13). Set- β is a predominantly nuclear protein (14), and its subcellular localization is regulated by a nuclear localization signal (NLS)³ and phosphorylation (15–18). Set- β functions have also been described in the cytoplasm (19–22), and its subcellular localization and expression are altered in Alzheimer disease (23, 24). We showed recently that Set- β is developmentally up-regulated in retinal ganglion cells (RGCs), a type of CNS projection neuron, and that, although it is predominantly nuclear in RGCs, the Set- β signal has also been detected in the cytoplasm and adjacent to the cellular membrane (7). Set- β activity at the cellular membrane is necessary for developmental axon growth in the *Xenopus* optic nerve (19), and we recently showed that recruiting full-length Set- β to cellular membranes in mammalian cells promotes axon regeneration *in vivo*, whereas nuclear Set- β suppresses neurite growth in primary CNS neurons (7).

Loss of Set function has also been implicated in cell death. Proteolytic cleavage of Set- β at Lys-176 by granzyme A in an immune system model (10, 25) or at the adjacent asparagine (Asn-175) by asparagine endopeptidase in a stroke model (26) results in the release of GAAD from a multiprotein complex. When released from inhibition by full-length Set- β , GAAD causes cell death by nicking DNA (10, 27). The changes in

* This work was supported, in whole or in part, by NEI/National Institutes of Health Grants EY020913 (to J. L. G.), P30-EY022589 (to the Shiley Eye Center, University of California San Diego), and P30 EY014801 (to the University of Miami) and NINDS/National Institutes of Health Grant T32NS007492 (to E. F. T.). This work was also supported by American Heart Association Grant 11PRE7310069 (to E. F. T.), by the Walter G. Ross Distinguished Chair in Ophthalmic Research (to J. L. G.), by an unrestricted grant from Research to Prevent Blindness, Inc., by the Lois Pope LIFE fellowship and research awards (to E. F. T.), and by a summer research scholarship and the CAS Summer Research Program for Underrepresented Minorities and Women (to S. G. F.). M. P. V. is an employee and stockholder in Oncotide Pharmaceuticals, Inc. and an employee of Duke University Medical Center.

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³ The abbreviations used are: NLS, nuclear localization signal; RGC, retinal ganglion cell; P, postnatal day; RNA-seq, RNA sequencing; IHC, immunohistochemistry; GAAD, granzyme A-activated DNase.

The N-terminal Set- β Protein Isoform Induces Neuronal Death

expression of Set- β in Alzheimer disease brains, its role in neuronal death after stroke, and the regulation of RGC and hippocampal neuron axon growth and regeneration motivated us to investigate Set- β isoforms and their role in neuronal survival. Here we used primary CNS neurons, RGCs, to characterize the neuronal diversity of Set- β isoforms; to identify a novel, alternatively spliced Set- β transcript lacking an NLS; and to show that an N-terminal Set- β cleavage product can induce RGC death.

Experimental Procedures

Animals—All animal procedures were approved by the University of Miami Institutional Animal Care and Use Committee and by the Institutional Biosafety Committee at the University of Miami and were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research. C57BL/6J mice were obtained from Charles River Laboratories, Inc., and Sprague-Dawley rats were obtained from Harlan Laboratories, Inc. Animals of both sexes were used through the study in similar numbers.

Cell Culture and Reagents—RGCs were purified from early postnatal C57BL/6J mice or Sprague-Dawley rats by immunopanning using antibodies against Thy1 (CD90, MCA02R, Serotec, for mice; T11D7, ATCC, for rats) as described previously (7, 28). RGCs were electroporated immediately after purification, as described previously (7, 29) and plated at low density on poly-D-lysine (10 mg/ml) and laminin (1 mg/ml) (Life Technologies) in 48-well tissue culture plates (Falcon). RGCs were cultured for 1–3 days in defined growth medium consisting of Neurobasal medium (Life Technologies) supplemented with sodium pyruvate, *N*-acetyl cysteine, L-glutamine, Sato supplement, insulin, BDNF, CNTF, and forskolin, as described previously (28), and homemade supplement similar to B2, as described previously (7).

Constructs—Mammalian pcDNA3.1 expression vectors tagged N-terminally with Xpress for wild-type Set- β (a gift from Dianbo Qu, University of Ottawa), myc for Set- $\beta\Delta C$ (C terminus deleted from position Leu-176), FLAG for Set- α (a gift from Changying Guo and Judy Lieberman, Boston Children's Hospital, and Department of Pediatrics, Harvard Medical School), and mCherry in the pSPORT-6 vector. Expression in all constructs was driven by a CMV promoter.

PCR with Blocking Primers and Sequencing—The Quick-RNA MicroPrep kit (R1050, Zymo Research) was used for RNA extraction from acutely purified C57BL/6J mouse RGCs according to the instructions of the manufacturer, and RNA was measured with a Nanodrop 2000 Spectrophotometer (Thermo Scientific). 510 ng of total RNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad) according to the instructions of the manufacturer, and 1 μ l was used as a template for PCR with Phusion DNA polymerase (New England Biolabs) performed on DNA-Engine (Bio-Rad) with Set- β (forward, 5'-GCTCAACTCCAATCACGACGGG-3'; reverse, 5'-CCTCTTCGTCGTCATCATC-3') primers alone or with addition of non-complementary primers against the NLS sequence modified at their 3' end by addition of a seven-carbon linker terminating with NH₂ (AC7) to block transcription

(forward, 5'-AAACGTTTCGAGTCAAACGCAG-3'-AC7; reverse, 5'-CCTCTTCCTGCTGGCTTTATT-3'-AC7) (Sigma). Blocking primers were added at 3 and 20 times the concentration of Set- β primers, respectively. The PCR product was run on the gel and imaged as above. The 158-base pair gel band was cut out and reamplified without the blocking primers, and its PCR-product was cloned into the pCR2.1 vector using the TA cloning kit (Life Technologies) and sequenced (Eurofins, Huntsville, AL). Visualization of the alignment to the mouse genome and transcripts was done using the Genomatix genome browser (Genomatix Inc.).

Immunocytochemistry, Imaging, and Immunofluorescence Intensity Quantification—Sprague-Dawley P8 rat eyes were dissected and fixed for 2 h in 4% paraformaldehyde after puncturing the cornea, washed three times in PBS, incubated in 30% sucrose at 4 °C, washed three times in PBS, cryopreserved in OCT with liquid nitrogen, and cryosectioned (30 μ m). Retinal cryosections were mounted on Superfrost Plus micro slides (VWR); blocked and permeabilized with 20% goat serum and 0.5% Triton X-100 for 1 h; incubated overnight at 4 °C with primary antibodies, Set- β (1:200, epitope 1, amino acids 3–14), or Set- α/β (epitope 2, amino acids 45–56, both gifts from Suzanne Specht, NCI/NIH), and Brn3A (1:100, catalog no. MAB1585, Millipore); washed three times in PBS; incubated overnight at 4 °C with DAPI (1:3000, Life Technologies) and Alexa fluorophore-conjugated secondary antibodies (1:500, Life Technologies); washed three times in PBS, mounted in ProLong Gold (catalog no. P36934, Life Technologies) with a glass coverslip; and imaged using a confocal microscope at $\times 40$ (LSM710, Zeiss). The nuclear and cytoplasmic average pixel intensity of Set immunofluorescence in RGCs was measured in Brn3A+ cells in the ganglion cell layer with AxioVision 4.8 (Zeiss). Three biological replicates with at least 85 randomly selected RGCs per experiment were subjected to statistical analysis with a two-tailed Student's *t* test (SPSS).

Immunostaining of cultured RGCs was performed similarly, with fixation for 30 min in 4% paraformaldehyde, and blocking and permeabilization with 10% goat serum and 0.2% Triton X-100 for 30 min. Primary antibodies included rabbit anti-Set- β (epitope 1) or anti-Set- α/β (epitope 2, as above), mouse anti-Xpress (1:300, catalog no. R91025, Life Technologies), mouse anti-myc (1:300, catalog no. sc-40, Santa Cruz Biotechnology), rabbit anti-Tuj1 (1:350, catalog no. MRB-435P, Covance, Emeryville, CA), and mouse anti-Tuj1 (1:350, catalog no. MMS-435P, Covance). Images of transfected cells were acquired at $\times 20$ on an AxioObserver.Z1 (Zeiss).

Survival Assay—Acutely purified P4 RGCs transfected with mCherry, Xpress-tagged wild-type Set- β , and myc-tagged Set- $\beta\Delta C$ were plated at low density in 48-well tissue culture plates in defined growth medium as above. Two hours after transfection, survival was assessed by counting calcein-positive (catalog no. C3100MP, Life Technologies) or sytox-positive (catalog no. S11368, Life Technologies) cells costained with Hoechst (catalog no. H1399, Life Technologies). Three biological replicates each were counted over 1300 cells/condition in each experiment. Statistical analysis was performed with analysis of variance and post-hoc LSD, with the day of the experiment treated as a random (blocking) factor. In parallel, at 1, 2, and 3 days, the

number of RGCs per unit area transfected with mCherry, Xpress-tagged wild-type Set- β , and myc-tagged Set- $\beta\Delta C$ (immunostained as above) were counted using AxioVision 4.8 (Zeiss) and normalized to 1 day (106 cells/condition). Statistical analysis was performed using analysis of variance with repeated measures and post hoc LSD (SPSS).

Cell Fractionation and Western Blots—10 million P5 rat RGCs acutely purified as above were separated into cytoplasmic and nuclear fractions, as described previously (30). Briefly, cells were washed twice in 1 ml of Dulbecco's PBS by centrifuging for 5 min at $600 \times g$ at 4°C , resuspended in $40 \mu\text{l}$ of cytoskeleton buffer (10 mM Pipes (pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl_2 , 1 mM EGTA, and 0.5% Triton X-100) per 1 million cells. The upper half of the supernatant was isolated as the cytoplasmic fraction, and the pellet was washed as above and resuspended in $20 \mu\text{l}$ of cell lysis buffer (catalog no. 9803S, Cell Signaling Technology) per 1 million cells as the nuclear fraction. Fractions were sonicated (Bioruptor UCD-200TN-EX, Diagenode, Liege, Belgium) at 4°C five times for 1 min with 20-s intervals with the output selector switched to high, and stored immediately at -80°C . Protease (catalog no. 11836153001, Roche) and phosphatase (catalog no. 78428, Pierce) inhibitors were added at 1 tablet/10 ml or $1\times$, respectively, recommended dilutions. Fraction lysates were immunoblotted with rabbit anti-GAPDH (1:2000, $^{14}\text{C}10$, Cell Signaling Technology) and rabbit anti-acetyl-histone H3 (1:3000, catalog no. 06599, Millipore). RGC fraction lysates were also immunoblotted with rabbit anti-Set- β (epitope 1) or rabbit anti-Set- α/β (epitope 2) (1:6000).

For Western blotting of whole cell protein, P5 rat RGCs were acutely purified as above. The retinal suspension cells that did not adhere to the anti-Tuj1-coated immunopanning dish were used as RGC-depleted retinal suspension cells. For P5 cortical homogenates, acutely dissected rat cortices were frozen in liquid nitrogen and homogenized using a cold mortar and pestle; resuspended in buffer (catalog no. 87787, Pierce) with protease inhibitors (catalog no. 11836153001, Roche) and phosphatase inhibitors (78428, Pierce) at 1 tablet/10 ml or $1\times$, respectively; sonicated as above; centrifuged at $12,000 \times g$ for 10 min at 4°C ; and then the supernatant was stored immediately at -80°C . Protein concentrations were measured using a BCA microassay, and $25 \mu\text{g}$ of protein extract from each sample was immunoblotted with rabbit anti-Set- β (epitope 1, 1:1000), mouse anti-Set- α (1:1000, see below), and GAPDH (1:2000, $^{14}\text{C}10$, Cell Signaling Technology). P5 RGC and cortical homogenates protein extracts were also immunoblotted using rabbit anti-Set- α/β (epitope 2, 1:6000).

For Western blotting, lysates in LDS sample buffer ($4\times$, catalog no. NP0007, Life Technologies) with 10 mM DTT were incubated at 98°C for 5 min, electrophoresed on 8–16% NuPAGE SDS-PAGE gradient gel (Life Technologies), transferred to posttransferred PVDF membranes (Millipore), blocked in 3% BSA and 0.1% Tween 20 (pH 7.6) for 1 h, incubated at 4°C overnight in block with primary antibodies, washed in PBST three times, incubated with HRP-conjugated anti-rabbit IgG (1:2500, Santa Cruz Biotechnology) for 2 h at room temperature, washed in PBST three times, and developed with SuperSignal West Pico chemiluminescent substrate (cat-

alog no. 34077, Thermo Scientific). The blots were imaged using LAS3000 (Fujifilm) and processed with Multi Gauge (Fujifilm).

Anti-Set- α monoclonal antibody was produced by standard methods. Briefly, the Set- α isoform-specific peptide KRQS-PLPPQKKKPRC in the N terminus was synthesized by solid phase methods, purified on HPLC, and coupled with a bis-maleimide to keyhole limpet hemocyanin (KLH). BALB/c mice were immunized with KLH-peptide conjugates and boosted until titers were sufficiently high, and then spleens were harvested and used for cell fusions. Limiting dilution cloning was used to isolate a single hybridoma per well on a 96-well tissue culture plate, and conditioned media from these hybridomas were screened for immunoreactivity with the KLH-peptide conjugates. The hybridoma (clone 5F4D9) that reacted with the Set- α but not with Set- β antigen (KELNSNHDGADETC) was expanded and used for ascites fluid production in naive BALB/c mice. IgG1 antibodies from ascites fluid were further concentrated by affinity chromatography on protein A/G-agarose and elution from the resin, and titers of greater than 1:50,000 were accepted. The resulting anti-Set- α (clone 5F4D9) was also tested (using 1:1000 dilution) on PC-3 and DU145 cell-line protein extracts in parallel with goat anti-Set- α/β (E-15, 1:1000; catalog no. 5655, Santa Cruz Biotechnology). For further validation of antibody specificity, HEK293 cells were cultured in DMEM (Life Technologies) with 5% inactivated FCS and penicillin/streptomycin to 70% confluence in a 6-well plate and transfected using Lipofectamine 3000 (catalog no. L3000001, Life Technologies) with $5 \mu\text{g}$ of Set- β , Set- α , or mCherry plasmids. The medium was changed at 24 h, and the protein was collected at 48 h. Cells were washed twice with PBS with 1 mM EDTA and lysed using cell lysis buffer (catalog no. 9803S, Cell Signaling Technology) with PMSF (catalog no. 8553, Cell Signaling Technology), an inhibitor of serine proteases. Immunoblotting with anti-Set- β , anti-Set- α , and anti-GAPDH antibodies was performed as described above for RGCs and cortical homogenates.

Results

The Set locus encodes multiple alternatively spliced isoforms (Fig. 1A). Set- β isoforms differ from the closely related Set- α isoforms by the 24-amino acid first exon, derived from an alternative promoter and start site (8, 31). RNA-seq data suggest that Set- β transcripts are expressed significantly higher in primary RGCs compared with Set- α on the basis of the relatively lower expression of the Set- α -specific exon compared with the Set- β -specific exon (Fig. 1A). To test whether Set- β is the primary isoform in RGCs on a protein level, we analyzed Set- α and Set- β protein expression in RGCs by Western blot analysis (Fig. 1B) using a validated Set- β -specific antibody (7, 14) against an epitope encoded by the Set- β -specific first exon (*epitope 1*, Fig. 1A) and a novel Set- α antibody against an epitope encoded by the alternative Set- α -specific first exon (*epitope 3*, Fig. 1A).

We validated the specificity of Set- α antibody on PC-3 and DU145 cell lines by showing that Set- α antibody detected only one of the two bands detected by the nonspecific Set- α/β antibody (Fig. 1C). The difference in molecular weight at which Set- α ran on the gel in cell lines and neurons could be due to

The N-terminal Set- β Protein Isoform Induces Neuronal Death

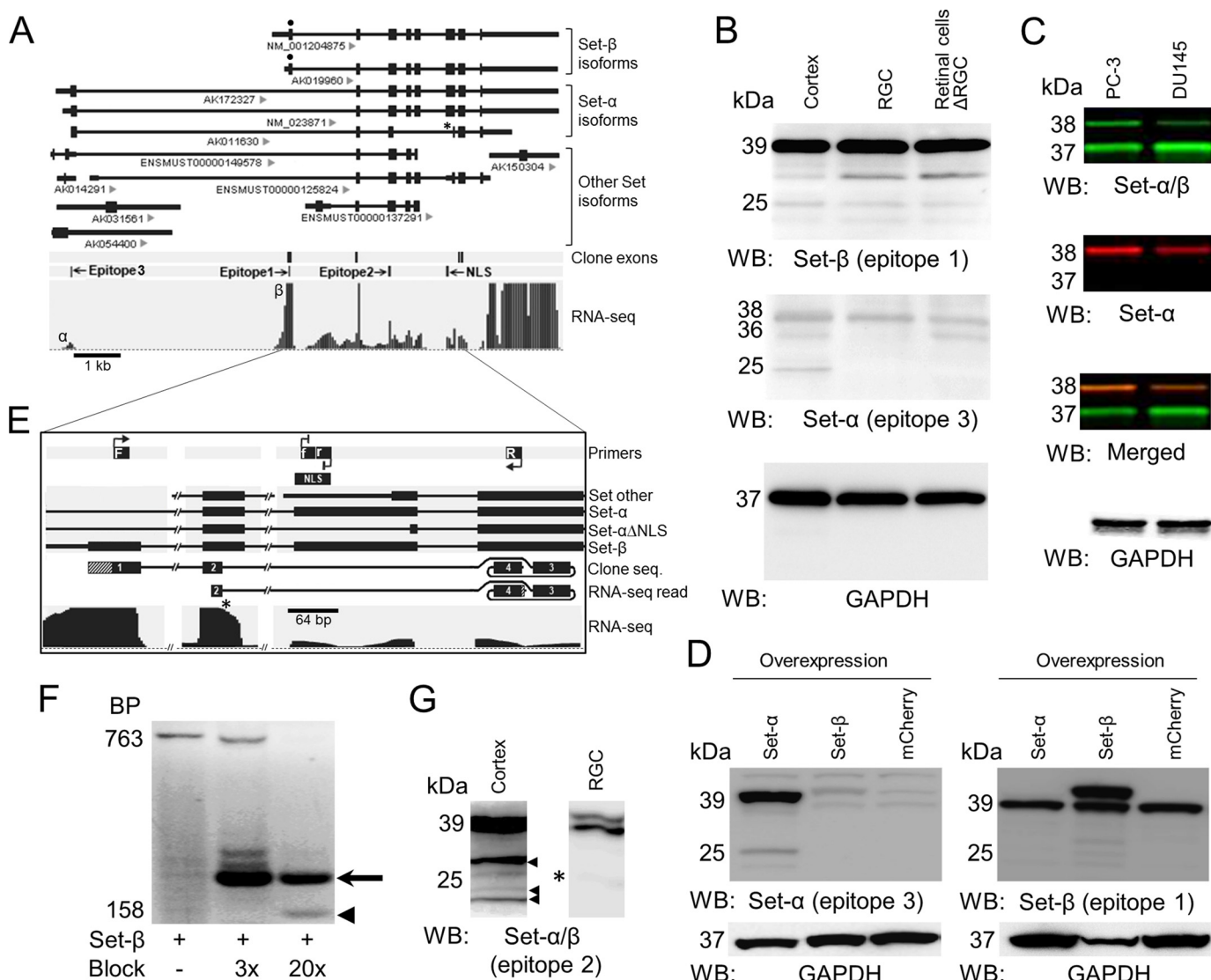


FIGURE 1. Expression of Set- α and Set- β isoforms in primary neurons and a novel alternatively spliced Set- β isoform lacking the NLS. *A*, alternatively spliced Set isoforms from public databases aligned to the genome. Identification is indicated under the transcripts. Epitopes for Set antibodies used through the study are indicated. Epitope 1 is unique to Set- β -specific exon (\bullet), epitope 3 is unique to the Set- α specific exon, and epitope 2 is found in both isoforms. The asterisk indicates a Set- α isoform lacking an NLS sequence (Set- $\alpha\Delta$ NLS). Poly(A)-selected RNA-seq reads from purified P5 RGCs mapped uniquely to the Set locus (the raw data reads used for construction of this figure are available upon request). Reads aligned to the Set- β -specific exon (β) showed a significantly higher peak compared with the Set- α -specific exon (α). Transcripts and alignment to the genome were visualized using the Genomatix genome browser. Set locus RNA-seq raw reads data are available upon request. *B*, immunoblotting of P5 cortical homogenates, purified RGCs, and retinal cell- Δ RGC (as marked) for Set- β (epitope 1) and Set- α (epitope 3) revealed the predominance of full-length Set- β isoforms at 39 kDa and a weaker 25-kDa signal across samples. Set- α (epitope 3) detected lower molecular weight bands in cortical homogenates and retinal cell- Δ RGC but not in RGCs. *WB*, Western blot. *C*, human cell-lines, PC-3 and DU145, probed with anti-Set- α (epitope 3) and anti-Set- α/β antibodies, demonstrate the specificity of anti-Set- α antibody, which identified only one of two isoforms detected by the nonspecific anti-Set- α/β antibody. *D*, HEK293 cell line transfected with Set- α , Set- β , or mCherry constructs, probed with anti-Set- α (epitope 3) and anti-Set- β (epitope 1) antibodies, demonstrate the specificity of, and lack of cross-reactivity between, anti-Set- α and anti-Set- β antibodies. *E*, schematic of RT-PCR using a Set- β -specific forward primer (*F*), a reverse primer (*R*) from the exon past the NLS, and internally nested forward and reverse blocking primers against the NLS sequence modified at their 3' ends to prevent PCR product elongation (*f* and *r*). RNA-seq read is 100% matching sequence identified in RNA-seq raw reads data. The lined area at 3' corresponds to the 8-bp sequence past the clone sequence, which also aligned 100% to the exon. *F*, RT-PCR on RNA from acutely purified P5 RGCs showed increasing detection of a 158-bp product (arrowhead, clone seq in *B*) over the 763-bp band corresponding to the full-length Set- β at increasing concentrations of blocking primers 3 times and 20 times more than the Set- β primers. A higher molecular weight band (arrow) resulted from transcription initiated by the blocking primers (confirmed by sequencing because of impurities or imperfect blockade). *G*, immunoblotting of P5 cortical homogenates and purified RGCs (as marked) for Set- α/β (epitope 2) indicated the predominance of 39-kDa band, consistent with the full-length Set- β isoforms, and a weaker 25-kDa signal (asterisk) in both samples. Additional lower molecular weight bands (arrowheads) were detected in cortical homogenates but not in RGCs.

differences in posttranslational modifications by species (human *versus* rat) or by cell type (prostate gland *versus* CNS). We also confirmed the specificity of Set- α and Set- β antibodies by immunoblotting protein extracts from HEK293 cells transfected with the Set- α , Set- β , and mCherry constructs (Fig. 1*D*). The Set- α antibody signal was not different between Set- β - and

mCherry-transfected cells, but a strong band of the expected molecular weight (\sim 38–39 kDa) was detected in Set- α -transfected cells. Conversely, the Set- β antibody signal was not different between Set- α - and mCherry-transfected cells, but a strong band of the approximate expected molecular weight (\sim 39.5 kDa) was detected in Set- β -transfected cells right above

the endogenously expressed Set- β band (the exogenous Set- β may have run slightly slower on the gel because of the Xpress fusion tag).

Using the validated antibodies, we found that Set- β signal intensity was stronger in purified P5 RGCs and P5 cortical homogenates. Although direct quantitative comparisons between different antibodies is not possible, the weak Set- α signal in CNS tissues using an antibody that demonstrated a strong signal in non-CNS tissues (Fig. 1C) is consistent with the literature reporting that Set- β is predominant in the brain relative to Set- α , whereas the opposite pattern was observed in tissues such as the spleen (8), and it is also consistent with our RNA-seq data detecting significantly higher Set- β -specific exon compared with Set- α -specific exon expression in primary RGC neurons (Fig. 1A).

Furthermore, we detected a lower molecular weight Set- β isoform (~25 kDa) in P5 RGCs and P5 cortical homogenates, but no Set- α lower molecular weight isoform was detected in RGCs, although a similarly sized species was detectable in cortical homogenates (Fig. 1B). Therefore, RGCs express at least two detectable Set- β species, the full-length 39-kDa and a smaller 25-kDa isoform but no detectable lower molecular weight Set- α isoform.

Because in our prior work we detected Set- β expression by *in situ* immunohistochemistry (IHC) in ganglion cells and the inner nuclear layers but not the outer nuclear layer of the retina (7), here we tested, by Western blot analysis, whether Set isoform expression differs between RGCs and other retinal cells. We did not find a notable difference in Set- β expression level or pattern between RGCs and other retinal cells (Fig. 1B). Set- α full-length isoform (~38 kDa) expression, although weak, was not different between RGCs and other retinal cells, but ~36 kDa and ~25 kDa isoforms were detectable in other retinal cells and cortical homogenate but not in RGCs (Fig. 1B).

Set- α transcript isoforms include splice variants lacking the NLS, which could result in their localization to the cytoplasm (Fig. 1A), but Set- β transcripts lacking the NLS sequence have not been reported to date. Although we and others have shown that Set- β is predominantly nuclear, Set- β has also been detected in RGC cytoplasm and at cellular membranes with the same Set- β -specific antibody we used here (7, 14). We therefore asked whether RGCs might express an alternatively spliced Set- β transcript lacking the NLS sequence similar to the Set- α isoform. To detect potentially rare transcripts using RT-PCR, we designed blocking primers against the NLS sequence with 3'-modified ends to prevent elongation by DNA polymerase (Fig. 1E). We then performed RT-PCR on purified RGC RNA using Set- β primers along with different concentrations of these NLS blocking primers. PCR with Set- β primers without blocking NLS showed that the full-length Set- β -encoding transcript (763-bp band) is the most highly expressed Set- β isoform in RGCs (Fig. 1F), consistent with our Western blot analysis (Fig. 1B). Several weaker signal bands were also detected lower on the gel, but their specificity is unknown. The addition of NLS blocking primers at increasing concentrations has enriched for a 158-bp band while preventing the amplification of the predominant 763-bp band (Fig. 1F). Sequencing of the 158-bp band confirmed that this was indeed a Set- β transcript lacking

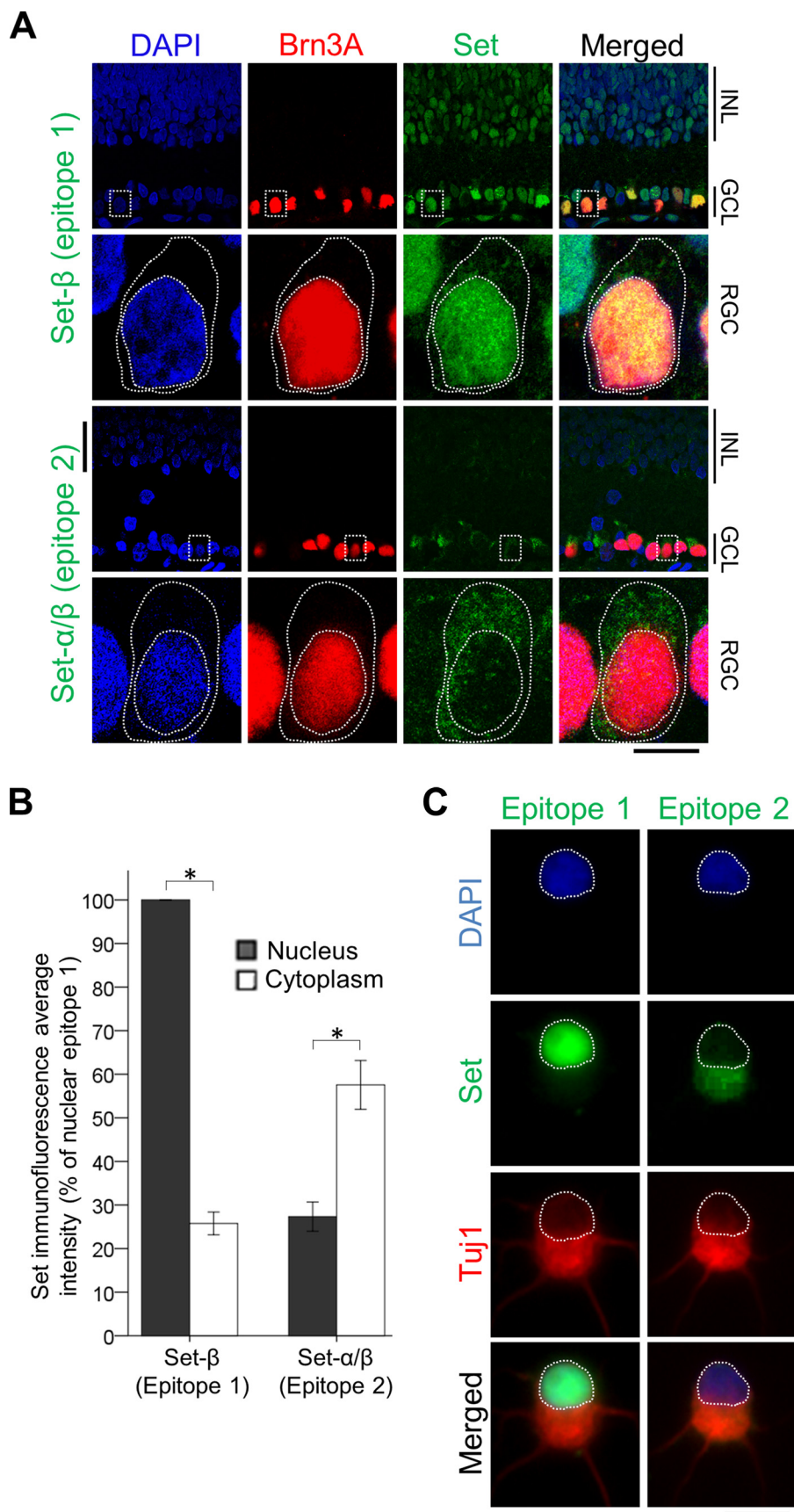
the NLS sequence (Fig. 1E, GenBank accession number KR054743), and a BLAST search of mouse databases did not find a significant match, confirming that this is a novel transcript.

We then manually searched raw RNA-seq reads from P5 RGCs and found an exact match that mapped uniquely to a part of this transcript and extended 8 bp 3' beyond the clone sequence (Fig. 1E, *RNA-seq read*). The low frequency of the matching reads in RNA-seq data and the low intensity of the 158-bp band on PCR without blocking primers suggest that this is a low-abundance isoform in RGCs. This novel transcript has several unique features that, along with its low abundance, may have hindered its identification in previous analyses: the 3' end of the second exon aligns to an onset of decline in RNAseq reads (Fig. 1E, *asterisk*), suggesting the existence of a short *de novo* exon within the annotated exon; the rearranged exons 3 and 4 aligned within the annotated Set exon and correspond to peaks of the bimodal distribution of reads within the annotated exon, supporting the expression of these shorter *de novo* rearranged exons; and our data suggest a *de novo* splice junction between the rearranged short exons (Fig. 1E). Because of these unique features we were not able to reliably reconstruct from RNA-seq the full-length sequence of this isoform. However, it could be a coding transcript because the RNA-seq data were generated from poly-A-selected transcripts.

We next examined the subcellular localization of Set- β protein using different antibodies. Set- β *in situ* IHC in RGCs detected a predominantly nuclear signal and a weaker signal in the cytoplasm and at cellular membranes (7). However, nuclear and cytoplasmic endogenous Set- β variants physically interact with different proteins in primary CNS neurons, which could differentially affect the accessibility to the N-terminal epitope for the antibody used in that study (*epitope 1*, Fig. 1A) (7). We therefore asked whether the low Set- β signal detected in the cytoplasm was specific to the N-terminal epitope 1. To address this question, we performed *in situ* IHC in RGCs using an antibody to a mid-region epitope of Set (*epitope 2*, Fig. 1A) (7), which is absent from the novel Set- β transcript lacking the NLS sequence (Fig. 1, A and E). We found that although the Set- β -specific N-terminal epitope 1 immunoreactivity was predominantly nuclear, with a weak signal also detected in the cytoplasm, the mid-region Set epitope 2 immunoreactivity was predominantly cytoplasmic, with only a weak signal detected in the nucleus (Fig. 2, A and B). Immunostaining of RGCs *in vitro* with these Set antibodies showed a similar pattern (Fig. 2C). This unexpected result suggests that either there is a difference in isoform localization or a difference in N-terminal and/or mid-region Set epitope masking in RGCs.

Therefore, we next examined protein expression with these antibodies on Western blot analysis. We found that full-length Set- β (~39 kDa) was the predominant isoform detected by both antibodies in P5 cortical homogenates and in P5 purified RGCs (Fig. 1, B and G). In cortical homogenates but not in purified RGCs, the epitope 2 antibody also detected lower molecular weight bands (Fig. 1G, *arrowheads*) not detected by the N-terminal antibody (Fig. 1B). The ~25-kDa lower molecular weight band was detected by both antibodies in cortical homogenates and in RGCs (Fig. 1, B and G, *asterisk*). The sim-

The N-terminal Set- β Protein Isoform Induces Neuronal Death



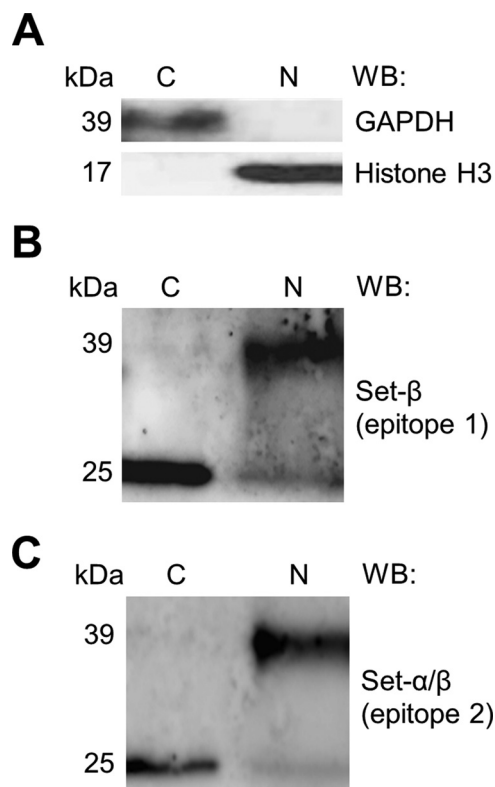


FIGURE 3. Set- β protein isoforms demonstrate differential localization. A–C, P5 RGC nuclear (N) and cytoplasmic (C) fractions immunoblotted for GAPDH (cytoplasmic marker), histone H3 (nuclear marker) (A), and Set- β (epitope 1) (B) and Set- α/β (epitope 2) (C) revealed that the full-length 39-kDa Set- β is predominantly nuclear, whereas the 25-kDa Set- β isoform is predominantly cytoplasmic in acutely purified RGCs. WB, Western blot.

ilarity between band patterns detected with the two antibodies in RGCs is consistent with the hypothesis that the full-length (~39 kDa) Set- β is the predominant isoform detected by both antibodies in RGCs, that the mid-region epitope 2 is not readily detectible by *in situ* IHC in RGC nuclei, and that a lower molecular weight (~25-kDa) N-terminal Set- β isoform is expressed in RGCs.

Epitope 2 is also present in Set- α , and although the Set- α signal in RGCs is weak, the epitope 2 signal in the cytoplasm may originate from the full-length Set- α . We therefore investigated the nuclear and cytoplasmic distribution of Set isoforms in RGCs using antibodies against epitopes 1 and 2. We separated nuclear and cytoplasmic fractions of purified P5 RGCs and analyzed them by Western blot, confirming successful fractionation with GAPDH (cytoplasmic) and histone H3 (nuclear) markers (Fig. 3A). Western blot analysis with either Set antibody showed that the full-length Set- β is predominantly nuclear, whereas the lower molecular weight (~25-kDa) isoform localized mostly to the cytoplasm (Fig. 3, B and C). The predominance of the lower molecular weight (~25-kDa) iso-

form in the cytoplasm, detected similarly with both epitopes, suggests that the epitope 2 signal detected in the cytoplasm by *in situ* IHC originates from the Set- β and not the Set- α isoform because Western blot analysis for Set- α did not detect a lower molecular weight (~25-kDa) isoform in RGCs (although it was detected in cortical homogenates, see above). Together, these results suggest that the full-length (~39-kDa) Set- β isoform is predominantly nuclear in RGCs and that a shorter (~25-kDa) N-terminal Set- β isoform is predominantly cytoplasmic in RGCs.

To investigate the localization and function of the truncated N-terminal Set- β isoform in neurons, we expressed an N-terminal Set- β fragment mimicking the N-terminal Leu-176 cleavage product (Set- $\beta\Delta C$, Fig. 4A) in purified primary RGCs *in vitro*. Full-length Set- β and mCherry constructs were used for controls. We found that, after 1 day in culture, Set- $\beta\Delta C$ distribution was similar in the cytoplasm and the nucleus compared with full-length Set- β , which localized predominantly in the nucleus (Fig. 4B). Therefore, although exogenously expressed full-length Set- β preferentially localizes to the nucleus, truncated Set- β localizes to both the nucleus and the cytoplasm, consistent with the nuclear/cytoplasmic fractionation and detection of the 39-kDa and 25-kDa isoforms noted in Fig. 3.

We next asked whether Set- $\beta\Delta C$ affects axon growth or survival. There was no difference in RGC survival 2 h after transfection with Set- $\beta\Delta C$, full-length Set- β , or mCherry control plasmids, demonstrating that acute transfection toxicity was not different between conditions (Fig. 4, B–D). Unexpectedly, we found that, by 3 days *in vitro*, all RGCs transfected with Set- $\beta\Delta C$ died (Fig. 4, B and E), demonstrating cytoplasmic swelling after 2 days *in vitro* (Fig. 4B, arrow) and leaving behind small pyknotic nuclei and fragmented cytoplasmic remnants (Fig. 4B, arrowhead). These data suggest that the N-terminal Set- β isoform resulting from a cleavage at Leu-176 localizes to the nucleus and the cytoplasm and is sufficient to induce neuronal death.

Discussion

Together, these data suggest that the Set locus encodes multiple isoforms expressed in the CNS, including Set- α and novel Set- β isoforms lacking an NLS. In RGCs, Set- β is the predominantly expressed isoform compared with Set- α , and full-length Set- β protein is localized predominantly in the nucleus. A shorter, ~25-kDa Set- β isoform detected in RGCs by Western blot analysis is expressed at significantly lower levels compared with full-length Set- β and is localized predominantly in the cytoplasm. A similar molecular weight, ~25-kDa Set- β isoform has been reported in K562 and HeLa cell lines as a byproduct of Set- β cleavage induced by infection or stroke injury (10, 25, 26). However, an ~25-kDa Set- β was also detected in HeLa cells

FIGURE 2. Set- β expression and developmental regulation in RGCs. A, P8 retinal sections were immunostained for Set- β (epitope 1), Set- α/β (epitope 2), and Brn3A (RGC marker), and counterstained with DAPI (nuclear marker) as marked. Example RGCs and their nuclei are outlined with dashed white lines in enlarged images. Scale bar = 100 μ m (main panels) and 20 μ m (enlarged images). GCL, ganglion cell layer; INL, inner nuclear layer. B, analysis of nuclear and cytoplasmic average pixel intensity of Set epitope 1 and 2 immunofluorescence in RGCs showed that Set- β (epitope 1) immunoreactivity is predominantly nuclear, whereas Set- α/β (epitope 2) immunoreactivity is predominantly cytoplasmic ($n = 3$, ≥ 85 randomly selected RGCs/experiment. Data are mean \pm S.E. *, $p < 0.01$ by two-tailed Student's *t* test). C, P8 RGCs immunostained after 1 day in culture for endogenous Set- β (epitope 1) and Set- α/β (epitope 2) (green), Tuj1 (neuronal marker, red), and DAPI (nuclear marker). Scale bar = 20 μ m. Nuclei are outlined with dashed white lines.

The N-terminal Set- β Protein Isoform Induces Neuronal Death

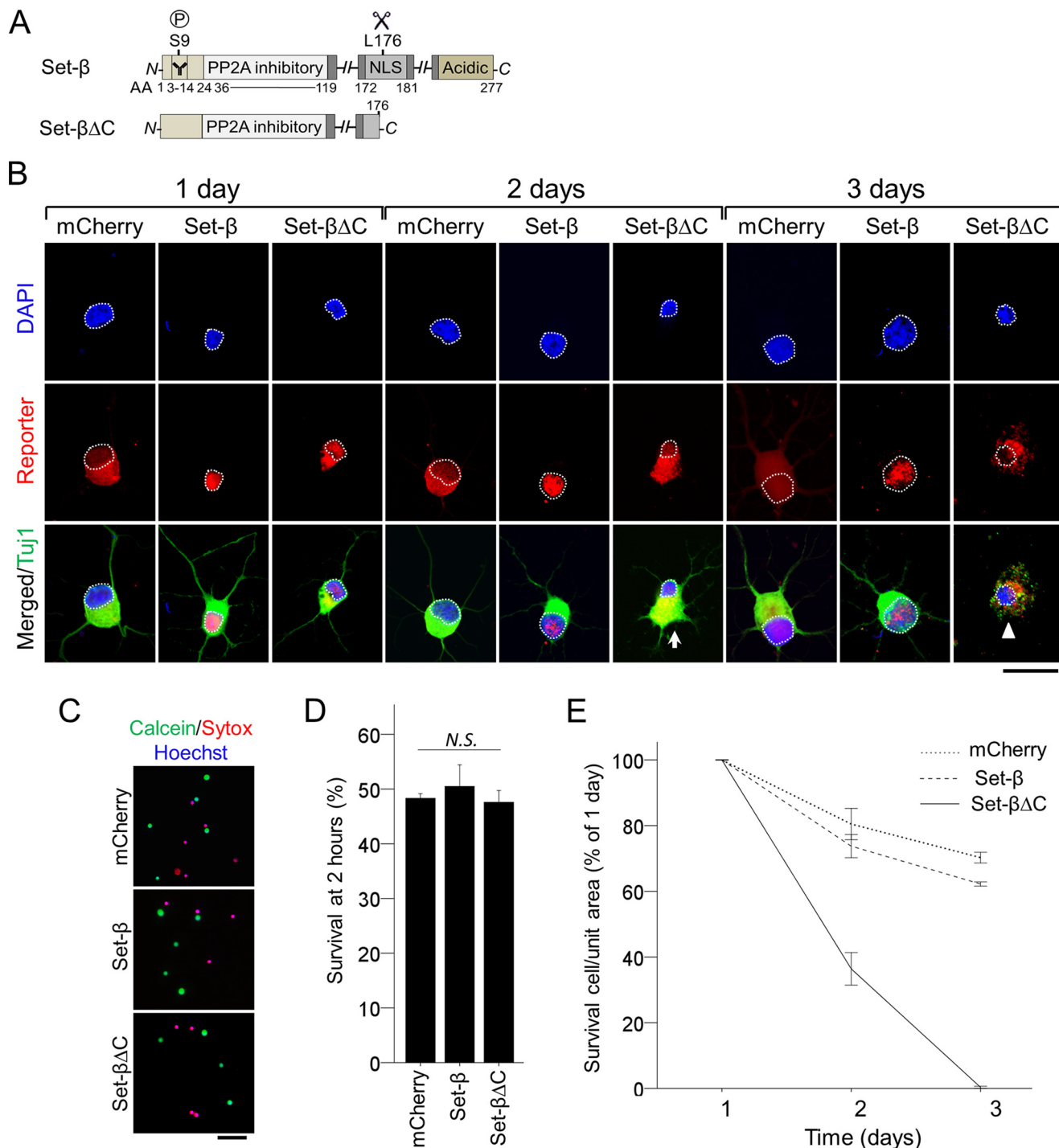


FIGURE 4. Set- $\beta\Delta C$ localizes to the cytoplasm and initiates cell death in cultured RGCs. *A*, full-length and truncated Set- β constructs, highlighting the serine 9 phosphorylation site (P), β isoform-specific antibody epitope (Y), PP2A inhibitory domain, NLS containing lysine 176 cleavage site (scissors), and acidic C-terminal domain deleted in the mutant construct (Set- $\beta\Delta C$). *B*, acutely purified P4 RGCs transfected with mCherry, full-length Set- β , or Set- $\beta\Delta C$ were immunostained at 1, 2, and 3 days for fusion reporter tags (red) and Tuj1 (neurite marker, green) and counterstained with DAPI (nuclear marker, blue). After 1 day in culture, full-length Set- β localized to the nucleus, whereas Set- $\beta\Delta C$ localized to both the nucleus and cytoplasm. By 2–3 days, morphologic characteristics of cell death, including cytoplasmic swelling (arrow) and cellular fragmentation (arrowhead), were apparent in Set- $\beta\Delta C$ -transfected RGCs. Nuclei are outlined with white dashed lines. Scale bar = 20 μ m. *C*, RGC survival after transfection was assessed by counting calcein-positive (live cell marker) or sytox-positive (dead cell marker) RGCs counterstained with Hoechst (nuclear marker). Example pictures from 2 h are shown. *D*, 2 h after transfection with full-length Set- β , Set- $\beta\Delta C$, or mCherry, survival was similar across conditions ($n = 3$, ≥ 1300 cells/condition in each experiment). Data are mean \pm S.E. N.S., not significant by analysis of variance. *E*, at 1, 2, and 3 days, transfected RGCs were counted per unit area normalized to 1 day (106 cells/condition). Set- $\beta\Delta C$ significantly increased cell death. Data are mean \pm S.E. $p < 0.01$ by analysis of variance with repeated measures with post hoc LSD.

that were not experimentally stressed or infected (8). Therefore, it remains to be investigated whether the low abundance, lower molecular weight Set- β isoform detected by Western blot

analysis in RGC cytoplasm is the cleavage product described previously (8, 10, 25, 26), the alternatively spliced isoform lacking an NLS identified here, or another isoform regulated by

transcriptional or posttranslational mechanisms. In any case, future work may be directed toward identifying what the neuronal function of the shorter is under normal physiological conditions and in response to insult.

In contrast to the results obtained by Western blot analysis, *in situ* IHC using the mid-region epitope 2 Set antibody detected a stronger signal in RGC cytoplasm. We showed previously that endogenous Set- β variants physically interact with different proteins in primary CNS neuron nuclear and cytoplasmic compartments using immunoprecipitation and mass spectrometry (7). Although this could be an artifact of technical nuances, an interesting possible explanation is that this mid-region Set epitope 2 is masked in RGC nuclei by Set- β nuclear binding partner(s). Exploring the relevance of the epitope 2 region through the use of deletion constructs or interfering peptides could lead to additional understanding of the potential importance of this region of the Set protein in neurons.

Our finding that exogenous expression of the N-terminal Set- β cleave product mimic (Set- β DC) is sufficient to induce RGC death is an intriguing addition to the prior literature. Cleavage of Set- β has been implicated previously in cell death as a loss-of-function phenotype: only full-length Set- β can inhibit the GAAD DNase. When GAAD is released from inhibition after enzymatic cleavage of the full-length Set- β in response to stress, unguarded GAAD nicks DNA and leads to cell death (10, 27). Our findings raise the possibility that the N-terminal Set- β cleavage product itself is sufficient to induce cell death in a gain-of-function fashion. It will be important to investigate in future studies whether this occurs in other cell types and *in vivo* after CNS insult or in neurodegeneration (*e.g.* in a glaucoma model). Two possible mechanisms could explain these data. Either the N-terminal Set- β cleavage product disrupts the full-length Set- β -GAAD complex, leading to cell death through a dominant-negative effect, or the N-terminal Set- β cleavage product leads to cell death through an entirely different molecular mechanism. Exogenous expression of the C-terminal Set cleavage product in the rat brain has also been shown to be neurotoxic (32) and may also act by disrupting the full-length Set- β -GAAD complex or a different molecular mechanism. Therefore, understanding the dominance or synergy of Set- β cleavage products and GAAD in inducing neurodegeneration and cell death would be important for the future development of neuroprotective therapies.

Considering the opposite roles full-length Set- β plays in neurons, promoting axon growth when localized to the cytoplasm/cellular membranes but suppressing axon growth when localized to the nucleus (7), it will be important to further investigate Set- β isoforms and dynamics after CNS injury and in neurodegenerative disease. For example, after optic nerve injury, nuclear Set- β levels do not appear to change, whereas cytoplasmic Set- β levels decrease slightly (7). However, in Alzheimer disease, Set- β translocates to the cytoplasm and may be cleaved as after stroke or infection (23, 24). The role of Set- β in Alzheimer disease is unknown. Cytoplasmic Set- β inhibits PP2A (23), which may lead to tau and amyloid precursor protein hyperphosphorylation and neurofibrillary degeneration (32–36), and Set- β ΔNLS exacerbated camptothecin-induced death of cortical neurons *in vitro* but had no effect without

camptothecin (15). Our studies showed that overexpressing PP2A-A (scaffold subunit) suppresses RGC neurite growth *in vitro*, and its localized inhibition may mediate axon regeneration induced by recruiting the full-length Set- β to neuronal membranes (7). Therefore, exploring to what degree these different cellular responses reflect neuroprotective or neurotoxic roles of Set- β *in vivo* and identifying the underlying isoforms and molecular mechanisms, particularly through the development of isoform-specific knockdown techniques, may lead to novel biological insights and/or therapeutic approaches.

Acknowledgments—We thank Dianbo Qu (University of Ottawa) for the Xpress-Set- β construct, Changying Guo and Judy Lieberman (Cellular and Molecular Medicine Program, Boston Children's Hospital, and Department of Pediatrics, Harvard Medical School) for the FLAG-Set- α construct, and Suzanne Specht, (NCI-Frederick) for Set antibodies. We also thank Eleut Hernandez for animal husbandry; George McNamara and Gabe Gaidosh for advanced imaging; William Hulme, Ryan Gentry, and Daniel Pita-Thomas for next-generation sequencing (NGS); Genomatix Software, Inc. for NGS analysis; and Lei Cao, Morton Terrell, Kapil Gupta, and Kristina Russano for technical assistance.

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