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(54) Titre : IDENTIFICATION DE LA PROTEINE CRMP4 EN TANT QUE REGULATEUR CONVERGENT DE
L'INHIBITION DE LA CROISSANCE DES AXONES

(54) Title: IDENTIFICATION OF CRMP4 AS A CONVERGENT REGULATOR OF AXON OUTGROWTH INHIBITION

(57) **Abrégé/Abstract:**

The present invention relates to the identification of the cytosolic phosphoprotein CRMP4b as a protein that physically and functionally interacts with RhoA to mediate neurite outgrowth inhibition. siRNA-mediated knockdown of CRMP4 promotes neurite outgrowth on myelin substrates indicating a critical role for CRMP4 in neurite outgrowth inhibition. Disruption of CRMP4b-RhoA binding with a competitive inhibitor attenuates neurite outgrowth inhibition on myelin and aggrecan substrates. Stimulation of neuronal growth cones with Nogo leads to co-localization of CRMP4b and RhoA at discrete regions within the actin-rich central and peripheral domains of the growth cone indicative of a potential function in cytoskeletal rearrangements during neurite outgrowth inhibition. Together these data indicate that a RhoA-CRMP4b complex forms in response to inhibitory challenges in the growth cone environment and regulate cytoskeletal dynamics at distinct sites necessary for axon outgrowth inhibition. Competitive inhibition of CRMP4b-RhoA binding suggests a novel, highly specific therapeutic avenue for promoting regeneration following CNS injury.



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**IDENTIFICATION OF CRMP4 AS A CONVERGENT REGULATOR OF AXON
OUTGROWTH INHIBITION**

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims the benefit of U.S. Provisional Application No. 60/783,030 filed on March 17, 2006, which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

10 The present invention relates to a novel application in the field of neurobiology. More specifically, the present invention concerns the discovery of a novel CRMP4-RhoA interaction and its role in myelin inhibition. It has been found that specific disruption of this CRMP4-RhoA interaction circumvents myelin-dependent inhibition of neuron regeneration. Modulation of the CRMP4-RhoA interaction and of CRMP4 activity may be useful for a
15 number of applications, including the repair of injured central nervous system (CNS) or peripheral nervous system (PNS) axons. A specific CRMP4-RhoA complex antagonist has been identified that renders neurons insensitive to myelin-dependent neurite outgrowth inhibition.

20 **BACKGROUND OF THE INVENTION**

 Trauma in the adult mammalian central nervous system (CNS) results in devastating clinical consequences due to the failure of injured axons to spontaneously regenerate. Chondroitin sulphate proteoglycans (CSPGs) and the myelin-associated inhibitors (MAIs)
25 myelin-associated glycoprotein (MAG) (McKerracher et al., 1994; Mukhopadhyay et al., 1994), Nogo-A (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000) and Oligodendrocyte-myelin glycoprotein (OMgp) (Kottis et al., 2002; Wang et al., 2002) bind receptor molecules on injured axons initiating intracellular signaling cascades which block axonal regrowth (Mandemakers and Barres, 2005). In part, CSPGs and MAIs inhibit axon
30 regeneration by disrupting Rho-GTPase-dependent cytoskeletal dynamics. Blockade of RhoA and a downstream effector Rho kinase (ROCK) promotes axon regeneration both *in vitro* and *in vivo* (Dergham et al., 2002; Borisoff et al., 2003; Fournier et al., 2003); however, the ability of RhoA and ROCK to affect multiple physiological processes in many cell types

(Riento and Ridley, 2003) highlights the need to identify novel intracellular signaling substrates of neurite outgrowth inhibition to develop more specific and potent therapeutic avenues.

5 The CRMPs are a family of cytosolic phosphoproteins with five vertebrate family members (CRMP1-5) (Goshima et al., 1995; Minturn et al., 1995; Byk et al., 1996; Gaetano et al., 1997; Inatome et al., 2000). CRMP1-4 alleles each produce two transcripts, a and b, and CRMPb variants are longer amino terminal variants of the originally identified CRMPa isoforms (Yuasa-Kawada et al., 2003). Although CRMPs share significant sequence similarity with dihydropyrimidinase (DHPase), an enzyme involved in pyrimidine catabolism, 10 no DHPase activity has been described for CRMPs (Wang and Strittmatter, 1997). Rather, a role for CRMPs in axon growth and pathfinding has been revealed. CRMPs are homologs of UNC-33, a protein that influences axon guidance and extension in *C. elegans* (Hedgecock et al., 1985; Siddiqui and Culotti, 1991). CRMP2 mediates growth cone collapse in response to the repulsive guidance cue Semaphorin3A (Goshima et al., 1995), and CRMP2 and CRMP4 15 influence neurite outgrowth (Minturn et al., 1995; Quinn et al., 1999; Quinn et al., 2003; Yoshimura et al., 2005). Mechanistically, CRMP2 can bind to tubulin heterodimers and organizes microtubule assembly to establish axon-dendrite fate during development (Fukata et al., 2002b; Arimura et al., 2005) and CRMP4 can promote F-actin bundling (Rosslenbroich et al., 2005). Further, a role for CRMP2 in endocytosis has been described (Nishimura et al., 20 2003) and an association between CRMP4b and intersectin, an endocytic-exocytic adaptor protein, is consistent with an endocytic role for this isoform (Quinn et al., 2003).

The role of the CRMP proteins in nervous system injury and regeneration has not been extensively studied; however CRMP2a does have a potent neurite elongating effect in nerve regeneration *in vivo* (Suzuki et al., 2003). CRMP1a, CRMP2a and CRMP5a mRNA 25 levels increase after hypoglossal nerve injury and CRMP4a expression is increased in regenerating adult sciatic motor neurons (Minturn et al., 1995; Suzuki et al., 2003) suggesting a more general role for CRMP proteins in the neuronal response to injury.

SUMMARY OF THE INVENTION

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In accordance with an embodiment of the present invention, there is provided a novel method to promote regeneration of neurons after injury to the CNS or PNS. More specifically, this method comprises the use (or administration) of an antagonist to CRMP4-RhoA

interaction in order to circumvent myelin-dependent inhibition of neuron regeneration. C4RIP has been identified as an antagonist of this type.

The surprising finding that CRMP4b interacts with RhoA in a Nogo-dependent manner led to the investigation of the potential role of this complex in the inhibition of neuron regeneration. This in turn resulted in the subsequent determination that antagonism of CRMP4 or of the CRMP4b-RhoA interaction attenuates neuron regeneration inhibition. This protein-protein interaction represents a novel, specific target for therapeutic intervention following CNS or PNS injury.

One of the advantages of the method of the present invention resides in the fact that the antagonist can be applied directly at the site of injury (i.e., *in situ*) in order to promote the regeneration of neurons.

The present invention further includes methods to identify compounds that disrupt CRMP4-RhoA interaction and that may thus be used to promote the regeneration of neurons.

In a screen to identify molecules that functionally interact with RhoA to mediate neuron regeneration inhibition, collapsin-response mediator protein 4b (CRMP4b) was identified as a molecule that interacts with Rho GTPase in a Nogo-dependent manner.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non restrictive description of preferred embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: A novel RhoA-CRMP4 interaction is enhanced by Nogo-66. (a) GSTRhoA63L pull down from PC12 cells stimulated with AP (+ lysate) or AP-Nogo-66 (+ Nogo lysate). Precipitation of a 75 kDa protein identified as CRMP4b by tandem mass spectrometry is enhanced in the AP-Nogo-66-stimulated lysate (arrow). The protein runs slightly below a non-specific protein from the GSTRhoA63L purification, which is visible in all lanes. The GSTRhoA63L lane represents beads that were not incubated with lysates. (b) GST and GSTRhoA63L pull downs from PC12 cell lysates immunoblotted with a pan-CRMP antibody. CRMPa (65 kDa) and CRMPb (75kDa) bands are indicated in the PC12 input and pull down lanes (arrows).

Figure 2: The RhoA-CRMP4 interaction is highly specific, nucleotide-independent, phospho-dependent and direct. (a-c) HEK293T cells co-transfected with

CRMP-V5 constructs and myc-tagged versions of wild type and mutant Rho GTPases and subjected to myc-immunoprecipitation. (a) CRMP4 preferentially binds to RhoA. Data from two separate blots are separated by a vertical line. (b) RhoA is the preferred binding partner for CRMP4. (c) CRMP4 binding to RhoA is nucleotide-independent. (d) CRMP-V5 was immunoprecipitated from control or Calyculin-treated HEK293T cells, separated by SDS-PAGE and overlaid with recombinant RhoA. RhoA binds directly to CRMP4a and CRMP4b but fails to bind to phosphorylated CRMP4b. IP, immunoprecipitation.

Figure 3: Nogo-P4 peptide enhances the interaction between CRMP4b and RhoA. (a, b) PC12 cells transfected with myc-RhoA and CRMP4b-V5 (a) or CRMP4a-V5 (b) and, stimulated for 0, 1 or 10 minutes with Nogo-P4 peptide and subjected to myc-immunoprecipitation. (c) P8 rat cerebellar cultures stimulated with Nogo-P4 peptide, subjected to RhoA immunoprecipitation and analyzed for RhoA and CRMP4b. IP, immunoprecipitation.

Figure 4: siRNA-mediated knockdown of CRMP4 expression promotes neurite outgrowth on myelin. (a) HEK293T cells co-transfected with CRMP4-V5 and scrambled- or CRMP4-targeted siRNA and analyzed by immunoblotting with anti-V5 antibody. (b) Dissociated rat DRG neurons infected with HSVCRMP4b-GFP and transfected with scrambled or CRMP4 siRNA. Scale bar, 100 μ m. (c) β III tubulin-stained dissociated rat DRG neurons transfected with scrambled or CRMP4 siRNA and seeded on laminin (control) or myelin substrates for an 18 hour neurite outgrowth assay. Scale bar, 100 μ m. (d) Quantitation of DRG outgrowth on control substrates with scrambled siRNA (Scram) or CRMP4 siRNA. (e) Quantitation of DRG neurite outgrowth on myelin from neurons transfected with scrambled or CRMP4 siRNA. Values are normalized to baseline outgrowth on the control laminin substrate for each experiment. Determinations are from 3 experiments performed in duplicate. * p <0.01 by student t-test compared to scrambled siRNA.

Figure 5: CRMP4b overexpression promotes an actin-based filopodial phenotype in the neuronal growth cone and neurite. (a) E13 chick DRG stained with an anti-CRMP4b antibody. (b) E7 chick DRG neurons infected with HSV-CRMP4b-V5 and stained with anti-V5 antibody. (c-d) E7 chick DRG neurons infected with HSV-CRMP4b-GFP and double stained with rhodamine phalloidin (c) to label F-actin or anti- β III tubulin antibody (d). (e) E13 chick DRG neurons infected with HSV-GFP or HSV-CRMP4b-V5. GFP and CRMP4b-infected growth cones were stained with rhodamine phalloidin (red). CRMP4b-V5

was stained with anti-V5 antibody (green). Scale bar, 10 μ m. **(f)** Magnification of boxed regions in panel (d) demonstrating enhanced neurite branching and filopodial extension in CRMP4b-V5-infected DRG neurons. **(g)** Quantitation of number of branches per neurite and filopodial length in GFP- or CRMP4b-V5-infected DRG neurons.

5 **Figure 6: CRMP4b and RhoA co-localize at discrete punctae during myelin-dependent growth cone collapse.** Immunofluorescent detection of endogenous CRMP4b and RhoA **(a)** or CRMP4b-V5 and RhoA **(b)** in control or myelin-stimulated E13 chick DRG growth cones. Arrows in the merged panels indicate areas of CRMP4b-V5-RhoA co-localization.

Figure 7: The amino terminal domain of CRMP4b is sufficient for RhoA binding and disrupts full length CRMP4b-RhoA binding when expressed as a recombinant fusion protein. **(a)** Schematic of CRMP constructs generated to assess CRMP4 domains involved in RhoA binding. **(b)** HEK293T cells co-transfected with myc-RhoA63L and CRMP-V5 constructs described in (a) and subjected to myc-immunoprecipitation. **(c)** HEK293T cells co-transfected with myc-RhoAWT, CRMP4-V5 and C4RIP-V5 to assess the effect of C4RIP on full length CRMP4 binding to RhoA. **(d)** HEK293T cells co-transfected with FLAG-RhoA63L, myc-ROCK and C4RIP-V5 to assess the effect of C4RIP on RhoA binding to ROCK.

Figure 8: C4RIP-V5 attenuates myelin inhibition. **(a)** E13 dissociated chick DRG neurons infected with HSV-C4RIP-V5 or HSV-CRMP4b-V5 and double-stained with anti-V5 and anti- β III tubulin antibodies to validate neuronal infection. **(b)** E13 dissociated chick DRG neurons infected with C4RIP-V5 or CRMP4b-V5 plated on laminin (control) or myelin substrates and stained with anti- β III tubulin to visualize neurite outgrowth. **(c-e)** Quantitation of neurite outgrowth from GFP-, C4RIP-, or CRMP4b-infected neurons on laminin (c), myelin (d) or aggrecan (e) substrates. In (c) neurite outgrowth per cell is normalized to outgrowth in GFP-infected neurons for each experiment (100%). In (d) and (e), neurite outgrowth is normalized to growth on the laminin substrate for each dose curve (100%). Determinations are from 4 separate experiments. Scale bar, 100 μ m. * p <0.05 and ** p <0.01 by student t-test compared to GFP. **(f)** Quantitation of growth cone collapse in E8 chick DRG neurons infected with HSV-GFP or HSV-C4RIP and stimulated for 20' with control AP ligand or AP-Sema3A ligand. Determinations are from 3 separate experiments performed in duplicate.

30 **Figure 9: Rat CRMP4b amino acid and cDNA Sequence Date.** **(a)** Rat CRMP4b amino acid sequence with C4RIP shaded in grey (SEQ ID NO: 1); **(b)** Rat C4RIP amino acid

sequence (SEQ ID NO: 2); (c) Rat CRMP4b cDNA sequence with C4RIP shaded in grey (SEQ ID NO: 3); (d) Rat C4RIP cDNA sequence (SEQ ID NO: 4); (e) Rat CRMP4a amino acid sequence (SEQ ID NO: 5); and (f) Rat CRMP4a cDNA nucleotide sequence (SEQ ID NO: 6).

5 **Figure 10: Examples of membrane-translocating sequences that may be suitable for therapeutic applications involving C4RIP or derivatives, analogs, variants or fragments of C4RIP. (a) C4RIP sequence with C3-05 internalizing sequence (SEQ ID NOS: 8 and 9); (b) C3-05 MTS (SEQ ID NO: 10); (c) C4RIP sequence with Kaposi MTS (SEQ ID NOS: 11 and 12); and (d) Kaposi MTS**

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DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

15 Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are now described. All publications mentioned herein are incorporated by
20 reference for the purpose of describing and disclosing the cell lines, vectors, and methodologies which are reported in the publications and which might be used in connection with the invention. The terminology used herein is for the purpose of describing particular embodiments only and is not intended to limit the scope of the present invention.

25 Use of the singular forms "a," "an," and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, reference to "a target polynucleotide" includes a plurality of target polynucleotides.

30 As used in this specification and claim(s), the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "include" and "includes") or "containing" (and any form of containing, such as "contain" and "contains"), are inclusive or open-ended and do not exclude additional, unrecited elements or process steps.

 The term "about" is used to indicate that a value includes an inherent variation of error for the device or the method being employed to determine the value.

The term "CRMP4", as used herein, is intended to include the native CRMP4 peptide as well as any biologically active fragment(s) or analog(s) thereof, including but not limited to CRMP4a and CRMP4b. The terms "fragment" and "analog" are used interchangeably herein to describe CRMP4 peptides useful in the methods of the present invention, as described
5 more particularly in the Detailed Description.

The term "CRMP4" also encompasses variants and functional analogs of CRMP4 having a homologous amino acid sequence with a CRMP4 peptide. The present invention thus includes pharmaceutical formulations comprising such CRMP4 variants and functional analogs, carrying modifications like substitutions, deletions, insertions, inversions or
10 cyclisations, but nevertheless having substantially the biological activity as CRMP4.

The term C4RIP means, in the context of the present invention, CRMP4b-RhoA Inhibitory Peptide, a peptide that represents the first 126 residues within the unique amino terminal domain of CRMP4b. (See Figure 9.) The term "C4RIP", as used herein, is intended to include the native C4RIP peptide as well as any biologically active fragment(s) or analog(s) thereof.

15 The terms "fragment" and "analog" are used interchangeably herein to describe C4RIP peptides useful in the methods of the present invention. The present invention thus includes pharmaceutical formulations comprising such C4RIP variants and functional analogs, carrying modifications like substitutions, deletions, insertions, inversions or cyclisations, but nevertheless having substantially the biological activity as C4RIP.

20 The term "analog" refers to a peptide that is related to CRMP4 but which has been modified. This modification does not, however, alter the biological activity of the interaction domain. Reasons for modifications include, but are not limited to, increasing the peptide's stability and solubility, decreasing the probability of denaturation, modifying its ability to be post-translationally modified, reducing manufacturing costs and enhancing large-scale
25 manufacturing.

The term "RhoA", as used herein, is intended to include the native RhoA peptide as well as any biologically active fragment(s) or analog(s) thereof. The terms "fragment" and "analog" are used interchangeably herein to describe RhoA peptides useful in the methods of the present invention.

30 The term "RhoA" also encompasses variants and functional analogs of RhoA having a homologous amino acid sequence with a RhoA peptide. The present invention thus includes pharmaceutical formulations comprising such RhoA variants and functional analogs, carrying

modifications like substitutions, deletions, insertions, inversions or cyclisations, but nevertheless having substantially the biological activity as RhoA.

The term “antagonist” refers, in the context of the present invention, to a molecule that inhibits the biological activity normally associated with CRMP4-RhoA complex formation or interaction or that inhibits CRMP4 function. Examples of antagonists include, but are not limited to C4RIP, C4RIP analogs and C4RIP derivatives.

The term “siRNA” means, in the context of the present application, small interfering RNA. Gene silencing or knockdown can be achieved through the use of synthetic, small interfering RNA.

The term “derivative” denotes, in the context of a functional derivative of a sequence whether a nucleic acid or amino acid sequence, a molecule that retains a biological activity (either function or structural) that is substantially similar to that of the original sequence. This functional derivative or equivalent may be a natural derivative or may be prepared synthetically. Such derivatives include amino acid sequences having substitutions, deletions, or additions of one or more amino acids, as well as chemical mimics, provided that the biological activity of the protein is conserved. The same applies to derivatives of nucleic acid sequences that can have substitutions, deletions, or additions of one or more nucleotides, provided that the biological activity of the sequence is generally maintained. When relating to a protein sequence, the substituting amino acid generally has chemico-physical properties that are similar to that of the substituted amino acid. The similar physicochemical properties include, similarities in charge, bulkiness, hydrophobicity, hydrophilicity and the like. The term derivative is intended to include fragments, segments, variants, analogs or chemical derivatives, including non-peptide chemical derivatives, of the subject matter of the present invention.

For purposes of the present invention, the terms “neurite outgrowth” and “neuron regeneration” are used synonymously.

The term “variant” as used herein is a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide, respectively. A typical variant of a polynucleotide differs in nucleotide sequence from another reference polynucleotide. Differences in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, insertions, deletions, fusions, and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a

polypeptide differs in amino acid sequence from another reference polypeptide. Generally, differences are such that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, insertion, deletions
5 in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be naturally occurring such as an allelic or pseudoallelic variant, including polymorphisms or mutations at one or more bases, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis
10 techniques or by direct synthesis. The term "mutant" is encompassed by the above definition of non-natural variants.

"splice variants" as referred to hereinafter are variants, which result from the differential or alternative splicing and assembly of exons present in a given gene. Typically, the encoded proteins will display total identity in most regions, but lower identity in the specific
15 region encoded by different exons.

A "deletion", as used herein, refers to a change in either amino acid or nucleotide sequence in which one or more amino acids or nucleotide residues, respectively, are absent, as compared to a reference polypeptide or polynucleotide.

An "insertion" or "addition", as used herein, refers to a change in an amino acid or
20 nucleotide sequence resulting in the addition of one or more amino acid or nucleotide residues, respectively, as compared to a reference polypeptide or polynucleotide.

A "substitution", as used herein, refers to the replacement of one or more amino acids or nucleotides by different amino acids or nucleotides, respectively, as compared to a reference polypeptide or polynucleotide.

The term "derivative", as used herein, refers to the chemical modification of CRMP4 or C4RIP. Illustrative of such modifications would be replacement of hydrogen by an alkyl, acyl, or amino group. A derivative of CRMP4 or C4RIP could also be generated by expression in a recombinant system using an appropriately modified DNA construct. Derivatives may or may not retain some of all of the essential biological characteristics of the natural molecule.
25

The term "identity" as used herein refers to a measure of the extent of identical nucleotides or amino acids that two or more polynucleotide or amino acid sequences have in common. In general, the sequences are aligned so that the highest order match is obtained, referred to as the "alignment". Such optimal alignments make use of gaps, which are inserted
30

to maximize the number of matches using local homology algorithms, such as the Smith-Waterman alignment. The terms "identity", or "similarity", or "homology", or "alignment" are well known to skilled artisans and methods to perform alignments and measure identity are widely described and taught in the literature: Dayhoff et al., Meth Enzymol 1983; 91: 524--
 5 Lipman D J and Pearson W R, Science 1985; 227: 1435--Altschul et al., J Mol Biol 1990; 215: 403.--Pearson W R, Genomics 1991; 11: 635.--Gribskov M and Devreux J, eds. (1992) Sequence Analysis Primer, WH Freeman & Cie, New-York.--Altschul et al., Nature Gen 1994; 6: 119. Furthermore, methods to perform alignments and to determine identity and similarity are codified in computer programs and software packages, some of which may also be web-
 10 based and accessible on the Internet. Preferred software includes but is not limited to BLAST (Basic Local Alignment Search Tools), including Blastn, Blastp, Blastx, tBlastn (Altschul et al., J Mol Biol 1990; 215: 403), FastA and TfastA (Pearson and Lipman, PNAS 1988; 85: 2444), Lasergene99 (DNASTAR, Madison Wis.), Omiga 2.0 or MacVector (Oxford Molecular Group, Cambridge, UK), Wisconsin Package (Genetic Computer Group (GCG), Madison, Wis.),
 15 Vector NTI Suite (InforMax Inc., N.Bethesda, Md.), GeneJockey II (Biosoft, Cambridge, UK).

As an illustration, by a polynucleotide having a nucleotide sequence with at least, for example, 95% "identity" to a reference nucleotide sequence of SEQ ID NO:3, SEQ ID NO:4 or SEQ ID NO:6, it is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five
 20 point mutations, or divergent nucleotides, per 100 nucleotides of the reference nucleotide sequence of SEQ ID NO:3, SEQ ID NO:4 or SEQ ID NO:6. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total
 25 nucleotides in the reference sequence may be inserted into the reference sequence.

These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more continuous groups within the reference sequence.

30 Similarly, by a polypeptide having an amino acid sequence having at least, for example, 95% "identity" to a reference amino acid sequence of SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:5, it is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino

acid alterations for every 100 amino acids of the reference amino acid sequence of SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:5. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence, or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more continuous groups within the reference sequence.

The term "biologically active" or "biological activity", as used herein, refer to a protein having structural, regulatory, biochemical, electrophysiological or cellular functions of a naturally occurring molecule. Likewise, "immunologically active" refers to the capability of the natural, recombinant, or synthetic CRMP4, C4RIP or RhoA, or any oligopeptide thereof, to induce a specific immune response in appropriate animals or cells and to bind with specific antibodies.

The term "modulate", as used herein, refers to a change or an alteration in the biological activity of CRMP4 or C4RIP. Modulation may be an increase or a decrease in protein activity, a change in binding characteristics, or any other change in the biological, functional or immunological properties of CRMP4 or C4RIP.

The term "portion", as used herein, with regard to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid. Thus, a protein "comprising at least a portion of the amino acid sequence of SEQ ID NO:1, SEQ ID NO:2 or SEQ ID: NO:5" encompasses the full-length CRMP4, C4RIP or fragments thereof.

In another embodiment of the invention, polynucleotide sequences or fragments thereof which encode CRMP4 or C4RIP, or fusion proteins or functional equivalents thereof, may be used in recombinant DNA molecules to direct expression of CRMP4 or C4RIP. Due to the inherent degeneracy of the genetic code, other DNA sequences that encode substantially the same or a functionally equivalent amino acid sequence may be produced and these sequences may be used to clone and express CRMP4 or C4RIP.

In another embodiment of the invention, a natural, modified, or recombinant polynucleotide encoding CRMP4 or C4RIP may be ligated to a heterologous sequence to

encode a fusion protein. For example, to screen peptide libraries for inhibitors of CRMP4-RhoA interaction, it may be useful to encode a chimeric CRMP4 or C4RIP protein that can be recognized by a commercially available antibody. A fusion protein may also be engineered to contain a cleavage site located between a CRMP4 or C4RIP encoding sequence and the
5 heterologous protein sequence, so that CRMP4 or C4RIP may be cleaved and purified away from the heterologous moiety.

In another embodiment, the coding sequence of CRMP4 or C4RIP may be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers et al., Nuc. Acids Res. Symp. Ser. 1980; 215-23; Horn et al., Nuc. Acids Res. Symp. Ser.
10 1980; 225-232). Alternatively, the protein itself may be produced using chemical methods to synthesize the amino acid sequence of CRMP4 or C4RIP, or portions thereof. For example, peptide synthesis can be performed using various solid-phase techniques (Roberge et al., Science 1995; 269: 202) and automated synthesis may be achieved, for example, using the ABI 431A Peptide Synthesizer (Perkin Elmer).

15 The newly synthesized peptide may be substantially purified by preparative high performance liquid chromatography e.g., Creighton T. (1983) "Proteins, Structures and Molecular Principles", W. H. Freeman & Co., New York, N.Y.). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; Creighton T (1983), supra). Additionally, the amino acid
20 sequences of CRMP4 or C4RIP, or any parts thereof, may be altered during direct synthesis and/or combined using chemical methods with sequences from other proteins, or any part thereof, to produce a variant polypeptide.

In order to express a biologically active CRMP4 or C4RIP, the nucleotide sequence encoding CRMP4 or C4RIP or functional equivalents may be inserted into an
25 appropriate expression vector, i.e., a vector, which contains the necessary elements for the transcription and translation of the inserted coding sequence.

Methods that are well known to those skilled in the art may be used to construct expression vectors containing a CRMP4 or C4RIP coding sequence and appropriate transcriptional and translational control elements. These methods include *in vitro* recombinant
30 DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Such techniques are described in "Molecular Cloning: A Laboratory Manual", Sambrook J, Ed., CSHL Press, 1989, Cold Spring Harbor, N.Y., and "Current Protocols in Molecular Biology", Ausubel et al., John Wiley & Sons, 1989, New York, N.Y.

A variety of expression vector/host systems may be utilized to contain and express a CRMP4 or C4RIP coding sequence. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems
5 infected with virus expression vectors (e.g., baculovirus); plant cell systems transformed with virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (e.g., Ti or pBR322 plasmids); or animal cell systems.

The "control elements" or "regulatory sequences" are those non-translated regions of the vector--enhancers, promoters, 5' and 3' untranslated regions--which interact with host
10 cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the Bluescript.RTM. phagemid (Stratagene, La Jolla, Calif.) or
15 pSport1.TM. plasmid (Gibco BRL) and ptrp-lac hybrids, and the like may be used. Other preferred prokaryotic vectors include but are not limited to pQE-9, pQE60, pQE70 (Quiagen), pNH8A, pNH16a, pNH18a, pNH46A (Stratagene) ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). The baculovirus polyhedrin promoter may be used in insect cells. Promoters or enhancers derived from the genomes of plant cells (e.g., heat shock,
20 RUBISCO; and storage protein genes) or from plant viruses (e.g., viral promoters or leader sequences) may be cloned into the vector. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are preferable. If it is necessary to generate a cell line that contains multiple copies of the sequence encoding CRMP4 or C4RIP, vectors based on SV40 or EBV may be used with an appropriate selectable marker.

25 In bacterial systems, a number of expression vectors may be selected depending upon the use intended for CRMP4 or C4RIP. For example, when large quantities of CRMP4 or C4RIP are needed for the induction of antibodies, vectors, which direct high-level expression of fusion proteins that are readily purified, may be used. Such vectors include, but are not limited to, the multifunctional E. coli cloning and expression vectors such as
30 Bluescript.RTM. (Stratagene, La Jolla, Calif.), in which the sequence encoding CRMP4 or C4RIP may be ligated into the vector in frame with sequences for the amino-terminal Methionine and the subsequent 7 residues of .beta.-galactosidase so that a hybrid protein is produced; pIN vectors (Van Heeke and Schuster, J. Biol. Chem. 1989; 264: 5503); and the

like; pGEX vectors (Promega, Madison, Wis.) may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems may be designed to include heparin, thrombin, or factor XA protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety at will.

In addition to bacteria, eukaryotic microbes, such as yeast, may also be used as hosts. Laboratory strains of *Saccharomyces cerevisiae*, Baker's yeast, are most often used although a number of other strains or species are commonly available. Vectors employing, for example, the 2 .mu. origin of replication of Broach et al. (Meth Enzymol 1983; 101: 307), or other yeast compatible origins of replication (see, for example, Stinchcomb et al. Nature 1979: 282; 39, Tschumper et al., Gene 1980: 10; 157, Clarke et al., Meth Enzymol 1983; 101: 300) may be used. Control sequences for yeast vectors include promoters for the synthesis of glycolytic enzymes (Hess et al. J Adv Enzyme Reg 1968; 7: 149; Holland et al., Biochemistry 1978; 17: 4900). Additional promoters known in the art include the promoter for 3-phosphoglycerate kinase (Hitzeman et al., J Biol Chem 1980; 255: 2073), alcohol oxidase, and PGH. Other promoters, which have the additional advantage of transcription controlled by growth conditions and/or genetic background are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, the alpha-factor system and enzymes responsible for maltose and galactose utilization. It is also believed that terminator sequences are desirable at the 3' end of the coding sequences. Such terminators are found in the 3' untranslated region following the coding sequences in yeast-derived genes. For reviews, see "Current Protocols in Molecular Biology", Ausubel et al., John Wiley & Sons, 1989, New York, N.Y. and Grant et al., Meth Enzymol. 1987; 153: 516.

In cases where plant expression vectors are used, the expression of a sequence encoding CRMP4 or C4RIP may be driven by any of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of CaMV may be used alone or in combination with the omega leader sequence from TMV (Takamatsu et al., EMBO J. 1987; 6: 307; Brisson et al., Nature 1984; 310: 511). Alternatively, plant promoters such as the small subunit of RUBISCO or heat shock promoters may be used (Coruzzi et al., EMBO J 1984; 3: 1671; Broglie et al., Science 1984; 224: 838; Winter et al., Results Probl. Cell Differ 1991; 17: 85). These constructs can be introduced into plant cells by direct DNA transformation or

pathogen-mediated transfection. Such techniques are described in a number of generally available reviews (see, for example, Hobbs S or Murry L E in "McGraw Hill Yearbook of Science and Technology" McGraw Hill, 1992, New York, N.Y.; pp. 191-196 or Weissbach and Weissbach in "Methods for Plant Molecular Biology", Academic Press, 1988, New York, N.Y.;
5 pp. 421-463).

An insect system may also be used to express CRMP4 or C4RIP. For example, in one such system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia* larvae. The sequences encoding CRMP4 or C4RIP may be cloned into a nonessential region of the virus,
10 such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of CRMP4 or C4RIP will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein. The recombinant viruses may then be used to infect, for example, *S. frugiperda* cells or *Trichoplusia* larvae in which CRMP4 or C4RIP may be expressed (Smith et al., J Virol 1983; 46: 584; Engelhard et al., Proc Natl Acad Sci 1994; 91:
15 3224).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, a sequence encoding CRMP4 or C4RIP may be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of
20 the viral genome may be used to obtain a viable virus, which is capable of expressing CRMP4 or C4RIP in infected host cells (Logan and Shenk, Proc Natl Acad Sci 1984; 81: 3655). In addition, transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, may be used to increase expression in mammalian host cells.

Specific initiation signals may also be used to achieve more efficient translation of a
25 sequence encoding CRMP4 or C4RIP. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences encoding CRMP4 or C4RIP, their initiation codons and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequence, or a portion thereof, is inserted, exogenous translational control
30 signals including the ATG initiation codon should be provided. Furthermore, the initiation codon should be in the correct reading frame to ensure the correct translation of the entire insert. Exogenous translational elements and initiation codons may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of

enhancers which are appropriate for the particular cell system which is used, such as those described in the literature (Scharf et al., *Results Probl Cell Differ* 1994; 20: 125; Bittner et al., *Meth Enzymol* 1987; 153: 516).

5 In addition, a host cell strain may be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" form of the protein may also be used to facilitate correct insertion, folding and/or function. Different host cells such as CHO, HeLa, MDCK,
10 HEK293, WI38, and COS, which have specific cellular machinery and characteristic mechanisms for such post-translational activities, may be chosen to ensure the correct modification and processing of the foreign protein.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines, which stably express CRMP4 or C4RIP, may be
15 transformed using expression vectors which may contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or separate vector. Following the introduction of the vector, cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and
20 recovery of cells, which successfully express the introduced sequences. Resistant clones of stably transformed cells may be proliferated using tissue culture techniques appropriate to the cell type.

Any number of selection systems may be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler et
25 al., *Cell* 1977; 11: 223) and adenine phospho-ribosyltransferase (Lowy et al., *Cell* 1980; 22: 817) genes which can be employed in tk.⁺ or aprt.⁺ cells, respectively. Also, antimetabolite, antibiotic, or herbicide resistance can be used as the basis for selection; for example, dhfr, which confers resistance to methotrexate (Wigler et al., *Proc Natl Acad Sci* 1980; 77: 3567); npt, which confers resistance to the aminoglycosides neomycin and
30 G418 (Colbere-Garapin et al., *J Mol Biol* 1981; 150: 1) and als or pat, which confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murry L E, *supra*). Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of tryptophan, or hisD, which allows cells to utilize histinol in

place of histidine (Hartman and Mulligan, Proc Natl Acad Sci 1988; 85: 8047). Recently, the use of visible markers has gained popularity with such markers as anthocyanins, .beta.-glucuronidase and its substrate GUS, and luciferase and its substrate luciferin, being widely used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system (Rhodes et al., Methods Mol Biol 1995; 55: 121).

Although the presence/absence of marker gene expression suggests that the gene of interest is also present, its presence and expression may need to be confirmed. For example, if the sequence encoding CRMP4 or C4RIP is inserted within a marker gene sequence, recombinant cells containing sequences encoding CRMP4 or C4RIP can be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with a sequence encoding CRMP4 or C4RIP under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the tandem gene as well.

Alternatively, host cells that contain the coding sequence for CRMP4 or C4RIP and that express CRMP4 or C4RIP may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridizations and protein bioassay or immunoassay techniques, which include membrane, solution, or chip-based technologies for the detection and/or quantification of the nucleic acid or protein.

The presence of the polynucleotide sequence encoding CRMP4 or C4RIP can be detected by DNA-DNA or DNA-RNA hybridization or amplification using probes or portions or fragments of polynucleotides encoding CRMP4 or C4RIP. Nucleic acid amplification based assays involve the use of oligonucleotides or oligomers based on the CRMP4- and C4RIP-encoding sequences to detect transformants containing DNA or RNA encoding CRMP4 and C4RIP. As used herein "oligonucleotides" or "oligomers" refer to a nucleic acid sequence of at least about 10 nucleotides and as many as about 60 nucleotides, preferably about 15 to 30 nucleotides, and more preferably about 20-25 nucleotides, which can be used as a probe.

A variety of protocols for detecting and measuring the expression of CRMP4 or C4RIP, using either polyclonal or monoclonal antibodies specific for the protein are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescent activated cell sorting (FACS). A two-site, monoclonal-based

immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on CRMP4 or C4RIP may be preferred, but a competitive binding assay may be employed. These and other assays are described, among other places, in "Serological Methods: A Laboratory Manual", Hampton et al., APS Press, 1990, St-Paul, Mich. and Maddox et al., J
5 Exp Med 1983; 158: 1211).

A wide variety of labels and conjugation techniques are known by those skilled in the art and may be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to polynucleotides encoding CRMP4 or C4RIP include oligolabeling, nick translation, end-labeling or PCR
10 amplification using a labeled nucleotide. Alternatively, the sequence encoding CRMP4 or C4RIP, or any portion of it, may be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes *in vitro* by addition of an appropriate RNA polymerase such as T7, T3 or SP6 and labeled nucleotides. These procedures may be conducted using a variety of
15 commercially available kits: from e.g. Pharmacia & Upjohn, (Kalamazoo, Mich.); Promega (Madison Wis.); and U.S. Biochemical Corp. (Cleveland, Ohio), or Ambion (Austin, Tex.). Suitable reporter molecules or labels, which may be used, include radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles, and the like.

Host cells transformed with a nucleotide sequence encoding CRMP4 or C4RIP may be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The protein produced by a recombinant cell may be secreted or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides, which encode CRMP4
25 or C4RIP may be designed to contain signal sequences that direct secretion of CRMP4 or C4RIP through a prokaryotic or eukaryotic cell membrane. Other recombinant constructions may be used to join sequences encoding CRMP4 or C4RIP to a nucleotide sequence encoding a polypeptide domain, which will facilitate purification of soluble proteins. Such purification facilitating domains include, but are not limited to, metal chelating peptides such
30 as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp., Seattle, Wash.).

In addition to recombinant production, fragments of CRMP4 or C4RIP may be produced by direct peptide synthesis using solid-phase techniques (see Stewart et al., "Solid-Phase Peptide Synthesis", WH Freeman & Co., 1969, San Francisco, Calif.; Merrifield et al., J Am Chem Soc 1963; 85: 2149). Chemical synthesis may be performed using manual techniques or by automation. Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Various fragments of CRMP4 or C4RIP may be chemically synthesized separately and combined using chemical methods to produce the full-length molecule.

In other embodiments of the invention, CRMP4 or C4RIP or fragments thereof may be used for therapeutic, diagnostic or screening purposes.

Antibodies may be generated using methods that are well known in the art. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments, and fragments produced by a Fab expression library. Neutralizing antibodies, (i.e., those which inhibit dimer formation) are especially preferred for therapeutic use.

For the production of antibodies, various hosts including goats, rabbits, rats, mice, humans, and others, may be immunized by injection with CRMP4 or C4RIP or any fragment or oligopeptide thereof which has immunogenic properties. Depending on the host species, various adjuvants may be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminum hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. Among adjuvants used in humans, BCG (bacilli Calmette-Guerin) and Corynebacterium parvum are especially preferable.

It is preferred that the peptides, fragments, or oligopeptides used to induce antibodies to CRMP4 or C4RIP have an amino acid sequence consisting of at least five amino acids, and more preferably at least 10 amino acids. It is also preferable that they are identical to a portion of the amino acid sequence of the natural protein, and they may contain the entire amino acid sequence of a small, naturally occurring molecule. Short stretches of CRMP4 or C4RIP amino acids may be fused with those of another protein such as keyhole limpet hemocyanin and antibody produced against the chimeric molecule.

Various immunoassays may be used for screening to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificities are well known in the art. Such immunoassays typically involve the measurement of complex formation

between CRMP4 or C4RIP and its specific antibody. A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering CRMP4 or C4RIP epitopes may be preferred, but a competitive binding assay may also be employed (Maddox, supra).

5 In another embodiment of the invention, the polynucleotides encoding CRMP4, C4RIP, or any fragment thereof, or antisense or siRNA sequences, may be used for therapeutic purposes. In one aspect, antisense or siRNA to the polynucleotide encoding CRMP4 or C4RIP may be used in situations in which it would be desirable to block the synthesis of the protein. In particular, cells may be transformed with sequences
10 complementary to polynucleotides encoding CRMP4 or C4RIP. Thus, antisense or siRNA sequences may be used to modulate CRMP4 or C4RIP activity, or to achieve regulation of gene function. Such technology is now well known in the art, and siRNA, sense or antisense oligomers or larger fragments, can be designed from various locations along the coding or control regions of sequences encoding CRMP4 or C4RIP.

15 Still in the realm of therapeutics, C4RIP or derivatives, analogs, variants or fragments of C4RIP, may be engineered to include an inherent cell membrane-translocating sequence (or MTS). The inclusion of such a MTS, also called an "internalization" or "internalizing" sequence, allows a translated peptide to be transported across a cell membrane (Rojas et al. 1998; Winton et al. 2002). This opens up the possibility of therapeutic applications in living
20 systems. Two example MTSs are illustrated with and without C4RIP in Figure 10. Figure 10 a) shows the C4RIP sequence with the C3-05 internalizing sequence, while Figure 10 c) illustrates the C4RIP sequence with the Kaposi MTS. Figure 10 b) and d) show the C3-05 MTS and Kaposi MTS, respectively. It is expected that other internalizing sequences would function with C4RIP, and therefore the scope of the present invention is meant to encompass
25 any MTS that would have the desired effect of transporting C4RIP or derivatives, analogs, variants or fragments of C4RIP across a cell membrane.

Expression vectors derived from retroviruses, adenovirus, herpes or vaccinia viruses, or from various bacterial plasmids may be used for delivery of nucleotide sequences to the targeted organ, tissue or cell population. Methods, which are well known to those skilled in
30 the art, can be used to construct recombinant vectors which will express antisense polynucleotides of the gene encoding CRMP4 or C4RIP. These techniques are described both in Sambrook et al. (supra) and in Ausubel et al. (supra).

Transient expression may last for a month or more with a non-replicating vector and even longer if appropriate replication elements are part of the vector system.

As mentioned above, modifications of gene expression can be obtained by designing antisense molecules, DNA, RNA or PNA, to the control regions of the gene encoding CRMP4
5 i.e., the promoters, enhancers, and introns. Oligonucleotides derived from the transcription initiation site, e.g., between positions -10 and +10 from the 5' end of the transcript, are preferred. Similarly, inhibition can be achieved using "triple helix" base-pairing methodology. Triple helix pairing is useful because it causes inhibition of the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or regulatory molecules.
10 Recent therapeutic advances using triplex DNA have been described in the literature (Gee, J. E. et al. (1994) In: Huber, B. E. and Carr, B. I. Molecular and Immunologic Approaches, Futura Publishing Co., Mt. Kisco, N.Y.). The antisense molecules may also be designed to block translation of mRNA by preventing the transcript from binding to ribosomes.

Ribozymes, enzymatic RNA molecules, may also be used to catalyze the specific
15 cleavage of RNA. The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Examples which may be used include engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of sequences encoding CRMP4 or C4RIP.

20 Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences: GUA, GUU, and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for secondary structural features which may render the oligonucleotide
25 inoperable. The suitability of candidate targets may also be evaluated by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays.

Antisense molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of RNA molecules. These include techniques for chemically synthesizing oligonucleotides such as solid phase phosphoramidite chemical synthesis.
30 Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding CRMP4 or C4RIP. Such DNA sequences may be incorporated into a wide variety of vectors with suitable RNA polymerase promoters such as T7 or SP6.

Alternatively, these cDNA constructs that synthesize antisense RNA constitutively or inducibly can be introduced into cell lines, cells, or tissues.

RNA molecules may be modified to increase intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the backbone of the molecule. This concept is inherent in the production of PNAs and can be extended in all of these molecules by the inclusion of nontraditional bases such as inosine, queosine, and wybutosine, as well as acetyl-, methyl-, thio-, and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine which are not as easily recognized by endogenous endonucleases.

Many methods for introducing vectors into cells or tissues are available and equally suitable for use *in vivo*, *in vitro*, and *ex vivo*. For *ex vivo* therapy, vectors may be introduced into stem cells taken from the patient and clonally propagated for autologous transplant back into that same patient. Delivery by transfection and by liposome injections may be achieved using methods that are well known in the art.

Any of the therapeutic methods described above may be applied to any suitable subject including, for example, mammals such as dogs, cats, cows, horses, rabbits, monkeys, and most preferably, humans.

An additional embodiment of the invention relates to the administration of a pharmaceutical composition, in conjunction with a pharmaceutically acceptable carrier, for any of the therapeutic effects discussed above. Such pharmaceutical compositions may consist of CRMP4 or C4RIP, antibodies to CRMP4 or C4RIP, mimetics, agonists, antagonists, or inhibitors of CRMP4 or C4RIP. The compositions may be administered alone or in combination with at least one other agent, such as stabilizing compound, which may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions may be administered to a patient alone, or in combination with other agents, drugs or hormones.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes.

The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation may be a lyophilized powder which may contain any or all of the following: 1-50 mM histidine, 0.1%-2% sucrose, and 2-7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. For administration of CRMP4 or C4RIP, such labeling would include amount, frequency, and method of administration. Pharmaceutical compositions suitable for use in the invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended purpose. The determination of an effective dose is well within the capability of those skilled in the art.

For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays, e.g., of neurite outgrowth, or in animal models, usually mice, rats or monkeys. The animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans. A therapeutically effective dose refers to that amount of active ingredient, for example CRMP4 or C4RIP or fragments thereof, antibodies of CRMP4 or C4RIP, agonists, antagonists or inhibitors of CRMP4 or C4RIP, which ameliorates the symptoms or condition. Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED₅₀ (the dose therapeutically effective in 50% of the population) and LD₅₀ (the dose lethal to 50% of the population). The dose ratio between therapeutic and toxic effects is the therapeutic index, and it can be expressed as the ratio, LD₅₀/ED₅₀.

Pharmaceutical compositions, which exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide

sufficient levels of the active moiety or to maintain the desired effect. Factors, which may be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting
5 pharmaceutical compositions may be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular formulation.

Normal dosage amounts may vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in
10 the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.

In one aspect, hybridization with PCR probes which are capable of detecting polynucleotide sequences, including genomic sequences, encoding CRMP4 or C4RIP or
15 closely related molecules, may be used to identify nucleic acid sequences which encode CRMP4 or C4RIP. The specificity of the probe, whether it is made from a highly specific region, e.g., 10 unique nucleotides in the 5' regulatory region, or a less specific region, e.g., especially in the 3' coding region, and the stringency of the hybridization or amplification (maximal, high, intermediate, or low) will determine whether the probe identifies only naturally
20 occurring sequences encoding CRMP4 or C4RIP, alleles, or related sequences.

Probes may also be used for the detection of related sequences, and should preferably contain at least 50% of the nucleotides from any of the CRMP4 or C4RIP encoding sequences. The hybridization probes of the subject invention may be DNA or RNA and
25 derived from the nucleotide sequence of SEQ ID NO:3, SEQ ID NO:4 or SEQ ID NO:6 or from genomic sequence including promoter, enhancer elements, and introns of the naturally occurring CRMP4 or C4RIP.

Means for producing specific hybridization probes for DNAs encoding CRMP4 or C4RIP include the cloning of nucleic acid sequences encoding CRMP4 or C4RIP or CRMP4
30 or C4RIP derivatives into vectors for the production of mRNA probes. Such vectors are known in the art, commercially available, and may be used to synthesize RNA probes *in vitro* by means of the addition of the appropriate RNA polymerases and the appropriate labeled nucleotides. Hybridization probes may be labeled by a variety of reporter groups, for example,

radionuclides such as ^{32}P or ^{35}S , or enzymatic labels, such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems, and the like. Polynucleotide sequences encoding CRMP4 may be used for the diagnosis of conditions or diseases that are associated with expression of CRMP4. Examples of such conditions or diseases include, but may not be limited to, injuries or damage to the central and peripheral nervous systems. The polynucleotide sequences encoding CRMP4 or C4RIP may be used in Southern or northern analysis, dot blot, or other membrane-based technologies; in PCR technologies; or in dip stick, pin, ELISA or chip assays utilizing fluids or tissues from patient biopsies to detect altered CRMP4 or C4RIP expression. Such qualitative or quantitative methods are well known in the art.

In a particular aspect, the nucleotide sequences encoding CRMP4 may be useful in assays that detect activation or induction of various neurological or other non-neurological disorders, particularly those mentioned above. The nucleotide sequence encoding CRMP4 may be labeled by standard methods, and added to a fluid or tissue sample from a patient under conditions suitable for the formation of hybridization complexes. After a suitable incubation period, the sample is washed and the signal is quantitated and compared with a standard value. If the amount of signal in the biopsied or extracted sample is significantly altered from that of a comparable control sample, the nucleotide sequence has hybridized with nucleotide sequences in the sample, and the presence of altered levels of nucleotide sequences encoding CRMP4 in the sample indicates the presence of the associated disease. Such assays may also be used to evaluate the efficacy of a particular therapeutic treatment regimen in animal studies, in clinical trials, or in monitoring the treatment of an individual patient.

In order to provide a basis for the diagnosis of disease associated with expression of CRMP4, a normal or standard profile for expression is established. This may be accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with a sequence, or a fragment thereof, which encodes CRMP4, under conditions suitable for hybridization or amplification. Standard hybridization may be quantified by comparing the values obtained from normal subjects with those from an experiment where a known amount of a substantially purified polynucleotide is used. Standard values obtained from normal samples may be compared with values obtained from samples from patients who are symptomatic for disease. Deviation between standard and subject values is used to establish the presence of disease.

Once disease is established and a treatment protocol is initiated, hybridization assays may be repeated on a regular basis to evaluate whether the level of expression in the patient begins to approximate that which is observed in the normal patient. The results obtained from successive assays may be used to show the efficacy of treatment over a period ranging from
5 several days to months.

With respect to neurological diseases, the presence of a relatively high amount of transcript in biopsied tissue from an individual may indicate a predisposition for the development of the disease, or may provide a means for detecting the disease prior to the appearance of actual clinical symptoms. A more definitive diagnosis of this type may allow
10 health professionals to employ preventative measures or aggressive treatment earlier thereby preventing the development or further progression of the disease.

Additional diagnostic uses for oligonucleotides encoding CRMP4 may involve the use of PCR. Such oligomers may be chemically synthesized, generated enzymatically, or produced from a recombinant source. Oligomers will preferably consist of two nucleotide
15 sequences, one with sense orientation and another with antisense, employed under optimized conditions for identification of a specific gene or condition. The same two oligomers, nested sets of oligomers, or even a degenerate pool of oligomers may be employed under less stringent conditions for detection and/or quantitation of closely related DNA or RNA sequences.

20 Methods which may also be used to quantitate the expression of CRMP4 or C4RIP include radiolabeling or biotinylating nucleotides, coamplification of a control nucleic acid, and standard curves onto which the experimental results are interpolated (Melby, P. C. et al. (1993) J. Immunol. Methods, 159:235-244; Duplaa, C. et al. (1993) Anal. Biochem. 229-236). The speed of quantitation of multiple samples may be accelerated by running the assay in an
25 ELISA format where the oligomer of interest is presented in various dilutions and a spectrophotometric or colorimetric response gives rapid quantitation.

In another embodiment of the invention, the nucleic acid sequence that encodes CRMP4 may also be used to generate hybridization probes that are useful for mapping the naturally occurring genomic sequence. The sequence may be mapped to a particular
30 chromosome or to a specific region of the chromosome using well-known techniques. Such techniques include FISH, FACS, or artificial chromosome constructions, such as yeast artificial chromosomes, bacterial artificial chromosomes, bacterial P1 constructions or single

chromosome cDNA libraries as reviewed by Price, C. M. (1993; Blood Rev. 7:127-134), and Trask, B. J. (1991; Trends Genet. 7:149-154).

Screening Assays

5 In other embodiments of the invention, CRMP4 and C4RIP, their catalytic or immunogenic fragments, or oligopeptides thereof can be used for screening libraries of compounds in any of a variety of drug screening techniques. The fragment employed in such screening may be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly. The formation of binding complexes, between CRMP4 or C4RIP and
10 the agent being tested, may be measured. Thus, the polypeptides of the invention may also be used to assess the binding of small molecule substrates and ligands in, for example, cells, cell-free preparations, chemical libraries, and natural product mixtures. These substrates and ligands may be natural substrates and ligands or may be structural or functional mimetics. In general, such screening procedures involve producing appropriate cells, which express the
15 receptor polypeptides of the present invention on the surface thereof. Such cells include cells from mammals, yeast, insects (i.e., *Drosophila*) or bacteria (i.e., *E. coli*). Cells expressing the receptor (or cell membranes containing the expressed receptor) are then contacted with a test compound to observe binding, or stimulation or inhibition of a functional response (for example, interference with the CRMP4-RhoA interaction).

20 The assays may simply test binding of a candidate compound wherein adherence to the cells bearing the receptor is detected by means of a label directly or indirectly associated with the candidate compound or in an assay involving competition with a labeled competitor. Further, these assays may test whether the candidate compound results in a signal generated by activation of the receptor, using detection systems appropriate to the cells
25 bearing the receptor at their surfaces (for example increased ion permeation measured by patch clamp or, preferably by ion imaging). Inhibitors of activation are generally assayed in the presence of a known agonist (for example, protons) and the effect of the candidate compound on the activation by the agonist is observed. Standard methods for conducting such screening assays are well understood in the art. Typically, the response may be
30 measured by use of a microelectrode technique accompanied by such measurement strategies as voltage clamping of the cell whereby activation of ion channels may be identified by inward or outward current flow as detected using the microelectrodes. ²²Na, ⁸⁶Rb, ⁴⁵Ca radiolabeled cations or ¹⁴C or ³H guanidine may be used to

assess such ion flux; a sodium, calcium or potassium ion sensitive dye (such as Fura-2, or Indo) may also be used to monitor ion passage through the receptor ion channel, or a potential sensitive dye may be used to monitor potential changes, such as in depolarization.

Another technique for drug screening, which may be used provides for high
5 throughput screening of compounds having suitable binding affinity to the protein of interest as described in published PCT application WO84/03564. In this method, as applied to CRMP4 or C4RIP large numbers of different small test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The test compounds are reacted with a CRMP4-RhoA conjugate and washed. Bound CRMP4-RhoA conjugate is then detected by
10 methods well known in the art. Purified CRMP4 or C4RIP can also be coated directly onto plates for use in the aforementioned drug screening techniques. Alternatively, non-neutralizing antibodies can be used to capture the peptide and immobilize it on a solid support.

In another embodiment, one may use competitive drug screening assays in which
15 neutralizing antibodies capable of binding to the CRMP4-RhoA conjugate specifically compete with a test compound for binding CRMP4 or C4RIP. In this manner, the antibodies can be used to detect the presence of any peptide that shares one or more antigenic determinants with CRMP4 or C4RIP.

The drug assays or screens described above, as well as any other that are known to
20 the person of skill in the art and that may be suitable to practice the present invention, may be packaged in a kit. All kits of this type are intended to be included within the scope of the present invention.

While the present invention is described with particular reference to the CNS, it is believed that it will also find application in the PNS because other antagonists targeting
25 intracellular signalling substrates that are convergent targets of MAIs and CSPGs have demonstrated efficacy in both CNS and PNS regeneration models (Madura et al. 2007; Hiraga et al. (2006); Dergham et al. 2002; Fournier et al. 2003).

Methods and Materials

30 *Plasmid construction*

To construct CRMP-V5 expression vectors, cDNA was amplified by polymerase chain reaction (PCR) from rat pEGFP-CRMP constructs (provided by Dr. Peter McPherson, McGill University). The PCR product was ligated into the *HindIII*- and *XhoI*- sites of pcDNA 3.1 V5-

His for CRMP1a, CRMP2a and CRMP3a and into *HindIII*- and *EcoRI*-site of pcDNA 3.1 V5-His for CRMP4a. The cDNA for CRMP4b was amplified from pcDNA 3.1CRMP4bV5-His TOPO (Quinn et al., 2003) and ligated into the *HindIII*- and *EcoRI*-site pcDNA 3.1 V5-His in frame with the V5-His tag. CRMP1b-V5 was constructed by amplifying the coding sequence
 5 by PCR from an EST clone (IMAGE:5686818) and ligated into *EcoRI*- and *XhoI*- sites of pcDNA 3.1 V5-His.

pcDNA myc-wild type RhoA (wt) was obtained from UMR cDNA Resource Center (<http://www.cdna.org/>). pRK5 myc-Rac1 (wt), pRK5 myc-Cdc42 (wt), pRK5 myc-RhoA63L, pRK5 myc-RhoAN19, pRK5 myc-RacQ61L, pRK5 myc-RacN17, pRK5 myc-Cdc42Q61L and
 10 pRK5 myc-Cdc42N17 constructs were provided by Dr. Nathalie Lamarche-Vane (McGill University). FLAG-RhoA63L was generated by sub-cloning RhoA63L into the *BamHI*- and *EcoRI*- sites of pcDNA3FLAG. pCAG-myc-mROCK II construct was provided by Dr. Shuh Narumiya (Kyoto University).

To generate C4RIP-V5, the unique amino terminal domain of CRMP4b (residues 1-
 15 126) was introduced into the *BamHI*- and *EcoRI*- sites of pcDNA 3.1V5-His. pHSVC4RIP was generated by subcloning C4RIP-V5 into the *HindIII*- and *Sall*- sites of pHSVPrPUC. pHSVCRMP4bGFP was generated by cloning CRMP4b into the *HindIII*- and *EcoRI*- sites of pEGFP N2 (Clontech) and subsequently sub-cloning into the *HindIII*- and *XbaI*- sites of pHSVPrPUC. Chimeric CRMP4bNCRMP2-V5 was constructed by PCR by ligating residues
 20 14-572 of CRMP2a into *EcoRI*- and *XhoI*- sites of C4RIP-V5. CRMP4ΔN consists of CRMP4a with the first 12 residues replaced by a single methionine. CRMP4ΔN was amplified by PCR and ligated into *HindIII*- and *EcoRI*- sites of pcDNA 3.1 V5-His.

Preparation of Herpes Simplex Viruses

25 pHSVPrPUC plasmids were transfected into 2-2 Vero cells which were superinfected with 5d/ 1.2 HSV helper virus 1 day later. Recombinant virus was amplified through three passages and stored at -80°C as described previously (Neve et al., 1997).

Preparation of recombinant proteins

30 Stimulations to examine inhibitory responses were performed with alkaline-phosphatase-conjugated Nogo-66 (AP-Nogo-66) purified from stably transfected 293 cells or Nogo-P4 peptide or myelin. AP-Nogo-66 or AP was purified by Ni²⁺ affinity chromatography as previously described (Nakamura et al., 1998; Fournier et al., 2001). For all treatments AP-

Nogo66-His (8nM) or AP (8nM) was preaggregated with 100 ng/ml anti-human AP (Niederost et al., 2002). Nogo-P4 (Alpha Diagnostics, San Antonio, TX) is a 25 aa inhibitory peptide sequence (residues 31-55 of Nogo-66) sufficient to mediate the inhibitory properties of Nogo-66, a potent inhibitory component of Nogo-A (GrandPre et al., 2000). Myelin extracts were prepared from bovine brain as described previously (Igarashi et al., 1993; Hsieh et al., 2006).

GST, GST-RhoAWT and GST-RhoA63L (construct provided by Dr. Keith Burridge, University of North Carolina, Chapel Hill) were expressed in *Escherichia coli* and purified on glutathione-Sepharose as previously described (Arthur et al., 2002; Wennerberg et al., 2002). For overlay assays, RhoA was cleaved from the GST moiety via thrombin cleavage. Aggrecan was purchased from Sigma Chemical Co. (Oakville, Ontario).

GST-RhoA Pull down assays

PC12 cells were grown to sub-confluence on collagen-coated plates in Roswell Park Memorial Institute Media (RPMI) 1640 supplemented with L-glutamine, and containing 10% horse serum, 5% fetal bovine serum (FBS), and 1% penicillin/streptomycin (Invitrogen, Burlington, Ontario), after which differentiation was induced for 24 hours with RPMI supplemented with 1% bovine serum albumin (BSA) fraction V and 50 ng/mL nerve growth factor (NGF) (Upstate Biotechnology, Waltham, MA). GST-RhoA63L pull downs were performed as described previously (Arthur et al., 2002). Briefly, after stimulation with AP-Nogo-66, cells were washed twice in ice cold Hepes-buffered saline (HBS), harvested in 1 mL of ice-cold lysis buffer containing 20 mM HEPES, pH 7.3, 150 mM NaCl, 5 mM MgCl₂, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), supplemented with Cømplete protease inhibitors (Roche Products, Laval, Quebec). GST-precleared lysates were then mixed with 30 µg of GST-RhoA63L fusion protein bound to sepharose beads for 1 hour at 4°C. Precipitated proteins were eluted with 2X sample buffer and analyzed by SDS-PAGE on a 4-15% gradient gel, followed by silver staining.

CRMP-RhoA Co-Immunoprecipitation Assays

HEK 293T cells were grown to sub-confluence and transfected with Lipofectamine 2000 according to manufacturer instructions (Invitrogen, Burlington, Ontario), washed twice with ice-cold PBS and lysed in lysis buffer A (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 1 mM Na₃VO₄, 1 mM NaF, 1 mM PMSF and Cømplete protease inhibitors (Roche Diagnostics, Laval, PQ)). Lysates were pre-cleared with protein A/G-

agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and subjected to immunoprecipitation with myc-agarose or V5-agarose (Sigma Chemical Co., Oakville, Ontario). After washing 3 times with ice-cold PBS, bound protein was eluted with SDS and immunoblotted with anti-Myc (9E10, 1:1000; Sigma Chemical Co., Oakville, Ontario) or anti-V5 (1:5000; Invitrogen, Burlington, Ontario). For time course experiments, PC12 cells were transfected for 24 hours using Lipofectamine 2000 (Invitrogen, Burlington, Ontario) and differentiated with 50 ng/ml nerve growth factor (NGF; Upstate Biotechnology, Waltham, MA) for 24 hours. Cells were treated with Nogo-P4 peptide for the indicated period of time at 37°C. Cells were then lysed and proteins were immunoprecipitated as described above.

10

Far Western

Overlay of CRMP4 with RhoA was performed as previously described (McPherson et al., 1994). Briefly, HEK293T cell lysates transfected with CRMP4a-V5, CRMP4b-V5 or empty vector were subjected to immunoprecipitation with V5-agarose (Sigma Chemical Co., Oakville, Ontario). Immunoprecipitates were separated by SDS-PAGE, transferred to PVDF membranes and overlaid with 10 µg/ml of bacterially-purified RhoA for 1 hour at room temperature. RhoA was detected with a rabbit anti-RhoA antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

20 *Neurite outgrowth and Growth Cone Collapse assays*

For neurite outgrowth assays, myelin was dried down on poly-L-lysine-coated substrates. Substrates were washed and coated with 10 µg/ml laminin for 1 hr. For outgrowth on aggrecan, poly-L-lysine-coated substrates were coated with aggrecan and 10 µg/ml laminin for 2 hr at 37°C. Dissociated E13 chick DRG neurons were cultured in DRG media (F-12 medium, 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 50 ng/ml NGF) in the presence of virus for 24 hours, fixed with 4% paraformaldehyde/20% sucrose in PBS and double stained with anti-βIII tubulin (Covance, Berkeley, CA) and anti-V5 antibody (Sigma Chemical Co., Oakville, Ontario). Neurite outgrowth lengths per cell were assessed using Image J, a public domain JAVA image processing program (<http://rsb.info.nih.gov/ij/>) as previously described (Fournier et al., 2003).

30

For growth cone collapse assays, chick E7 DRGs were cultured in DRG media for 18 hours on 4 well glass chamber slides sequentially coated with PLL and laminin as above. Sema3A-AP or AP conditioned media was prepared as described previously (Takahashi et

al., 1998). Explants were stimulated with Sema3A-AP- or AP- conditioned media at indicated concentrations for 20 min and fixed with 4% paraformaldehyde, 20% sucrose and 0.1M NaPO₄. Explants were stained with rhodamine phalloidin and assessed for growth cone collapse as described (Luo et al., 1993).

5

CRMP-4 siRNA

For knockdown of CRMP-4a and CRMP-4b, silencer pre-designed siRNA against rat CRMP-4 was used (siRNA ID: 48833; Ambion, Austin, TX). Controls were with a scrambled siRNA (CAGCAUGGUGGUACGCUUGUAAGCA) (SEQ ID NO: 7) CRMP4b-targetted siRNA
10 designed with the BLOCK-IT algorithm (Invitrogen, Burlington, Ontario). siRNAs were validated by co-transfecting siRNAs with CRMP4-V5 in HEK293T cells for 24 hours. Cell lysates were separated by SDS-PAGE and analyzed with an anti-V5 antibody. To validate siRNA efficacy in neurons, siRNA-transfected DRGs were co-infected with HSVCRMP4b-GFP. Twenty-four hours following infection GFP fluorescence was evaluated by fluorescence
15 microscopy. For neurite outgrowth assays, P5 dissociated rat DRGs were prepared and seeded on laminin substrates as described previously (Hsieh et al., 2006). After 4 hours, DRGs were serum starved (in F-12 medium and 50 ng/ml NGF) and transfected with indicated siRNAs using Lipofectamine 2000. After 5 hours of transfection, media was replaced with fresh DRG media. Twenty-four hours following transfection, DRGs were
20 removed from the plate with EDTA and re-seeded on myelin substrates. Neurons were left to grow for an additional 18 hours and processed for neurite outgrowth analysis as described above.

Immunofluorescence

25 E7-13 chick DRG explants were cultured in DRG media on poly-L-lysine- and laminin-coated substrates for 18 hours. For viral infections, recombinant viral preparations were added to the media 1 hour after plating. After 18 hours, cultures were treated with myelin and fixed with 4% paraformaldehyde/20% sucrose/PBS, permeabilized in 0.2% triton X-100 and double stained with polyclonal anti-RhoA antibody (Santa Cruz Biotechnology, Santa Cruz,
30 CA) and anti-CRMP4b or V5 antibody. For filopodia and branch analysis, GFP and CRMP4bV5-infected growth cones were double stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR) and anti-V5 antibody. Filopodial length per growth cone was assessed using Image J, by measuring total filopodial length per growth cone averaged by the total

number of filopodia per growth cone. Neurite branches were quantified by counting total branch buds per neurite.

Results

5 A novel Nogo-dependent interaction between CRMP4b and RhoA

To identify molecules that functionally interact with RhoA to mediate neurite outgrowth inhibition, proteins that have enhanced affinity for a constitutively active, GTPase deficient mutant of RhoA were screened ((RhoA63L; Khosravi-Far et al., 1994) following treatment with Nogo-66, a potent inhibitory fragment of Nogo-A (GrandPre et al., 2000). Bacterially-purified
10 *glutathione-S-transferase*-RhoA63L (GST-RhoA63L) was used as bait to precipitate proteins from PC12 cells following stimulation with Nogo-66 fused to alkaline phosphatase (AP-Nogo-66). PC12 cells were chosen for the biochemical screen based on their expression of MAI receptors (Hsieh et al., 2006), and their responsiveness to Nogo in neurite outgrowth assays (GrandPre et al., 2000) and biochemical assays evaluating RhoGTP levels (Fournier et al.,
15 2003). Proteins interacting with GST-RhoA63L were separated by SDS-PAGE and visualized by silver staining. A 75 kDa protein with enhanced affinity for GST-RhoA63L following Nogo-66 treatment (Fig. 1a) was identified as CRMP4b by tandem mass spectrometry. The enhanced CRMP4-RhoA interaction was validated with a pan-CRMP antibody (provided by Dr. Peter McPherson, McGill University), which recognizes the 75 kDa CRMPb isoforms and
20 the 65 kDa CRMPa isoforms (Fig. 1b). Both 75 kDa and 65 kDa CRMP isoform/s specifically precipitate with GST-RhoA63L while the interaction between the 75 kDa CRMPb isoform/s and RhoA is enhanced by Nogo stimulation (Fig. 1b).

To assess the specificity of the CRMP4b-RhoA interaction, the ability of other CRMP members to interact with RhoA was analyzed. Wild type myc-RhoA and CRMP-V5 constructs
25 were co-transfected in HEK293T cells. Myc-RhoA was immunoprecipitated from cell lysates and immune complexes were analyzed for CRMP-V5. RhoA interacts preferentially with CRMP4 family members with stronger binding to CRMP4b when compared to CRMP4a (Fig. 2a). RhoA fails to bind to other CRMPa isoforms and weakly associates with CRMP1b (Fig. 2a). Amino-terminally-tagged GFP-CRMP4b fails to associate with RhoA suggesting that
30 amino-terminal tags interfere with this binding interaction (data not shown).

The specificity of the interaction between CRMP4 and RhoA was assessed through the binding of CRMP4 to Rac and Cdc42, two other members of the small Rho GTPases that regulate the actin cytoskeleton and positively regulate neurite outgrowth (Bishop and Hall,

2000). CRMP4a-V5 and CRMP4b-V5 were tested for binding to myc-tagged, GTP-bound active forms of RhoA (myc-RhoA63L), Rac1 (myc-Rac1Q61L) and Cdc42 (myc-Cdc42Q61L). CRMP4 binding to RhoA is markedly stronger than to Rac1 or Cdc42 (Fig. 2b). Similarly, CRMP4 binding to wild type and GDP-bound forms Rac and Cdc42 is negligible (data not shown). Taken together, these results demonstrate that both CRMP4a and CRMP4b specifically bind to RhoA with CRMP4b binding more strongly than CRMP4a.

CRMP4b-RhoA binding is nucleotide-independent, phospho-dependent and direct

Rho GTPases cycle between an inactive GDP-bound state and an active GTP-bound state. Downstream effectors of Rho GTPases bind to Rho GTPases in the active GTP-bound state while guanine nucleotide exchange factors prefer Rho GTPases in the nucleotide free or GTP-bound state (Hall, 1994). To study the nucleotide-dependence of the CRMP4-RhoA interaction, CRMP4 binding to constitutively active myc-RhoA63L or dominant negative myc-RhoAN19 was assessed (Feig and Cooper, 1988). CRMP4a-V5 and CRMP4b-V5 interact with both myc-RhoA63L and myc-RhoAN19 (Fig. 2c) indicating that the interaction between CRMP4 and RhoA is nucleotide-independent.

To assess if the binding interaction between CRMP4 and RhoA is direct, a RhoA overlay assay was performed on CRMP-V5 immunoprecipitated from 293T cells (Fig. 2d). RhoA specifically binds to CRMP4b-V5 and CRMP4a-V5 and fails to bind to CRMP2a-V5 in the overlay assay indicating that the interaction between CRMP4b and RhoA is direct and specific.

The phospho-dependence of the interaction was evaluated by stimulating CRMP4b-V5-expressing 293T cells with Calyculin A, a serine/threonine phosphatase inhibitor, prior to immunoprecipitating CRMP4b-V5. Calyculin-dependent CRMP4b-V5 phosphorylation is indicated by an upward mobility shift of the CRMP4b-V5 protein (Fig 2d, V5 immunoblot). RhoA fails to bind to the phosphorylated species of CRMP4b-V5 in the overlay demonstrating that phosphorylated CRMP4b fails to bind to RhoA.

Nogo specifically modulates the CRMP4b-RhoA interaction

To further define the specificity and the time course of the Nogo effect on the CRMP4b-RhoA interaction, PC12 cells were co-transfected with wild type myc-RhoA and CRMP4-V5 and stimulated with Nogo-P4 peptide, the minimal sequence of Nogo-66 required

for Nogo-66-dependent responses (GrandPre et al., 2000). Myc-RhoA was immunoprecipitated and immune complexes were analyzed for CRMP4-V5. A rapid increase in CRMP4b-V5 immunoprecipitating with myc-RhoA is detected by 1 minute following Nogo stimulation, which is maintained by 10 minutes (Fig. 3a). Nogo stimulation does not regulate the interaction between myc-RhoA and CRMP4a-V5 (Fig. 3b). Similar results were obtained with AP-Nogo-66 stimulation and with the active form of RhoA (data not shown) suggesting that CRMP4b is regulated independently of Nogo-dependent RhoA cycling to the GTP-bound state. The Nogo-regulated RhoA-CRMP4b interaction was further confirmed by co-immunoprecipitation of endogenous proteins in Nogo-P4-treated P8 rat cerebellar cultures, where CRMP4b and RhoA form a complex 1 and 10 minutes following stimulation (Fig. 3c).

siRNA-mediated knockdown of CRMP4 attenuates neurite outgrowth inhibition

To determine if CRMP4 function is necessary for Nogo-dependent responses, neurite outgrowth on myelin substrates was assessed in the context of CRMP4-specific siRNA. The efficacy of CRMP4-specific siRNAs was validated in transfected 293T cells. One siRNA was identified, which robustly inhibits both CRMP4a and CRMP4b expression (Fig. 4a). Lipofectamine-mediated transfection of siRNAs efficiently targets P4 rat dorsal root ganglion neurons (DRGs) and CRMP4 siRNA introduced in this manner diminishes the expression of CRMP4b-GFP introduced by HSV-mediated infection (Fig. 4b). Rat DRGs were grown for 24 hours following siRNA transfection, removed from the substrate with EDTA and re-seeded on myelin substrates for an additional 18 hours. Rat DRG neuronal outgrowth is inhibited by approximately 50% on a substrate coated with 1 μ g/ml of myelin using this protocol (Fig. 4e), a more modest inhibitory response than when dissociated DRG neurons are plated immediately on myelin substrates (Fig. 8; (Hsieh et al., 2006)). Neuronal outgrowth on the control laminin substrate is not significantly affected by the introduction of CRMP4 siRNA (Fig. 4d). However, CRMP4 siRNA-transfected P4 rat DRGs grow significantly better on myelin substrates than those transfected with scrambled siRNA (Fig. 4c, e) indicating that CRMP4 is necessary for myelin-dependent inhibition. The efficacy of the CRMP4 siRNA (Fig. 4e) may be underestimated due to the failure to transfect 100% of the DRG neurons (Fig. 4b).

CRMP4b affects the growth cone actin cytoskeleton

CRMP proteins have been broadly implicated in the regulation of microtubule polymerization, actin bundling and endocytosis, three processes that influence growth cone

dynamics and neurite outgrowth (Fukata et al., 2002b; Nishimura et al., 2003; Rosslenbroich et al., 2005). To gain insight into how CRMP4b may influence neurite outgrowth inhibition, the distribution of CRMP4b in DRG growth cones was assessed. As previously described, endogenous CRMP4b has a punctate pattern within the growth cone extending throughout the central and peripheral domains (Fig. 5a)(Quinn et al., 2003). CRMP4b-V5 (Fig. 5b) and CRMP4b-GFP (Fig. 5c, d) fusion proteins also label the entire growth cone with a less distinct punctate profile, likely due to elevated cytosolic CRMP4b levels in the overexpression paradigm. Carboxy-terminally tagged CRMP4b-GFP labels the growth cone more broadly than tubulin (Fig. 5d) and extends into the actin-rich peripheral domain (Fig. 5c) co-localizing with actin at a subset of punctae within the growth cone (Fig. 5c, arrows). Intriguingly, CRMP4b-V5 overexpression promotes the extension of filopodia from the growth cone that are on average 70% longer than filopodia in GFP-infected growth cones (Fig. 5e, f, g). This phenotype is also manifested in the DRG neurite where ectopic actin-rich branches are formed (Fig. 5 e, f, g; arrowheads). A similar phenotype was promoted by CRMP4b-GFP overexpression (data not shown). On average, 8 branches per neurite can be detected on CRMP4b-infected neurites compared to 2 branches per neurite on GFP-infected neurites (Fig. 5g). The localization of CRMP4b within the growth cone is consistent with a role in microtubule or actin dynamics. However, the filopodial and branching phenotypes promoted by CRMP4b overexpression suggest that CRMP4b may affect neuronal phenotype through an actin-based mechanism, an important observation since CRMPs have been implicated in modulating both microtubule and actin dynamics (Fukata et al., 2002a; Rosslenbroich et al., 2005). While the possibility that the fusion proteins may behave differently from native CRMP4b cannot be ruled out, both CRMP4b-V5 and CRMP4b-GFP have similar distributions to endogenous CRMP and promote similar growth cone phenotypes, suggesting that CRMP4b functionally interacts mainly with the actin cytoskeleton.

To further address the potential role for CRMP4b-RhoA complexes in neurite outgrowth inhibition, the distribution of CRMP4b and RhoA during Nogo-dependent growth cone collapse was examined. Uninfected and CRMP4b-V5-infected DRG growth cones were fixed and stained for endogenous RhoA and CRMP4b or the V5 epitope tag. Prior to stimulation, endogenous RhoA and CRMP4b have distinct distributions within the growth cone with negligible colocalization (Fig. 6a). In unstimulated (control) growth cones, overexpressed CRMP4b-V5 also has a distinct distribution compared to endogenous RhoA (Fig. 6b). Following myelin stimulation, RhoA and CRMP4b-V5 co-localize at a subset of

distinct punctae within the growth cone central and peripheral domain (Fig. 6b, arrows) suggesting that a RhoA-CRMP4b complex forms in the growth cone where it may regulate actin cytoskeletal dynamics in response to inhibitory challenges.

5 **C4RIP-V5 attenuates CRMP4b-RhoA binding**

Nogo regulation of the CRMP4b-RhoA interaction raises the possibility that CRMP4b-RhoA complex formation is critical for inhibitory signaling. The specific enhancement of CRMP4b-RhoA binding suggests that the increased affinity may be mediated by the CRMP4b amino terminal extension. The ability of the CRMP4b amino terminus to mediate RhoA
10 binding was therefore assessed. A chimeric CRMP molecule consisting of the CRMP4b amino terminus fused to CRMP2 (CRMP4bNCRMP2) was generated as well as a CRMP4 construct consisting of the common region of CRMP4a and CRMP4b (CRMP4aΔN; Fig. 7a). Both CRMP4bNCRMP2 and CRMP4aΔN co-immunoprecipitate with RhoA, however both proteins bind RhoA more weakly than full length CRMP4b (Fig. 7b). This indicates that two
15 independent RhoA binding sites within the CRMP4b molecule are sufficient for RhoA binding and that the two sites may co-operate to mediate maximal RhoA binding; however the amino-terminal region of CRMP4b appears to be the critical site mediating Nogo-dependent recruitment to RhoA (Fig. 3).

To specifically disrupt the Nogo-dependent interaction between full length CRMP4b
20 and RhoA, a construct was generated to express the unique amino terminal domain of CRMP4b (C4RIP – CRMP4b-RhoA Inhibitory Peptide; Fig. 7a) fused to a V5-epitope tag. As predicted, co-transfection of C4RIP-V5 significantly diminishes RhoA binding to full length CRMP4b without affecting binding to CRMP4a (Fig. 7c), presumably by competing for the RhoA binding site/s.

The specificity of C4RIP-V5 was then evaluated by examining its effect on binding
25 between FLAG-RhoA63L and myc-Rho kinase (myc-ROCK), a critical RhoA effector molecule for myelin inhibition. C4RIP-V5 does not diminish binding between FLAG-RhoA63L and myc-ROCK (Fig. 7d). Similarly, overexpression of CRMP4b-V5 does not affect the binding between FLAG-RhoA63L and myc-ROCK indicating that CRMP4b does not alter the ability of
30 RhoA to interact with this downstream effector.

C4RIP-V5 attenuates neurite outgrowth inhibition

The ability of C4RIP-V5 to attenuate CRMP4b-RhoA binding provides a valuable tool

to determine if CRMP4b-RhoA complex formation is necessary for its role in myelin inhibition.

CRMP4b-V5 or C4RIP-V5 was introduced into dissociated E13 chick DRG neurons via recombinant HSV virus (Fig. 8a) and neurite outgrowth was assessed on myelin substrates (Fig. 8b, d). CRMP4b-V5 is not sufficient to mimic myelin inhibitory responses (Fig. b, c),
5 presumably due to its failure to increase its interaction with RhoA (Fig. 6b). C4RIP significantly attenuates myelin-dependent outgrowth inhibition compared to GFP- or full length CRMP4b-infected neurons (Fig. 8b, d). Unlike previously characterized Rho and ROCK antagonists, C4RIP does not promote basal DRG outgrowth on permissive control substrates (Fig. 8b, c; Lehmann et al., 1999; Fournier et al., 2003). DRG neurons infected with
10 HSV-C4RIP or with HSV-dominant negative ROCK (HSV-DNROCK; Alabed et al., 2006), are both protected from myelin inhibition; however, HSV-DNROCK promotes basal outgrowth by approximately 50%.

An advantage to targeting intracellular mediators of neurite outgrowth inhibition is their potential as convergent targets, which may attenuate multiple inhibitory influences. To
15 evaluate if C4RIP-V5 may block additional inhibitory signals associated with CNS injury, the effect of C4RIP-V5 on neurite outgrowth inhibition mediated by aggrecan, an inhibitory chondroitin sulphate proteoglycan of the glial scar, was examined. Intriguingly, it was found that C4RIP also promotes neurite outgrowth on aggrecan (Fig. 8e).

To assess the specificity of C4RIP, its ability to block Sema3A-dependent growth cone
20 collapse in E8 chick DRG neurons was tested. Sema3A-stimulation of neurons engages Rac1 GTPase and the CRMP2 isoform (Huber et al., 2003). It was found that C4RIP has no effect on Sema3A-dependent growth cone collapse (Fig. 8f). Taken together, these results indicate that disruption of the CRMP4b-RhoA interaction protects neurons from inhibitory influences that signal through RhoA GTPase.

Discussion

Regeneration following CNS trauma is limited by the activation of intracellular pathways within the injured neuron that block axonal extension through targeted modifications to the cytoskeleton (Hsieh et al., 2006). Development of antagonists to intracellular targets of
30 axon outgrowth inhibitors is an effective approach to circumvent the inhibitory influence of the astroglial scar and MAIs (Dergham et al., 2002; Niederost et al., 2002; Borisoff et al., 2003; Fournier et al., 2003). Here, it was demonstrated that CRMP4b is a necessary intracellular mediator of neurite outgrowth inhibition. The present findings suggest that complex formation

between CRMP4b and RhoA is critical for outgrowth inhibition and that this inhibition may be mediated through an actin-dependent phenotype. By targeting the critical CRMP4b-RhoA binding interaction, a competitive antagonist of CRMP4b-RhoA binding was developed which specifically promotes neurite outgrowth on inhibitory substrates suggesting an exciting new therapeutic target for nerve repair following CNS injury.

Dynamics of the CRMP4b-RhoA interaction

The present invention describes a specific protein interaction between RhoA and CRMP4, which does not extend to other closely related family members of the Rho GTPases, nor to other CRMP family members. In the absence of Nogo stimulation the baseline interaction between endogenous CRMP4b and RhoA is negligible in cerebellar neurons and DRG growth cones. In transfected 293T and PC12 cells, CRMP4b and RhoA do interact in the absence of Nogo; however, this interaction could be a function of protein overexpression.

Intriguingly, the RhoA-CRMP4b interaction is not dependent on the nucleotide binding state of RhoA, rather the interaction is dependent on the phosphorylation status of CRMP4. This raises the additional possibility that the strength of the baseline interaction may vary in different cells types as a function of the complement of kinases and phosphatases.

Since the RhoA used to screen for protein interactors was purified in bacteria, it is also conceivable that dephosphorylated RhoA is a favored binding partner for CRMP4b. This raises the possibility of a novel level of RhoA regulation in response to myelin inhibitors where the focus has largely been on the ability of MAIs to convert RhoA to the GTP-bound form (Dergham et al., 2002; Niederost et al., 2002; Fournier et al., 2003). This idea is consistent with finding that RhoA is phosphorylated at Ser188 by PKA and that RhoA phosphorylation modifies its binding to its endogenous inhibitor, Rho guanine nucleotide dissociation factor (RhoGDI) (Ellerbroek et al., 2003; Nusser et al., 2006). CRMP4 is subject to phosphorylation by glycogen synthase kinase-3 β and dephosphorylation by PP2A phosphatase (Hill et al., 2006) and it is reasonable to hypothesize that phosphorylation alters its binding properties based on similarities to CRMP2 (Uchida et al., 2005). Together this suggests a model whereby Nogo-dependent engagement of a CRMP- and/or Rho-directed phosphatase may promote formation of a CRMP4b-RhoA complex. A prediction of this model would be that CRMP4b over-expression may fail to mimic myelin inhibition in DRG neurons because its association with RhoA is not adequately enhanced in the absence of appropriate post

translational modifications, and this is what was observed (Fig. 6, 8).

Role of N-terminal variants of the CRMP proteins

The recent discovery of novel amino-terminal variants of the original CRMP members
5 in rat and in chick (Quinn et al., 2003; Yuasa-Kawada et al., 2003) has revealed additional
potential functions for CRMPs. The unique amino-terminal extensions of the CRMPb
isoforms could impart supplementary functions to each CRMP variant by mediating additional
protein interactions. A reasonable possibility is that CRMP4a and CRMP4b are capable of
mediating similar cytoskeletal rearrangements, however the amino terminal region of
10 CRMP4b is necessary for recruitment to the appropriate cytoskeletal elements within the
growth cone. This is consistent with the finding that overexpression of CRMP4b but not
CRMP4a leads to an increase in neurite branching (Quinn et al., 2003).

C4RIP as a therapeutic agent

15 Targeting CRMP4-RhoA with C4RIP is a potential avenue for therapeutic intervention.
The ability of C4RIP to attenuate inhibition in response to both MAIs and CSPGs is an
obvious advantage in the complex inhibitory environment following CNS injury. The
enrichment of CRMP expression in the nervous system raises the possibility that additional
side effects on other cell types may be limited when compared to targeting ubiquitous
20 molecules such as RhoA or ROCK. A unique characteristic of C4RIP is its failure to affect
basal neurite outgrowth. This is believed to be the first example of an intracellular molecule,
which can be specifically ascribed to an inhibitor-dependent neurite outgrowth pathway.

The function of CRMP4b in myelin-dependent inhibition

25 Reasonable hypotheses for CRMP4 function in myelin inhibition include effects on
microtubule dynamics, actin dynamics and/or endocytosis. CRMP2 can bind to tubulin
heterodimers and is an important organizer of microtubule assembly for establishing axon-
dendrite fate during development (Fukata et al., 2002b; Arimura et al., 2005). This is partly
mediated by binding to tubulin heterodimers and promoting microtubule assembly (Fukata et
30 al., 2002b). In fact, CRMP2 undergoes ROCK-dependent phosphorylation in response to
Nogo-66 and MAG and has been hypothesized to subsequently regulate microtubule
dynamics (Mimura et al., 2006). Although all CRMP members can bind tubulin, CRMP4 is not
a substrate for ROCK and no change in affinity between CRMP4 and tubulin in response to

Nogo-66 stimulation was detected by co-immunoprecipitation analysis in PC12 cells (data not shown). This suggests that Nogo-66 may not affect CRMP4-dependent microtubule dynamics; however, if the interaction is locally regulated within the neurite or growth cone, detection of this by co-immunoprecipitation may not have been possible.

5 The above data suggests that a CRMP4b-RhoA complex may participate in neurite outgrowth inhibition through actin-dependent processes. An association between CRMP4b and intersectin, an endocytic-exocytic adaptor protein (Quinn et al., 2003) raises the possibility that CRMP4b could play a role in regulating endocytosis. Endocytosis during neurite inhibition may be important for regulating membrane dynamics (Fournier et al., 2000),
10 or may target the internalization of cell surface receptors or cell adhesion molecules enabling their temporal and spatial regulation in response to MAIs. A plausible model is one in which CRMP4b-RhoA complexes regulate localized actin rearrangements from the growth cone periphery to the central domain and subsequent endocytic events necessary for growth cone collapse and neurite withdrawal.

15 Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified without departing from the spirit, scope and nature of the subject invention, as defined in the appended claims.

List of References

1. Alabed YZ, Grados-Munro E, Ferraro GB, Hsieh S, Fournier AE (2006) Neuronal responses to myelin are mediated by ROCK. *J Neurochem* 96:1616-1625.
- 5 2. Arimura N, Menager C, Kawano Y, Yoshimura T, Kawabata S, Hattori A, Fukata Y, Amano M, Goshima Y, Inagaki M, Morone N, Usukura J, Kaibuchi K (2005) Phosphorylation by Rho kinase regulates CRMP-2 activity in growth cones. *Mol Cell Biol* 25:9973-9984.
- 10 3. Arthur WT, Ellerbroek SM, Der CJ, Burrridge K, Wennerberg K (2002) XPLN, a guanine nucleotide exchange factor for RhoA and RhoB, but not RhoC. *Journal of Biological Chemistry* 277:42964-42972.
- 15 4. Bishop AL, Hall A (2000) Rho GTPases and their effector proteins. *Biochem J* 348 Pt 2:241-255.
- 20 5. Borisoff JF, Chan CC, Hiebert GW, Oschipok L, Robertson GS, Zamboni R, Steeves JD, Tetzlaff W (2003) Suppression of Rho-kinase activity promotes axonal growth on inhibitory CNS substrates. *Mol Cell Neurosci* 22:405-416.
- 25 6. Byk T, Dobransky T, Cifuentes-Diaz C, Sobel A (1996) Identification and molecular characterization of Unc-33-like phosphoprotein (Ulip), a putative mammalian homolog of the axonal guidance-associated unc-33 gene product. *Journal of Neuroscience* 16:688-701.
7. Chen MS, Huber AB, van der Haar ME, Frank M, Schnell L, Spillmann AA, Christ F, Schwab ME (2000) Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. *Nature* 403:434-439.
- 30 8. Dergham P, Ellezam B, Essagian C, Avedissian H, Lubell WD, McKerracher L (2002) Rho signaling pathway targeted to promote spinal cord repair. *J Neurosci* 22:6570-6577.

9. Ellerbroek SM, Wennerberg K, Burridge K (2003) Serine phosphorylation negatively regulates RhoA in vivo. *J Biol Chem* 278:19023-19031.
10. Feig LA, Cooper GM (1988) Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP. *Mol Cell Biol* 8:3235-3243.
11. Fournier AE, GrandPre T, Strittmatter SM (2001) Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. *Nature* 409:341-346.
- 10 12. Fournier AE, Takizawa BT, Strittmatter SM (2003) Rho Kinase inhibition enhances axonal regeneration in the injured CNS. *J Neurosci* 23:1416-1423.
13. Fournier AE, Nakamura F, Kawamoto S, Goshima Y, Kalb RG, Strittmatter SM (2000) Semaphorin3A enhances endocytosis at sites of receptor-F-actin colocalization during growth cone collapse. *J Cell Biol* 149:411-422.
- 15 14. Fournier AE, Takizawa BT, Strittmatter SM (2003) Rho Kinase inhibition enhances axonal regeneration in the injured CNS. *J Neurosci* 23: 1416-1423.
- 20 15. Fukata Y, Itoh TJ, Kimura T, Menager C, Nishimura T, Shiromizu T, Watanabe H, Inagaki N, Iwamatsu A, Hotani H, Kaibuchi K (2002a) CRMP-2 binds to tubulin heterodimers to promote microtubule assembly. *Nat Cell Biol* 4:583-591.
16. Fukata Y, Itoh TJ, Kimura T, Menager C, Nishimura T, Shiromizu T, Watanabe H, Inagaki N, Iwamatsu A, Hotani H, Kaibuchi K (2002b) CRMP-2 binds to tubulin heterodimers to promote microtubule assembly. *Nature Cell Biology* 4:583-591.
- 25 17. Gaetano C, Matsuo T, Thiele CJ (1997) Identification and characterization of a retinoic acid-regulated human homologue of the unc-33-like phosphoprotein gene (hUlip) from neuroblastoma cells. *J Biol Chem* 272:12195-12201.
- 30 18. Goshima Y, Nakamura F, Strittmatter P, Strittmatter SM (1995) Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33. *Nature*

376:509-514.

19. GrandPre T, Nakamura F, Vartanian T, Strittmatter SM (2000) Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. *Nature* 403:439-444.
20. Hall A (1994) Small GTP-binding proteins and the regulation of the actin cytoskeleton. *Annu Rev Cell Biol* 10:31-54.
21. Hedgecock EM, Culotti JG, Thomson JN, Perkins LA (1985) Axonal guidance mutants of *Caenorhabditis elegans* identified by filling sensory neurons with fluorescein dyes. *Developmental Biology* 111:158-170.
22. Hill JJ, Callaghan DA, Ding W, Kelly JF, Chakravarthy BR (2006) Identification of okadaic acid-induced phosphorylation events by a mass spectrometry approach. *Biochem Biophys Res Commun* 342:791-799.
23. Hiraga A, Kuwabara S, Doya H, Kanai K, Fujitani M, Taniguchi J, Arai K, Mori M, Hattori T, Yamashita T. Rho-kinase inhibition enhances axonal regeneration after peripheral nerve injury. *J. Peripher Nerv Syst.* 2006 Sep; 11(3):217-24.
24. Hsieh S, Ferraro GB, Fournier AE (2006) Myelin-associated inhibitors regulate cofilin phosphorylation and neuronal inhibition through Lim kinase and Slingshot phosphatase. *J Neurosci* 26:1006-1015.
25. Huber A, Kolodkin A, Ginty D, Cloutier J-F (2003) Signaling at the growth cone: Ligand-receptor complex and the control of axon growth and guidance. *Ann Rev Neurosci* 26:509-563.
26. Igarashi M, Strittmatter SM, Vartanian T, Fishman MC (1993) Mediation by G proteins of signals that cause collapse of growth cones. *Science* 259:77-79.
27. Inatome R, Tsujimura T, Hitomi T, Mitsui N, Hermann P, Kuroda S, Yamamura H, Yanagi S (2000) Identification of CRAM, a novel unc-33 gene family protein that

associates with CRMP3 and protein-tyrosine kinase(s) in the developing rat brain. J Biol Chem 275:27291-27302.

- 5 28. Jurney WM, Gallo G, Letourneau PC, McLoon SC (2002) Rac1-mediated endocytosis during ephrin-A2- and semaphorin 3A-induced growth cone collapse. J Neurosci 22:6019-6028.
- 10 29. Khosravi-Far R, Chrzanowska-Wodnicka M, Soltski PA, Eva A, Burrridge K, Der CJ (1994) Dbl and Vav mediate transformation via mitogen-activated protein kinase pathways that are distinct from those activated by oncogenic Ras. Mol Cell Biol 14:6848-6857.
- 15 30. Kottis V, Thibault P, Mikol D, Xiao ZC, Zhang R, Dergham P, Braun PE (2002) Oligodendrocyte-myelin glycoprotein (OMgp) is an inhibitor of neurite outgrowth. J Neurochem 82:1566-1569.
- 20 31. Lehmann M, Fournier A, Selles-Navarro I, Dergham P, Sebok A, Leclerc N, Tigyi G, McKerracher L (1999) Inactivation of Rho signaling pathway promotes CNS axon regeneration. J Neurosci 19:7537-7547.
32. Luo Y, Raible D, Raper JA (1993) Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell 75:217-227.
- 25 33. Mandemakers WJ, Barres BA (2005) Axon regeneration: it's getting crowded at the gates of TROY. Curr Biol 15:R302-305.
- 30 34. Madure T, Kubo T, Tanag M, Matsude K, Tomita K, Yano K, Hosokawa K. The Rho-associated kinase inhibitor fasudil hydrochloride enhances neural regeneration after axotomy in the peripheral nervous system. Plast Reconstr Surg. 2007 Feb; 119(2): 526-35.
35. McKerracher L, David S, Jackson DL, Kottis V, Dunn RJ, Braun PE (1994) Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of

neurite growth. Neuron 13:805-811.

36. McPherson PS, Czernik AJ, Chilcote TJ, Onofri F, Benfenati F, Greengard P, Schlessinger J, De Camilli P (1994) Interaction of Grb2 via its Src homology 3 domains with synaptic proteins including synapsin I. Proc Natl Acad Sci U S A 91:6486-6490.
37. Mimura F, Yamagishi S, Arimura N, Fujitani M, Kubo T, Kaibuchi K, Yamashita T (2006) Myelin-associated Glycoprotein Inhibits Microtubule Assembly by a Rho-kinase-dependent Mechanism. J Biol Chem 281:15970-15979.
38. Minturn JE, Fryer HJ, Geschwind DH, Hockfield S (1995) TOAD-64, a gene expressed early in neuronal differentiation in the rat, is related to unc-33, a C. elegans gene involved in axon outgrowth. Journal of Neuroscience 15:6757-6766.
39. Mukhopadhyay G, Doherty P, Walsh FS, Crocker PR, Filbin MT (1994) A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. Neuron 13:757-767.
40. Nakamura F, Tanaka M, Takahashi T, Kalb RG, Strittmatter SM (1998) Neuropilin-1 extracellular domains mediate semaphorin D/III-induced growth cone collapse. Neuron 21:1093-1100.
41. Neve RL, Howe JR, Hong S, Kalb RG (1997) Introduction of the glutamate receptor subunit 1 into motor neurons in vitro and in vivo using a recombinant herpes simplex virus. Neuroscience 79:435-447.
42. Niederost B, Oertle T, Fritsche J, McKinney RA, Bandtlow CE (2002) Nogo-A and myelin-associated glycoprotein mediate neurite growth inhibition by antagonistic regulation of RhoA and Rac1. J Neurosci 22:10368-10376.
43. Nishimura T, Fukata Y, Kato K, Yamaguchi T, Matsuura Y, Kamiguchi H, Kaibuchi K (2003) CRMP-2 regulates polarized Numb-mediated endocytosis for axon growth.

Nature Cell Biology 5:819-826.

44. Nusser N, Gosmanova E, Makarova N, Fujiwara Y, Yang L, Guo F, Luo Y, Zheng Y, Tigyi G (2006) Serine phosphorylation differentially affects RhoA binding to effectors: implications to NGF-induced neurite outgrowth. Cell Signal 18:704-714.
45. Prinjha R, Moore SE, Vinson M, Blake S, Morrow R, Christie G, Michalovich D, Simmons DL, Walsh FS (2000) Inhibitor of neurite outgrowth in humans. Nature 403:383-384.
46. Quinn CC, Gray GE, Hockfield S (1999) A family of proteins implicated in axon guidance and outgrowth. Journal of Neurobiology 41:158-164.
47. Quinn CC, Chen E, Kinjo TG, Kelly G, Bell AW, Elliott RC, McPherson PS, Hockfield S (2003) TUC-4b, a novel TUC family variant, regulates neurite outgrowth and associates with vesicles in the growth cone. Journal of Neuroscience 23:2815-2823.
48. Riento K, Ridley AJ (2003) Rocks: multifunctional kinases in cell behaviour. Nat Rev Mol Cell Biol 4:446-456.
49. Rojas M, Donahue JP, Tan Z, Lin Y-Z (1998) Genetic engineering of proteins with cell membrane permeability Nature Biotechnology 16:370.
50. Rosslenbroich V, Dai L, Baader SL, Noegel AA, Gieselmann V, Kappler J (2005) Collapsin response mediator protein-4 regulates F-actin bundling. Exp Cell Res 310:434-444.
51. Siddiqui SS, Culotti JG (1991) Examination of neurons in wild type and mutants of *Caenorhabditis elegans* using antibodies to horseradish peroxidase. Journal of Neurogenetics 7:193-211.
52. Suzuki Y, Nakagomi S, Namikawa K, Kiryu-Seo S, Inagaki N, Kaibuchi K, Aizawa H, Kikuchi K, Kiyama H (2003) Collapsin response mediator protein-2 accelerates axon

regeneration of nerve-injured motor neurons of rat. *Journal of Neurochemistry* 86:1042-1050.

53. Takahashi T, Nakamura F, Jin Z, Kalb RG, Strittmatter SM (1998) Semaphorins A and E act as antagonists of neuropilin-1 and agonists of neuropilin-2 receptors. *Nat Neurosci* 1:487-493.
54. Uchida Y, Ohshima T, Sasaki Y, Suzuki H, Yanai S, Yamashita N, Nakamura F, Takei K, Ihara Y, Mikoshiba K, Kolattukudy P, Honnorat J, Goshima Y (2005) Semaphorin3A signalling is mediated via sequential Cdk5 and GSK3beta phosphorylation of CRMP2: implication of common phosphorylating mechanism underlying axon guidance and Alzheimer's disease. *Genes Cells* 10:165-179.
55. Wang KC, Koprivica V, Kim JA, Sivasankaran R, Guo Y, Neve RL, He Z (2002) Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. *Nature* 417:941-944.
56. Wang LH, Strittmatter SM (1997) Brain CRMP forms heterotetramers similar to liver dihydropyrimidinase. *J Neurochem* 69:2261-2269.
57. Wennerberg K, Ellerbroek SM, Liu RY, Karnoub AE, Burridge K, Der CJ (2002) RhoG signals in parallel with Rac1 and Cdc42. *J Biol Chem* 277:47810-47817.
58. Winton MJ, Dubreuil C, Lasko D, Leclerc N, McKerracher L (2002) Characterization of New Cell Permeable C3-like Proteins that Inactivate Rho and Stimulate Neurite Outgrowth on Inhibitory Substrates *J. Biol Chem* 277:32820-32829.
59. Yoshimura T, Kawano Y, Arimura N, Kawabata S, Kikuchi A, Kaibuchi K (2005) GSK-3beta regulates phosphorylation of CRMP-2 and neuronal polarity. *Cell* 120:137-149.
60. Yuasa-Kawada J, Suzuki R, Kano F, Ohkawara T, Murata M, Noda M (2003) Axonal morphogenesis controlled by antagonistic roles of two CRMP subtypes in microtubule organization. *European Journal of Neuroscience* 17:2329-2343.

CLAIMS:

1. A polypeptide having the amino acid sequence of SEQ ID NO: 2, or a derivative,
5 analog, variant or fragment thereof.
2. A polypeptide as defined in claim 1, which is encoded by the nucleotide sequence of
SEQ ID NO: 4 or a degenerate variant thereof.
- 10 3. A polypeptide as defined in claim 1, further comprising a membrane-translocating
sequence (MTS).
4. A polypeptide as defined in claim 3, wherein said MTS is encoded by the nucleotide
sequence of SEQ ID NO: 10 or SEQ ID NO:13, or degenerate variants thereof.
15
5. An expression vector comprising the nucleotide sequence of SEQ ID NO: 4 or a
degenerate variant thereof operably-linked to an expression control sequence.
6. An expression vector comprising the nucleotide sequence of SEQ ID NO:4 or a
20 degenerate variant thereof and a membrane-translocating sequence (MTS) operably-linked to
an expression control.
7. An expression vector as described in claim 6, wherein said MTS has the nucleotide
sequence of SEQ ID NO: 10 or SEQ ID NO:13, or degenerate variants thereof.
25
8. An expression vector as defined in any one of claims 5-7, wherein said vector is
derived from the herpes simplex virus (HSV).
9. A cultured cell comprising an expression vector as defined in any one of claims 5-8.
30
10. A method of producing a protein, wherein said method comprises culturing the cell of
claim 9 under conditions permitting expression of said protein.

11. A method as defined in claim 10, further comprising purifying the protein from the cell or the medium of the cell.

12. Use of a compound that disrupts CRMP4-RhoA interaction or other functions of CRMP4 so as to promote the regeneration of neurons after injury to or disease of the central or peripheral nervous systems.

13. A use as defined in claim 12, wherein said compound is C4RIP or a derivative, analog, variant or fragment thereof.

14. A use as defined in claim 12, wherein said compound has the amino acid sequence of SEQ ID NO: 2 or is a derivative, analog, variant or fragment thereof.

15. A method of repairing central or peripheral nerve damage in a subject in need thereof comprising the administration to the site of the damage an effective amount of a compound that disrupts CRMP4-RhoA interaction or other functions of CRMP4 in order to promote the regrowth of neurons.

16. A method as defined in claim 15, wherein said compound is C4RIP or a derivative, analog, variant or fragment thereof.

17. A method as defined in claim 15, wherein said compound has the amino acid sequence of SEQ ID NO:2 or is a derivative, analog, variant or fragment thereof.

18. A method of suppressing the myelin-dependent inhibition of neuron regeneration, comprising the step of delivering, to the nerve growth environment, an effective amount of a CRMP4-RhoA antagonist or CRMP4 antagonist.

19. A method as defined in claim 18, wherein said compound is C4RIP or a derivative, analog, variant or fragment thereof.

20. A method as defined in claim 18, wherein said compound has the amino acid sequence of SEQ ID NO:2 or is a derivative, analog, variant or fragment thereof.

21. A method of identifying a compound that inhibits CRMP4-RhoA interaction or other functions of CRMP4, the method comprising:

- 5 a. providing a cell expressing CRMP4 and RhoA;
 b. contacting the cell with a test compound to see whether the compound disrupts interaction between CRMP4 and RhoA or other functions of CRMP4.

22. A method of identifying a compound that inhibits CRMP4-RhoA interaction or other
10 functions of CRMP4, the method comprising:

- a. providing a cell expressing CRMP4 and RhoA;
 b. contacting the cell with a quantity of compound known to inhibit either
15 CRMP4-RhoA interaction or other functions of CRMP4, and measuring the effect;
 c. in a separate vessel, contacting the cell with a test compound and measuring the effect
 d. comparing the results from (b) and (c) to determine whether the test compound is also an inhibitor.

20

23. A method as defined in claim 22, wherein said compound known to inhibit CRMP4-RhoA interaction or other functions of CRMP4 is C4RIP or a derivative, analog, variant or fragment thereof or where the inhibitor is expressed endogenously via a construct inserted into the DNA of the cell.

25

24. A method as defined in claim 23, wherein said compound known to inhibit CRMP4-RhoA interaction or other functions of C4RIP has the amino acid sequence of SEQ ID NO: 2 or is a derivative, analog, variant or fragment thereof.

30

25. A method as defined in any one of claims 21-24 which is included in a kit.

26. A method of treating an injury to or a disease of the central or peripheral nervous system, said method comprising administration of a therapeutically effective amount of a

compound that disrupts CRMP4-RhoA interaction or other functions of CRMP4.

27. A method as described in claim 26, wherein said compound is C4RIP or a derivative, analog, variant or fragment thereof.

5

28. A method as described in claim 26, wherein said compound has the amino acid sequence of SEQ ID NO: 2 or is a derivative, analog, variant or fragment thereof.

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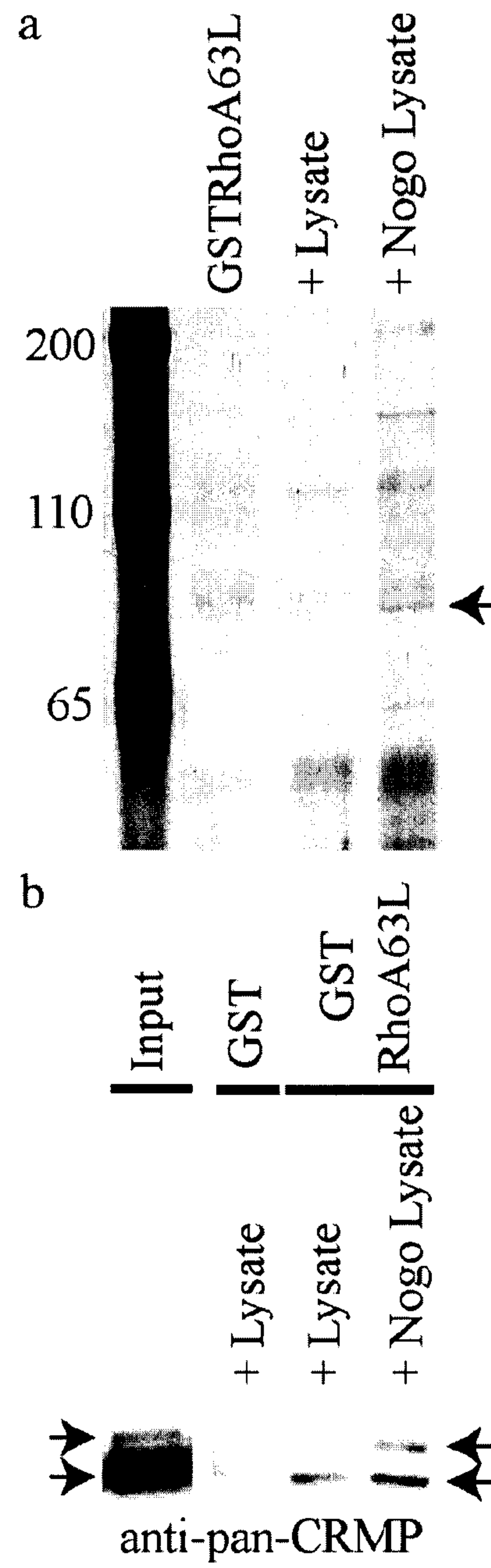


Figure 1

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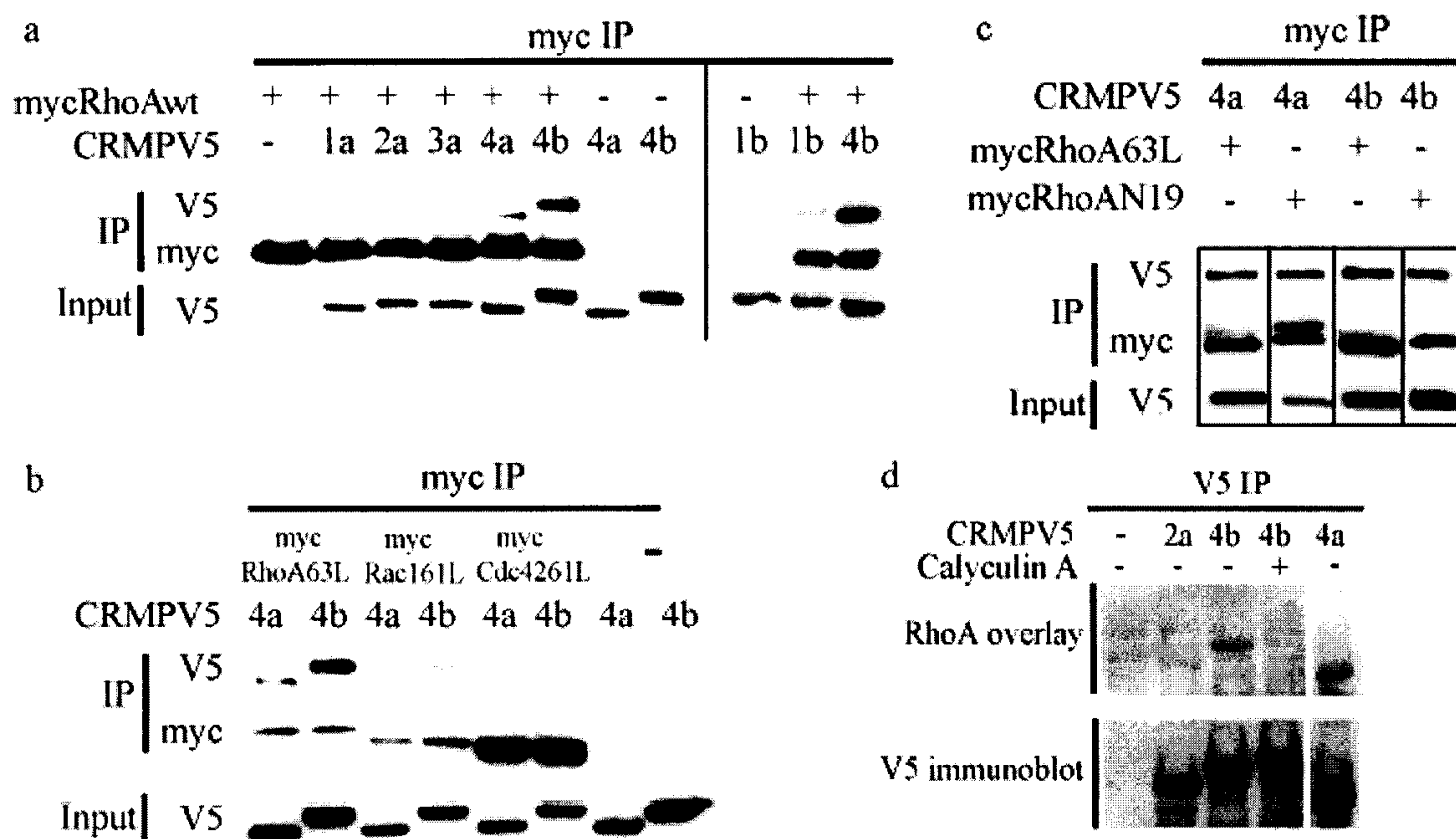


Figure 2

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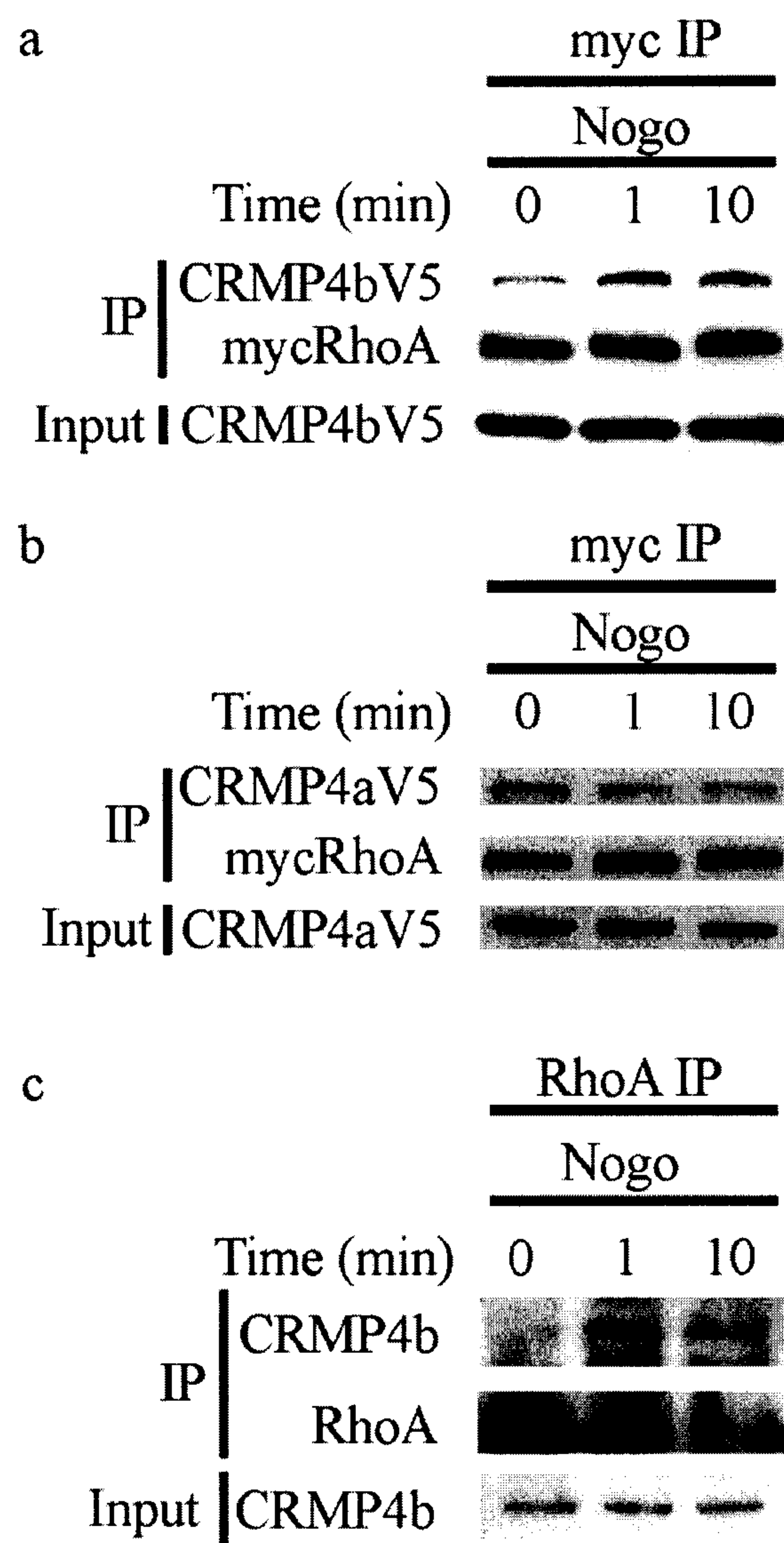


Figure 3

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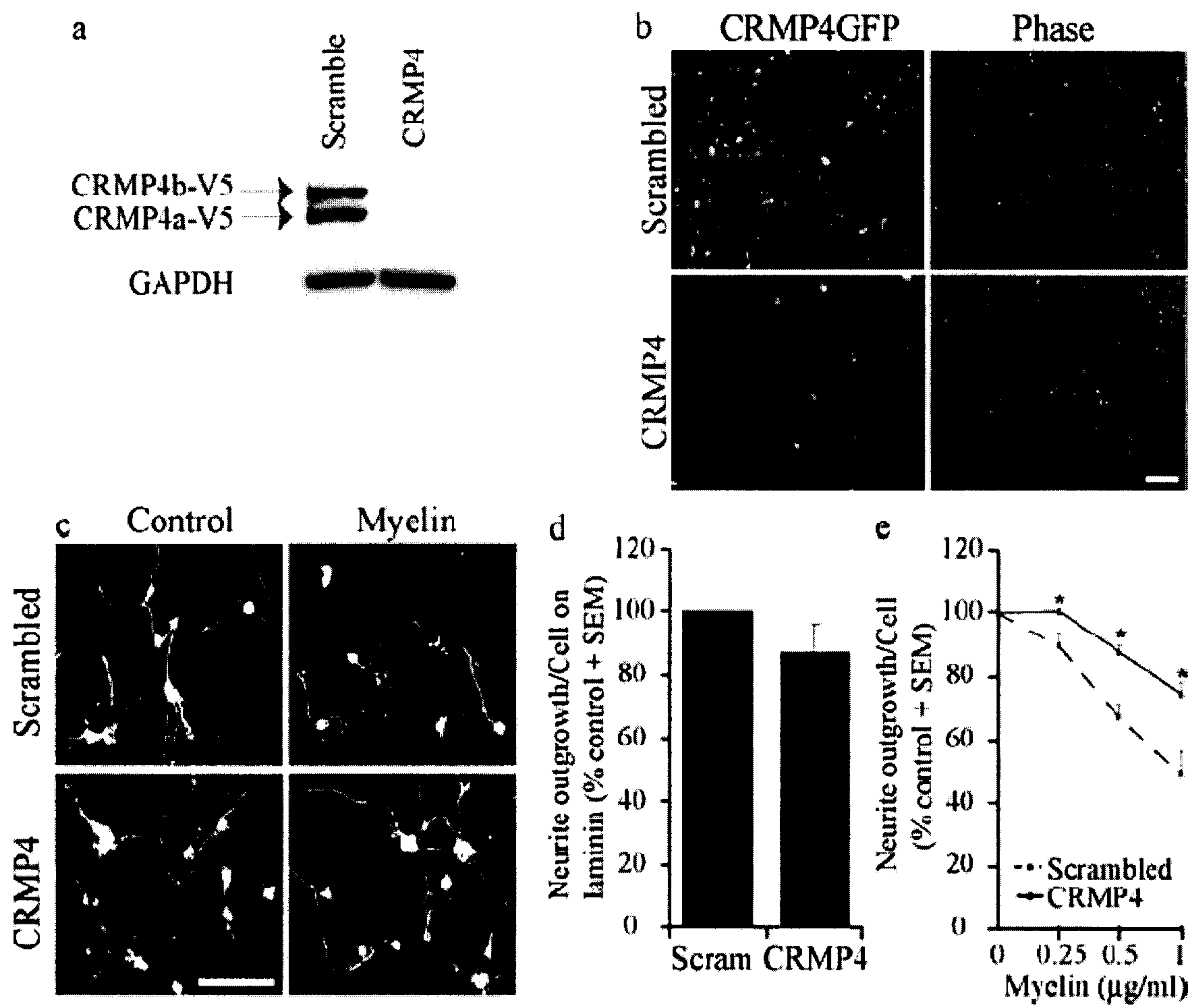


Figure 4

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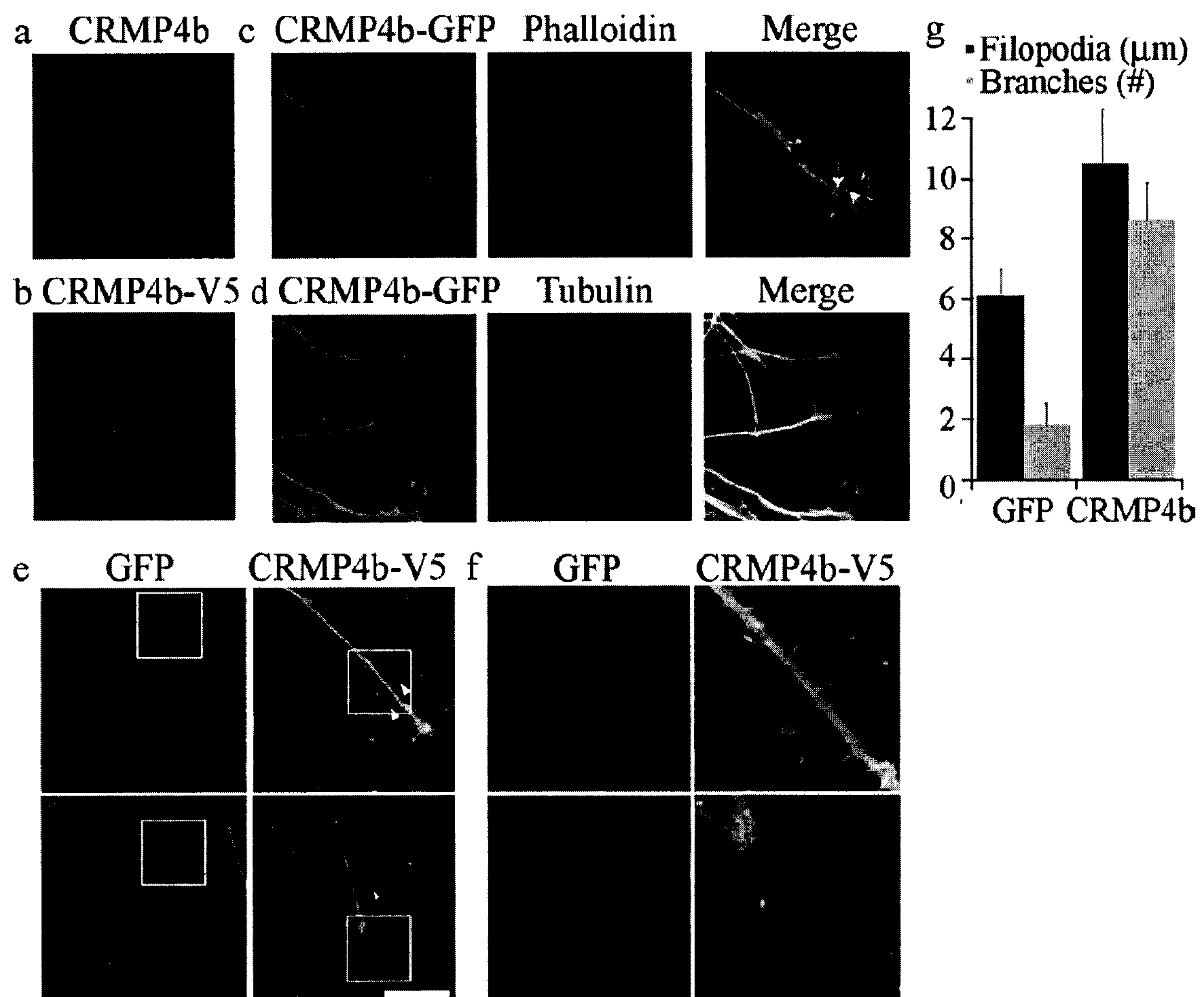


Figure 5

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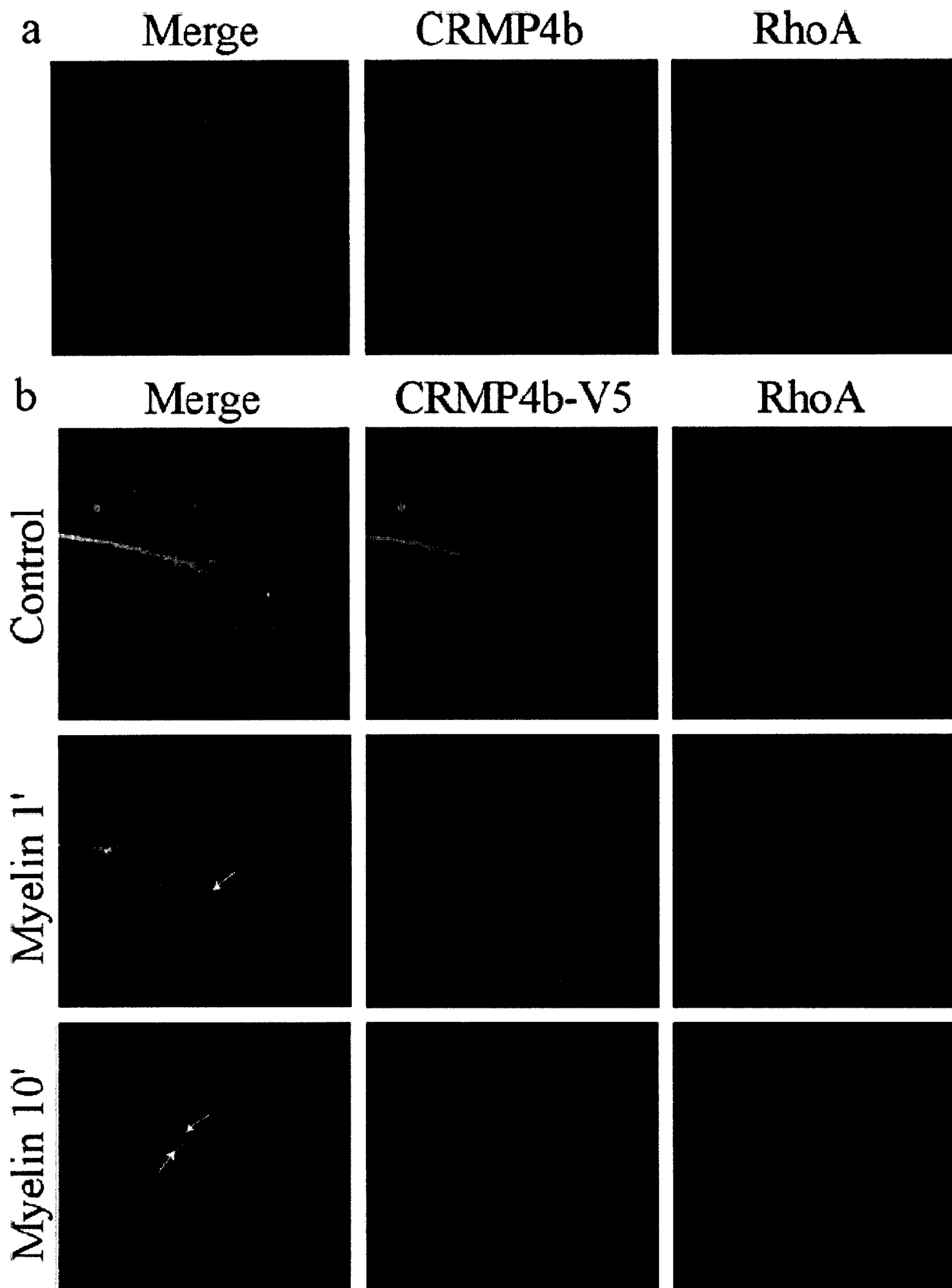


Figure 6

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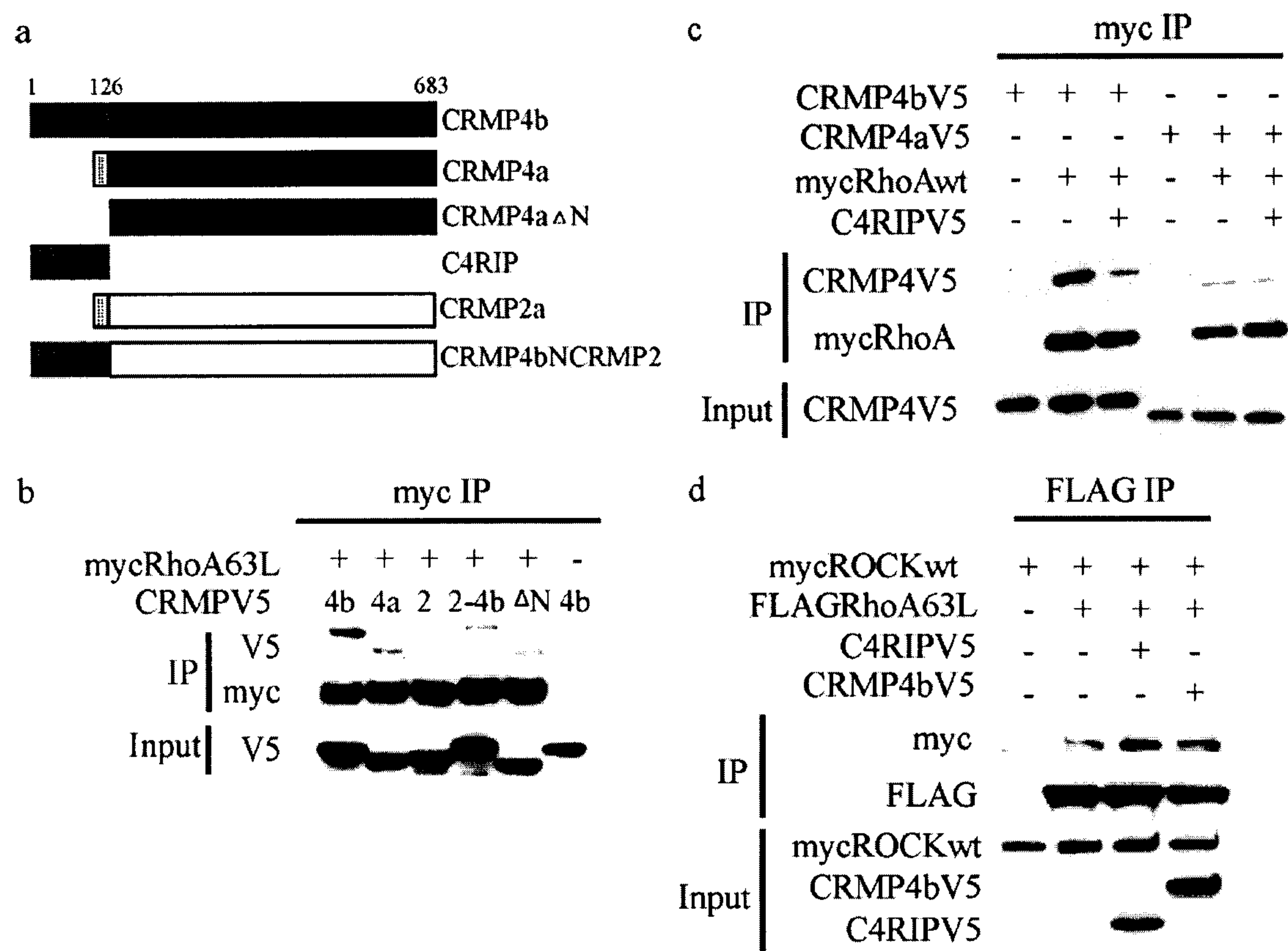


Figure 7

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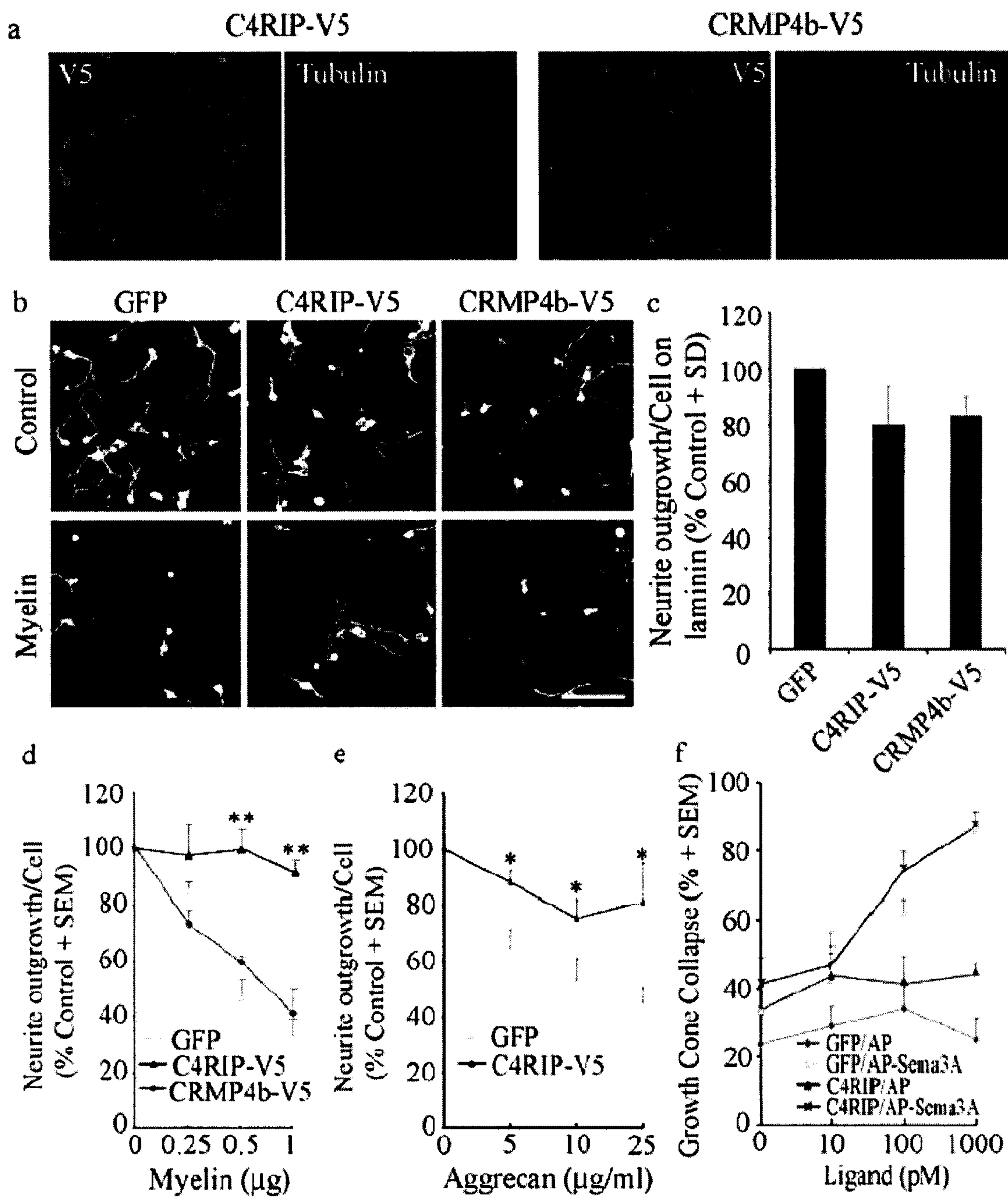


Figure 8

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Rat CRMP4b Amino Acid and cDNA Sequence Data

(a) Rat CRMP4b amino acid sequence with C4RIP sequence shaded in grey (SEQ ID NO: 1)

MASGRRGWDSSHEDDLPVYLARPGTTDQVPRQKYGGMFCNVEGA
 FESKTLDFDALSVGQRGAKTPRSSQSGRGAGNRPGVEGDTRRGPGREESREPVPESP
 KPAGVEIRSATGKEVLQNLGPKDKSDRLLIKGGRIVNDDQSFYADIYMEDGLIKQIGD
 NLIVPGGVKTIEANGKMVMPGGIDVHTHFQMPYKGMTTVDDFFQGTKAALAGGTTMII
 DHVVPEPESSLTEAYEKWREWADGKSCCDYALHVDITHWNDSVKQEVQNLKSKEKGVNS
 FMVYMAYKDLYQVSNTELYEIFTC LGELGAI AQVHAENGDI IAQEQARMLEMGITGPE
 GHVLSRPEELEAEAVFRAITVASQTNCPLYVTKVMSKSAADLISQARKKGNVVFGEPI
 TASLGIDGTHYWSKNWAKAAAFVTSPPLSPDPTTPDYINSL LASGDLQLSGSAHCTFS
 TAQKAIGKDNFTAIPEGTNGVEERMSVIWDKAVATGKMDENQFVAVTSTNAAKIFNLY
 PRKGRIAVGSDSDLVIWDPDAVKIVSAKNHQSVAEYNIFEGMELRGAPLVVICQGKIM
 LEDGNLHVTQGAGRFI PCSPFSDYVYKRIKARRKMADLH AVPRGMYDGPVFDLT TTPK
 GGTPAGSTRGSPTRPNPPVRNLHQSGFSLSGTQVDEGVR SASKRIVAPPGGRSNITSL
 S

(b) Rat C4RIP Amino Acid Sequence (SEQ ID NO: 2)

MASGRRGWDSSHEDDLPVYLARPGTTDQVPRQKYGGMFCNVEGA
 FESKTLDFDALSVGQRGAKTPRSSQSGRGAGNRPGVEGDTRRGPGREESREPVPESP
 KPAGVEIRSATGKEVLQNLGPKDK

(c) Rat CRMP4b cDNA sequence with C4RIP coding region shaded in grey (SEQ ID NO: 3)

1 atggcttcgg gccgaagggg ttgggacagc toccacgagg acgacctgcc tgtgtacttg
 61 gcccggccgg gcaccacaga tcagggtccc cggcagaagt acggtggcat gttctgcaac
 121 gtggagggcg ccttcgagag caagacattg gatatttgatg ccttgagtgt gggacagegc
 181 ggcgcacaaa ctccccggag cagccagggc agcggccgcg gcgcggggaa ccggcccggg
 241 gtggaagggg acacgcgcag gggcccgggc cgggaggaga gcagggagcc cgtgcctgag
 301 tcgccaagc ccgcccgggt agagatccgg agcggccactg gcaaggaggt cttgcagaac
 361 ctcggtccc aaggacaagag tgaccgtctt ctaatcaagg gagggagaat cgtcaacgat
 421 gatcagtcct tttatgctga tatttacatg gaggatggct tgataaagca aattggagac
 481 aatctgattg tccctggagg tgtgaagacc attgaggcca atgggaagat ggtgatgcct
 541 ggaggcattg atgtccatac ccacttcag atgccttaca aggggatgac cacagtggac
 601 gatttcctcc aagggacaaa ggctgcctta gcgggaggaa ccaccatgat cattgaccat
 661 gtggtacctg aacctgagtc tagcctgacc gaggcctatg aaaagtggcg tgagtgggct

Figure 9-9

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```

721 gacgggaaga gctgctgtga ctatgctttg catgtggaca tcacccactg gaatgacagc
781 gtcaagcaag aggtgcagaa cctcagtaag gaaaaaggcg ttaactcctt catggtttac
841 atggcataca aggatttata tcaagtgtcc aacacagagc tctatgagat cttcacctgc
901 ctgggagaaac tggggggccat tgctcaagtt catgccgaga atggagacat cattgcccag
961 gagcaggcac gaatgctgga aatgggaata acaggcccag aaggatcatgt tctgagcaga
1021 ccggaagagc tggaagctga ggctgtgttc cgtgccatca ccgtcgccag ccagaccaac
1081 tgcccccttt atgtcaccaa ggtcatgagc aagagcgcgg ctgatctcat ctcacaagcc
1141 aggaagaaaag gaaatgtggt ctttggcgag cccatcactg ccagcctggg aatagatgga
1201 acccattact ggagtaagaa ctgggccaag gcagctgcat ttgtgacatc cccacctctg
1261 agccctgacc caaccacacc tgactacatc aactccttgc tggccagtgg agatctgcag
1321 ctctctggaa gtgcccactg taccttcagc actgcccaga aagccattgg gaaggacaac
1381 ttcacggcta tccctgaggg caccaatggc gtggaggagc gtatgtctgt catctgggac
1441 aaggctgtgg ccacaggga gatggatgaa aaccagtttg tggctgtgac aagtaccaac
1501 gctgccaaaga tattcaacct gtaccctcgc aaggggagaa tagctgtggg ttctgacagc
1561 gaccttgtca tctgggatcc agatgccgtg aagatcgtct ctgccaagaa ccaccagtgc
1621 gttgcggaat acaacatctt tgaagggatg gagctgcgtg gggcacctct ggtggttata
1681 tgccagggca agatcatgct ggaagatggt aacctgcacg tgaccagggg ggccggccgc
1741 ttcattccct gcagcccatt ctctgactat gtctataagc gcattaaagc aaggagggaag
1801 atggcggaac tgcatgcagt cccaagaggg atgtatgatg gaccagtgtt tgacttgacc
1861 accaccccca agggggggcac cccagctggc tctactcgag gctctccac tcggccaaac
1921 ccaccagtga ggaacctcca tcagtcggga ttagtctgt caggcaccca agtgatgag
1981 ggtgtccgct cagccagcaa acgcattgtg gcgccccctg gaggccgttc taacatcaca
2041 tccctgagtt aa

```

(d) Rat C4RIP cDNA sequence (SEQ ID NO: 4)

```

1 atggcttcgg gccgaagggg ttgggacagc tcccacgagg acgacctgcc tgtgtacttg
61 gcccggccgg gcaccacaga tcaggtccca cggcagaagt acggtggcat gttctgcaac
121 gtggagggcg ccttcgagag caagacattg gattttgatg ccttgagtgt gggacagcgc
181 ggcgcaaaa ctccccggag cagccagggc agcgccgcg gcgcggggaa cggcccggg
241 gtggaagggg acacgcgcag gggcccgggc cgggaggaga gcaggagacc cgtgcctgag
301 tcgccaagc ccgccggggg agagatccgg agcgccactg gcaaggaggt cttgcagaac
361 ctcggtccca aggacaag

```

(e) Rat CRMP4a amino acid sequence (SEQ ID NO: 5)

```

MSYQGKKNIPRITSDRLLIKGGRIVNDDQSFYADIYMEDGLIKQ
IGDNLIVPGGVKTIEANGKMVMPGGIDVHTHFQMPYKGMTTVDDFFQGTKAALAGGTT
MIIDHVVPEPESSLTEAYEKWREWADGKSCCDYALHVDITHWNDSVKQEVQNLSKEKG
VNSFMVYMAYKDLYQVSNTELYEIFTCLGELGAIAQVHAENGDI IAQEQARMLEMGIT
GPEGHVLSRPEELEAEAVFRAITVASQTNCPLYVTKVMSKSAADLISQARKKGNVVF
EPITASLGIDGTHYWSKNWAKAAAFVTSPPLSPDPTTPDYINSL LASGDLQLSGSAHC
TFSTAQKAIGKDNFTAIPEGTNGVEERMSVIWDKAVATGKMDENQFVAVTSTNAAKIF
NLYPRKGRIAVGSDSDLVIWDPDAVKIVSAKNHQSVAEYNIFEGMELRGAPLVVICQG
KIMLEDGNLHVTQGAGRFI PCSPFSDYVYKRIKARRKMADLHAVPRGMYDGPVFDLTT
TPKGGTPAGSTRGSPTRPNPPVRNLHQSGFSLSGTQVDEGVR SASKRIVAPPGGRSNI
TSL S

```

Figure 9-10

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(f) Rat CRMP4a cDNA nucleotide sequence (SEQ ID NO:6)

```

1 atgtcctacc agggcaagaa gaacattcct cggatcacga gtgaccgtct tctaatacaag
61 ggagggagaa tcgtcaacga tgatcagtcc ttttatgctg atatttacat ggaggatggc
121 ttgataaagc aaattggaga caatctgatt gtccctggag gtgtgaagac cattgaggcc
181 aatgggaaga tggatgatgcc tggaggcatt gatgtccata ccacttcca gatgccttac
241 aaggggatga ccacagtga cgatttcttc caagggacaa aggtgcctt agcgggagga
301 accaccatga tcattgacca tgtggtacct gaacctgagt ctgacctgac cgaggcctat
361 gaaaagtggc gtgagtgggc tgacgggaag agctgctgtg actatgcttt gcatgtggac
421 atcacccact ggaatgacag cgtcaagcaa gaggtgcaga acctcagtaa ggaaaaaggc
481 gttaactcct tcatggttta catggcatac aaggatttat atcaagtgtc caacacagag
541 ctctatgaga tcttcacctg cctgggagaa ctggggggcca ttgctcaagt tcatgccgag
601 aatggagaca tcattgcca ggagcaggca cgaatgctgg aaatgggaat aacaggccca
661 gaaggatcatg ttctgagcag accggaagag ctggaagctg aggtgtgtt ccgtgccatc
721 accgtcgcca gccagaccaa ctgccccctt tatgtcacca aggtcatgag caagagcgcg
781 gctgatctca tctcacaagc caggaagaaa ggaaatgtgg tctttggcga gcccatcact
841 gccagcctgg gaatagatgg aaccattac tggagtaaga actgggcca ggcagctgca
901 tttgtgacat cccacctct gagccctgac ccaaccacac ctgactacat caactccttg
961 ctggccagtg gagatctgca gctctctgga agtgccact gtaccttcag cactgccag
1021 aaagccattg ggaaggacaa cttcacggct atccctgagg gcaccaatgg cgtggaggag
1081 cgtatgtctg tcatctggga caaggctgtg gccacaggga agatggatga aaaccagttt
1141 gtggctgtga caagtaccaa cgctgccaa agatattcaacc tgtacctcgc caaggggaga
1201 atagctgtgg gttctgacag cgacctgtgc atctgggatc cagatgccgt gaagatcgtc
1261 tctgccaaga accaccagtc ggttgcgga tacaacatct ttgaagggat ggagctgcgt
1321 ggggcacctc tgggtggttat ctgccagggc aagatcatgc tggaagatgg taacctgcac
1381 gtgaccagcagg gggccggccg cttcattccc tgcagcccat tctctgacta tgtctataag
1441 cgcattaaag caaggaggaa gatggcggac ctgcatgcag tcccaagagg catgtatgat
1501 ggaccagtgt ttgacttgac caccacccc aaggggggca cccagctgg ctctactcga
1561 ggctctccca ctcgggccaa cccaccagt aggaacctcc atcagtcggg atttagtctg
1621 tcaggcaccc aagtggatga ggggtgtccg tcagccagca aacgcattgt ggcgccccct
1681 ggaggccggt ctaacatcac atccctgagt taa

```

Figure 9-11

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Sample Membrane Translocating-Sequences with and without C4RIP**a) C4RIP sequence with C3-05 MTS (SEQ ID NOS: 8 and 9)**

Bam HI

ATGGCTTCGGGCCGAAGGGGTTGGGACAGCTCCCACGAGGACGACCTGCCTGTGTACTTGGCC
 CGGCCGGGCACCACAGATCAGGTCCCACGGCAGAAGTACGGTGGCATGTTCTGCAACGTGGAGGGCGCC
 TTCGAGAGCAAGACATTGGATTTTGATGCCCTGAGTGTGGGACAGCGCGGCCAAAACCTCCCCGGAGC
 AGCCAGGGCAGCGGCCGCGGCGCGGGGAACCGGCCCGGGGTGGAAGGGGACACGCGCAGGGGCCCGGGC
 CGGGAGGAGAGCAGGGAGCCCGTGCCTGAGTCGCCCCAAGCCCGCCGGGGTAGAGATCCGGAGCGCCACT
 GGCAAGGAGGTCTTGCAGAACCTCGGTCCCAAGGACAAG GTGATGAATCCCGCAAACGCGCAA
 GGCAGACATACACCCGGTACCAGACTC Eco RI C3-05

permeability sequence

Xho I

MASGRRGWDS SHEDDLPVYL ARPGTTDQVP RQKYGGMFCN VEGAFESKTL DFDALSVGQR
 GAKTPRSSQG SGRGAGNRPG VEGDTRRGPG REESREPVPE SPKPAGVEIR SATGKEVLQN
 LGPKDKEFVM NPANAQGRHT PGTRLL

b) C3-05 MTS (SEQ ID NO: 10)

GTGATGAATCCCGCAAACGCGCAAGGCAGACATACACCCGGTACCAGACTC

c) C4RIP sequence with Kaposi MTS (SEQ ID NOS: 11 and 12)

Bam HI

ATGGCTTCGGGCCGAAGGGGTTGGGACAGCTCCCACGAGGACGACCTGCCTGTGTACTTGGCC
 CGGCCGGGCACCACAGATCAGGTCCCACGGCAGAAGTACGGTGGCATGTTCTGCAACGTGGAGGGCGCC
 TTCGAGAGCAAGACATTGGATTTTGATGCCCTGAGTGTGGGACAGCGCGGCCAAAACCTCCCCGGAGC
 AGCCAGGGCAGCGGCCGCGGCGCGGGGAACCGGCCCGGGGTGGAAGGGGACACGCGCAGGGGCCCGGGC
 CGGGAGGAGAGCAGGGAGCCCGTGCCTGAGTCGCCCCAAGCCCGCCGGGGTAGAGATCCGGAGCGCCACT
 GGCAAGGAGGTCTTGCAGAACCTCGGTCCCAAGGACAAG GCAGCCGTTCTTCTCCCTGTTCTT
 CTTGCCGACCC Eco RI mts kaposi

permeability sequence

Xho I

ASGRRGWDS SHEDDLPVYL ARPGTTDQVP RQKYGGMFCN VEGAFESKTL DFDALSVGQR
 GAKTPRSSQG SGRGAGNRPG VEGDTRRGPG REESREPVPE SPKPAGVEIR SATGKEVLQN
 LGPKDKEFAA VLLPVLLAAP LE

d) Kaposi MTS (SEQ ID NO: 13)

GCAGCCGTTCTTCTCCCTGTTCTTCTTGCCGACCC

Figure 10