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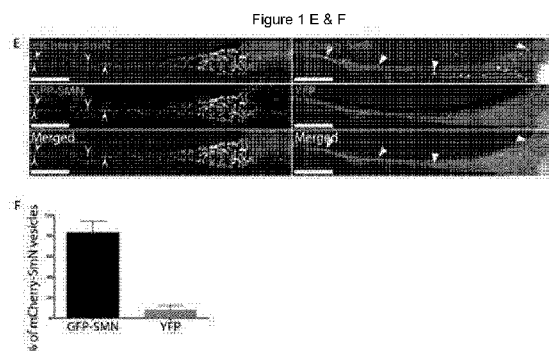
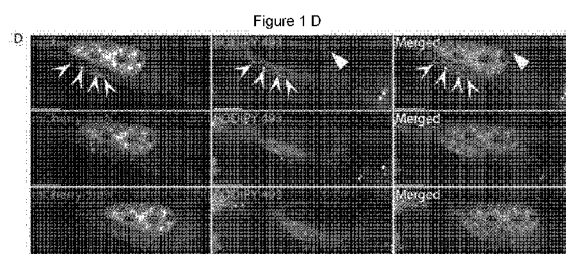
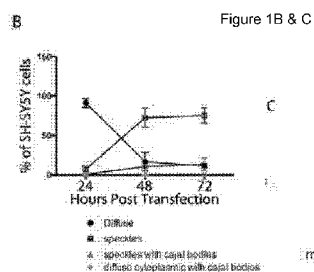
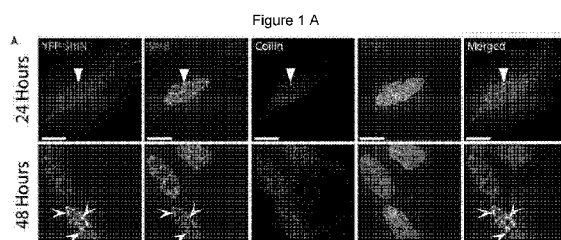
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(57) **Abstract:** The present invention is based on the finding that the essential neural protein, neurochondrin (NCDN), interacts with the Survival Motor Neurone (SMN) protein. In neurodegenerative disorders, novel interactions such as this provide new insights into disease pathology and/or additional or alternate therapeutic options. Thus the disclosure provides neurochondrin (NCDN) modulators for use in the treatment and/or prevention of a neurodegenerative disorder.





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NOVEL TREATMENTS

FIELD OF THE INVENTION

The disclosure provides modulator compounds for use in the treatment and/or prevention of neurodegenerative diseases and methods of identifying agents useful in the treatment
5 and/or prevention of the same.

BACKGROUND OF THE INVENTION

The neurodegenerative inherited disease, Spinal Muscular Atrophy (SMA), is the leading genetic cause of infant mortality, affecting 1:6000 live births. SMA causes a loss of spinal
10 motor neurons and is characterised by progressive muscle atrophy.

The cause of SMA, in the vast majority of cases, is a reduction in the amount of functional Survival Motor Neuron (SMN) protein through the deletion or mutation of the gene, *SMN1*; this resulting in expression of too little of the SMN protein. A second gene, *SMN2*, is present in varying numbers of copies and can produce a small amount of full-length SMN protein.
15 However, the *SMN2* gene contains a point mutation in an exon splicing enhancer resulting in the truncation of most of the SMN protein produced by *SMN2*.

It is not currently clear how a deficiency in functional SMN leads to the specific symptoms of SMA. In particular, the differing sensitivity of cell types to lowered SMN levels, with motor neurons most severely affected, is difficult to explain as SMN is an essential protein and
20 complete deletion is lethal at the cellular level (Schrack et al, 1997; Hsieh-Li et al, 2000). Recent drug trials using anti-sense oligonucleotides designed to increase protein production from the *SMN2* genes in paediatric patients have been successful, with the novel treatment nusinersen/spinraza.

Nevertheless, research into new therapeutic strategies and new and additional drugs is a
25 priority.

SUMMARY OF THE INVENTION

The present invention is based on the finding that the essential neural protein, neurochondrin (NCDN), interacts with the Survival Motor Neurone (SMN) protein. In diseases such as Spinal Muscular Atrophy, novel interactions such as this provide new
30 insights into disease pathology and/or additional or alternate therapeutic options.

While the genetic cause of SMA is clear (deletion or mutation of the SMN1 gene affecting SMN expression), precisely how low or reduced levels of SMN lead to SMA (or the symptoms thereof) is not properly understood. Further, efforts to decipher the complex cellular pathology of SMA are complicated by the fact that the SMN protein has numerous functions within the cell. An additional and poorly understood phenomenon is that while it is now thought that many cell types are affected in SMA, motor neurons appear particularly sensitive to low levels of SMN protein.

SMN is localised to nuclear gems and within the cytoplasm and has been implicated in trafficking, which is likely to be of key importance in large and highly polarised cells such as motor neurons. A subsequent proteomic screen in a neural cell line identified neurochondrin (NCDN) as a protein interacting with SMN and the Sm proteins, most likely within cytoplasmic trafficking vesicles. NCDN is an essential protein linked to promotion of neural outgrowth, cell polarity and the regulation of synaptic plasticity.

Without wishing to be bound by theory, it is suggested that if an increase in both NCDN and/or SMN is required for enhanced cytoplasmic vesicle localisation, or an alternative shared function of SMN and NCDN, then therapeutics increasing NCDN expression could be useful in the treatment and/or prevention of neurodegenerative diseases such as SMA. Therapeutics of this type may be used together with current, existing or developing therapeutics, which, in the case of SMA treatment, often target the *SMN2* gene.

It should also be noted (again without wishing to be bound by theory) that because motor neurons are unexpectedly sensitive to low levels of SMN protein, any associated or downstream effect of a SMN deficiency on other cells is hard to notice. As new drugs and treatments for diseases such as SMA are developed, the prognosis associated with SMA improves and it is suggested that a consequence of this may be the rise or development of other SMN deficiency associated problems in other cells and/or systems that are not so exquisitely sensitive to an SMN deficiency.

Thus, a first aspect of this disclosure provides neurochondrin (NCDN) modulators for use in the treatment and/or prevention of a neurodegenerative disorder.

The term "neurodegenerative disorder" may include the class of diseases and/or conditions referred to as neuromuscular disorders. In particular, the term embraces spinal muscular atrophy (SMA: also referred to as autosomal recessive proximal spinal muscular atrophy and/or 5q spinal muscular atrophy).

As such this disclosure provides neurochondrin (NCDN) modulators for use in the treatment and/or prevention of a spinal muscular atrophy.

The term "neurodegenerative disorder" may not embrace a "neurological autoimmune disease". The term "neurodegenerative disorder" may not embrace one or more of the diseases, conditions or syndromes selected from the group consisting of Alzheimer's Disease, Autism, Aspergers's Syndrome, Apraxia, Aphasia, Cerebellar syndrome, Cerebellitis, Chorea, Encephalitis, Movement disorder, spinocerebellar ataxia, preferably a non-progressive form, Paralysis, Paraplegia, Gaucher's disease, Myopathy, Myasthenia gravis, Multiple Sclerosis, Parkinsons's disease, Polyneuropathy and Dementia, preferably Cerebellar syndrome, Cerebellitis, Movement disorder and Dementia.

A neurochondrin (NCDN) modulator shall be referred to hereinafter as a "NCDN modulator". The term "NCDN modulator" embraces any substance or compound (or indeed a composition comprising the same) which increases or decreases any aspect of the expression, function and/or activity of the NCDN protein.

A NCDN modulator may comprise one or more active NCDN modulator(s). Additionally or alternatively, a NCDN modulator may comprise two or more compounds which individually are not NCDN modulators but when used together modulate one or more aspects of NCDN expression, function and/or activity.

In the context of this disclosure, the term "NCDN expression" relates to the amount of NCDN protein produced in a cell and/or the quantity, amount or rate of NCDN gene transcription/translation in a cell. The term "NCDN function" relates to the role of the NCDN protein in a cell, including for example its normal or usual (wildtype) roles and/or intracellular interactions and the like. The term "NCDN activity" relates the amount or quantity of normal or wildtype function of the NCDN protein in a cell. As stated, a useful NCDN modulator may increase or decrease any of these aspects of NCDN expression, function and/or activity.

A NCDN modulator for use in the treatment and/or prevention of a neurodegenerative disorder such as SMA may increase (directly or indirectly) NCDN expression, function and/or activity.

Any increase or decrease in NCDN expression, function and/or activity may be assessed relative to a control, predetermined or known amount of NCDN expression, function or activity. For example, an increase or decrease in NCDN expression, function and/or activity may be assessed relative to the level of NCDN expression, function and/or activity in a

wildtype or diseased cell. A wildtype cell may be a cell which exhibits a normal amount of NCDN expression, function and/or activity. A wildtype cell may be a "healthy" cell - that is a cell which is free of any symptoms related to, or associated with, neurodegenerative diseases (for example SMA) and/or genetic mutations associated or causative of the same.

- 5 A diseased cell may be any cell harbouring a genetic mutation associated with or causative of a neurodegenerative condition (including SMA) and/or exhibiting one or more symptoms associated therewith and in which the level of NCDN expression, function and/or activity is reduced below that known to occur in a wildtype cell. The control cell may be derived from a cell line, for example a neural cell line or neuroblastoma cell line. Exemplary cell line cells
10 may include the SH-SY5Y cells.

A NCDN modulator can be, for example, a protein, a peptide, an oligonucleotide, a small molecule, an antibody (or some epitope binding fragment thereof), oligosaccharide or a lipid.

- A NCDN modulator may increase the activity or function of the native or wildtype NCDN protein by supplementing, replicating and/or enhancing or promoting its effect in a cell. For
15 example, a suitable NCDN modulator may mimic or replicate wildtype NCDN function in a cell. One of skill will appreciate that a substance or compound which has an NCDN-like effect can be used to enhance, supplement or replicate the function and/or activity of NCDN in a cell. Modulators of this type (i.e. modulators which are compounds which replicate some aspect of NCDN function or activity) may be used to ablate any NCDN loss (or partial loss)
20 of function in a diseased cell.

A NCDN modulator may increase the expression of a gene or genes involved in NCDN expression, NCDN processing and/or NCDN production. For example, a useful NCDN modulator may increase expression of the *NCDN* gene. One of skill will appreciate that any increase in *NCDN* gene expression may lead to an increase in intracellular levels of NCDN.

- 25 Useful modulator compounds may be derived from wildtype or native NCDN. For example a NCDN modulator compound may comprise, for example a recombinant or synthetically generated NCDN molecule and/or a functional fragment of a NCDN protein. It should be understood that the term "functional" as applied to "fragment" is intended to mean that the fragment possesses at least some of the function or activity associated with the complete,
30 native NCDN protein.

An exemplary (canonical) *Homo sapiens* neurochondrin (NCDN) sequence is provided as SEQ ID NO: 1 below:

MSCCDLAAAG QLGKASIMAS DCEPALNQAE GRNPTLERYL GALREAKNDS EQFAALLLV
 KAVKAGDIDA KTRRRIFDAV GFTFPNRLLT TKEAPDGCPD HVLRALGVAL LACFCSDPEL
 AAHPQVLNKI PILSTFLTAR GDPDDAARRS MIDDTYQCLT AVAGTPRGPR HLIAGGTVSA
 LCQAYLGHGY GFDQALALLV GLLAAAETQC WKEAEPDLA VLRGLSEDFQ KAEDASKFEL
 5 CQLLPLFLPP TTVPPECYRD LQAGLARILG SKLSSWQRP ALKLAARLAH ACGSDWIPAG
 SSGSKFLALL VNLACVEVRL ALEETGTEVK EDVVTACYAL MELGIQECTR CEQSLLKEPQ
 KVQLVSVME AIGAVIHILL QVGSEKQKEP FVFASVRILG AWLAEETSSL RKEVCQLLPF
 LVRYAKTLYE EAEEANDLSQ QVANLAISPT TPGPTWPGDA LRLLLPGWCH LTVEDGPREI
 LIKEGAPSLI CKYFLQWEL TSPGHDTSVL PDSVEIGLQT CCHIFLNLVV TAPGLIKRDA
 10 CFTSLMNTLM TSLPALVQQQ GRLLLAANVA TLGLLMARLL STSPALQGTP ASRGFFAAAI
 LFLSQSHVAR ATPGSDQAVL ALSPEYEGIW ADLQELWFLG MQAFTGCVPL LPWLAPAALR
 SRWPQELLQL LGSVSPNSVK PEMVAAYQGV LVELARANRL CREAMRLQAG EETASHYRMA
 ALEQCLSEP

One of skill will appreciate that other sequences may also encode functional NCDN proteins.
 15 All mammalian NCDN proteins (including human NCDN proteins) are to be included within
 the scope of the term "NCDN protein". NCDN sequences included within the scope of the
 term NCDN protein may exhibit anywhere from about 60% or 65% to about 95% or 99%
 sequence identity or homology to SEQ ID NO: 1. For example, identical or homologous.
 NCDN proteins may have primary amino acid sequences which are at least about 70%,
 20 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 96%, 97% or 98% identical or homologous to
 the sequence of SEQ ID NO: 1.

A NCDN modulator may comprise all or part of the sequence provided by SEQ ID NO: 1. For
 example, a NCDN modulator may comprise a fragment, for example a functional fragment,
 of SEQ ID NO: 1. It should be noted that the term "NCDN modulator", "NCDN fragment" or
 25 "functional fragment" as used herein, applies to modulators or fragments which are derived
 from any sequence which is identical or homologous to SEQ ID NO: 1

A "functional fragment" may be any fragment derived from a NCDN protein (for example
 SEQ ID NO: 1 or sequence identical or homologous thereto) which exhibits a function and/or
 activity which is the same, substantially the same or similar to a native or wild-type NCDN
 30 protein (such as that provided by SEQ ID NO: 1). For example, and without wishing to be
 bound by theory, a functional fragment of an NCDN protein or of SEQ ID NO: 1 may exhibit
 an ability to control or modulate (for example inhibit, suppress, increase, enhance or
 supplement), one or more of the following:

- (i) signaling pathways (in neurons);

- (ii) signal transduction events (in neurons);
- (iii) neural outgrowth;
- (iv) synaptic plasticity;
- (v) dendrite morphogenesis; and
- 5 (vi) cell polarity (of neurons).

A NCDN fragment (for example a fragment of SEQ ID NO: 1) for use may comprise anything from between about 5-15 residues and N-1 residues, where N is the total number of residues present in the NCDN protein. In the case of SEQ ID NO: 1, the total number of residues (N) is 729. For example, useful fragments may contain about 10, 20, 30, 40, 50, 100, 200, 300,
 10 400, 500, 600 or 700 residues from, for example a NCDN protein or from SEQ ID NO: 1. In one embodiment, a useful (but functional) fragment may comprise one or more (functional) domains, sections or regions from an NCDN protein or from SEQ ID NO: 1.

The term "functional fragments" may also embrace those fragments which are antagonists or agonists of NCDN function or activity. For example, a useful NCDN fragment may be one
 15 which mimics the effect of NCDN and which serves to increase the amount of functional NCDN in a cell or which neutralises some protein or cellular component which might otherwise suppress, inhibit or interfere with native/wildtype NCDN expression, activity or function.

Useful fragments may be made recombinantly and thus the disclosure provides nucleic acids
 20 which encode SEQ ID NO: 1. These nucleic acids or fragments thereof, may be used to produce recombinant forms of the NCDN protein or functional fragments thereof. Thus, where the disclosure provides fragments of a nucleic acid sequence that encodes SEQ ID NO: 1, those fragments may themselves encode functional fragments (as defined above) of SEQ ID NO: 1. Nucleic acids of this type will be referred to hereinafter as "NCDN nucleic"
 25 acids. Thus, the disclosure further provides vectors (for example plasmids and other constructs) comprising a NCDN nucleic acid. Further, there is provided a host cell comprising a vector as described herein.

The term "NCDN modulator" may embrace allosteric type modulators that indirectly modulate some aspect of NCDN function, activity and/or expression. For example an
 30 allosteric NCDN modulator may modulate the expression function and/or activity of some

cellular component which itself usually modulates some aspect of NCDN expression, function and/or activity.

A NCDN modulator may comprise a substance or compound which mimics the wildtype or normal function/activity of NCDN. Modulators of this type may supplement, enhance or
5 improve native intracellular NCDN activity and may engage in or complete those intracellular interactions which are normally or usually involve NCDN.

As stated, a NCDN modulator may comprise an antibody which binds to or which has affinity for NCDN, or an epitope thereof. Useful antibodies may exhibit affinity and/or specificity for other cellular components, for example the SMN (survival Motor Neurone) protein and/or
10 members of the Sm protein family. For example antibodies for use, may exhibit a specificity and/or affinity for sites usually bound by the NCDN protein. One of skill will appreciate that antibodies with specificity/affinity of this sort may act as "agonists" in that they replicate the function of NCDN and instigate NCDN associated cellular events.

One of skill will appreciate that the term "antibody" may embrace any suitable form of
15 antibody or antibody fragment. For example, included within the scope of the term antibody are monoclonal and/or polyclonal antibodies and antibodies of any particular isotype (IgG, IgM, IgE, IgD, IgA etc.).

Useful antibodies and/or fragments may include, for example, humanised antibodies, chimeric antibodies, re-surfaced antibodies, camelid antibodies (including hclgG type
20 antibodies), shark antibodies (IgNAR), rodent antibodies (including murine antibodies), Fab fragments, F(ab')₂ fragments, monospecific Fab₂ fragments, Bispecific Fab₂ fragments, trispecific Fab₂ fragments, diabodies, triabodies, scFv-Fc fragments, minibodies and the like.

Thus any or all of the antibodies described herein may have affinity and/or specificity for particular epitopes, including, for example, specificity and/or affinity for NCDN binding sites
25 present on proteins which interact with NCDN. As described herein, this would include antibodies which exhibit specificity and/or affinity for the NCDN binding/interaction sites on the SMN protein.

One of skill will be familiar with the various processes used to make antibodies, which processes may include the use of antigens (for example SMN and/or NCDN based antigens
30 (for example immunogenic fragments thereof)) as immunogens to elicit antibody responses.

Further information regarding antibody manufacture, purification and use may be derived from, for example, Antibodies: A Laboratory Manual, Second edition, Edited by Edward A. Greenfield (Cold Spring Harbor Laboratory Press) – the entire contents of which is incorporated herein by reference.

- 5 Antibodies for use may be conjugated. For example, an antibody may be conjugated to a therapeutic or cytotoxic moiety. In some cases, the antibody (and its target specificity/affinity) are used to direct or target a specific conjugate to a specific cell type.

With the knowledge that NCDN interacts with the SMN protein, it is possible to develop assays which exploit this observation in order to test agents for NCDN modulator function.

- 10 Thus NCDN modulators for any of the uses described herein may be identified by means of assays which test agents for NCDN modulator activity.

For example a suitable cell, such as a cell from an established cell line, including, for example neural or neuroblastoma cell lines, may be subjected to antisense or RNA interference technology (for example siRNA based protocols) in which NCDN and/or SMN
15 expression is inhibited, ablated or reduced. A test agent may then be contacted with the cell in order to determine whether or not the test agent ablates any of the effects associated with NCDN or SMN loss of function and/or replaces or replicates any aspect of native/wildtype NCDN or SMN function, expression or activity.

Thus the disclosure provides a method of determining whether or not a test agent possesses

- 20 NCDN activity, said method comprising;

 providing a cell with reduced levels of NCDN and/or SMN gene and/or protein expression;

 contacting the cell with a test agent; and

- determining whether or not the test agent replicates or replaces any aspect of NCDN
25 or SMN function or activity.

- In order to determine whether or not the test agent replicates any aspect of NCDN and/or SMN function, expression or activity (or any of the effects associated with NCDN or SMN loss of function), once the test agent has been contacted with the cell, the cell may be assessed to determine whether or not any of the effects associated with NCDN or SMN loss
30 of function have been modulated (for example, improved) by the test agent.

A suitable cell for use in the method described above may be a SH-SY5Y cell.

An assay according to this disclosure may identify agents which modulate (for example increase, enhance, promote or improve) one or more of the events selected from the group consisting of:

- 5 (i) NCDN::SMN interactions;
- (ii) localisation of SMN to cytoplasmic vesicles
- (iii) localisation of both the SMN and NCDN within the cell; and
- (iv) the intracellular formation of cytoplasmic foci containing endogenous NCDN.

10 Test agents shown to possess some aspect of NCDN/SMN function or activity (perhaps as evidenced by their ability to modulate one or more of the effects listed as (i)-(iv) above) may find application in the treatment and/or prevention of neurodegenerative disorders, including, for example SMA.

15 It should be noted that agents which modulate (for example increase, enhance, promote or improve) one or more of the events selected from the group consisting of (i)-(iv) above, may be assessed relative to a wildtype or normal level of any of (i)-(iv) above. A wildtype or normal level of any of the events identified as (i)-(iv) above may be determined from a normal or wildtype cell – that is a cell not affected by SMA or any mutations which (substantially) affect NCDN or SMN expression, function or activity.

20 The provision of a cell with reduced or ablated levels of NCDN expression, function and/or activity may be achieved by reducing intracellular protein expression by using siRNA (or similar, for example shRNA). Suitable siRNA sequences may be transfected into the appropriate cell lines.

25 By way of example, one or more (for example any combination or all) of the following siRNA may be used - these sequences being effective to modulate or reduce NCDN expression in a cell:

- i) GUUCAUUGGUGACGAGAAA (SEQ ID NO: 2);
- ii) AGACCUCAUCCUUGCGUAA (SEQ ID NO: 3);
- iii) AGGCCAAGAAUGACAGCGA (SEQ ID NO: 4); and

iv) GGCCAUGAUAUCGCAGUU (SEQ ID NO: 5).

As stated, assays such as those described herein may be used to identify agents that are (potentially) useful in the treatment and/or prevention of neuromuscular disorders such as, for example SMA.

5 Other assays may take a different approach to the identification of NCDN modulators. For example it may be possible to exploit cells in which the expression, function and/or activity of one or more components which interact with NCDN have been modulated. For example, a cell may be contacted with, for example, si/shRNA constructs which reduce the expression of SMN. In reducing SMN expression, the number of cytoplasmic foci containing
10 endogenous NCDN may also be reduced. Cells of this type (in which SMN expression is modulated) may be used in order to determine whether or not a test agent has an effect on SMN expression and, in turn, the intracellular formation of cytoplasmic foci containing endogenous NCDN.

For example, a siRNA with the sequence CAGUGGAAAGUUGGGGACA (SEQ ID NO: 6)
15 may be used to reduce SMN expression in a cell.

Reduction in protein expression using sh/siRNA may be achieved by transfecting cell lines (for example SH-SH5Y cell lines) with vectors (for example pSUPER-GFP.Neo plasmids, oligoengine) expressing the relevant sh/siRNA (for example sh/siRNA to NCDN and/or SMN).

20 Any of the NCDN modulators described herein, including those identified by the assays and methods described herein, may be combined with one or more other therapeutic agents. For example, a NCDN modulator described herein may be combined with one or more existing treatments (drugs/therapeutics) for neurodegenerative disorders including, for example, SMA therapeutics.

25 Existing SMA treatments include the use of anti-sense oligonucleotides which increase the production of protein from the *SMN2* gene (the other gene producing a small amount of full-length SMN protein). The treatment known as nusinersen (spinraza) is in development and expected to be available soon. Thus, the NCDN modulators described herein may be used together with existing SMA treatments such as anti-sense oligonucleotide based treatments
30 including those comprising the use of nusinersen.

Thus, a NCDN modulator of this invention may be administered prior to the administration of another SMA therapeutic or together (or concurrently) with, or at the same time as, another SMA therapeutic. Additionally or alternatively, any of the NCDN modulators described herein may be administered after the administration of another SMA therapeutic. Further any of the
5 NCDN modulator(s) described herein may be administered before, concurrently and/or after administration of some other therapeutic, for example a therapeutic useful in the treatment of a disease other than SMA.

For example, one or more of the NCDN modulators described herein may be used together with a *SMN2* modulator type SMA therapeutic (a *SMN2* modulator being a substance,
10 compound or composition which increases (or decreases) *SMN2* expression and/or the rate or amount of SMN protein expressed therefrom), to enhance localisation of the increased SMN to cytoplasmic vesicles and to allow an increase in mRNA transport to growth cones. Without wishing to be bound by theory, this may lead to more normal development and the survival of motor neurons. It is also suggested (again without wishing to be bound by theory),
15 that the interaction between SMN and NCDN may be important to the correct localisation of both proteins; indeed, in SMA, several signalling pathways may be disrupted through this mis-localisation of NCDN. Compounds capable of enhancing or increasing NCDN expression and increasing the level of interaction between NCDN and SMN, may be useful in the treatment of SMA.

20 Also provided is a method of treating SMA, said method comprising administering to a subject in need thereof a therapeutically effective amount of a NCDN modulator. The NCDN modulator may comprise (or consist essentially of or consist of) any of the modulator compounds described herein.

Further described is the use of a NCDN modulator as described herein in the manufacture of
25 a medicament for the treatment of SMA.

The NCDN modulators described herein may be provided in composition form. A NCDN modulator may be combined or formulated with some form of excipient, diluent or carrier in order to provide a NCDN composition.

A composition of this disclosure may be a pharmaceutical composition comprising one or
30 more NCDN modulators (as described herein) and, for example, pharmaceutically acceptable excipients, diluents and/or carriers. The list of possible pharmaceutically acceptable carrier ingredients for use with an NCDN modulator of this disclosure, may further include, for example, buffers, flavouring agents, binders, surface active agents,

thickeners, lubricants, preservatives (including anti-oxidants) and the like, and substances included for the purpose of rendering the formulation isotonic with the blood of the intended recipient.

5 A range of pharmaceutically acceptable carriers are well known to those skilled in the art and include, but are not limited to, 0.1 M and preferably 0.05 M phosphate buffer or 0.8% saline.

10 Additionally, pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

15 A composition suitable for topical formulation may be provided, for example, as a gel, a cream or an ointment.

A NCDN modulator may be provided in an encapsulated form. For example, if taken orally, the NCDN modulator may be provided as an enteric coated tablet or composition. Suitable encapsulation coatings may include polysaccharide, wax and/or gelatin based coatings. Additionally or alternatively, a NCDN modulator may be provided encapsulated in a vesicle, 20 for example a lipid vesicle, this may make entry of the NCDN modulator into a cell easier.

Compositions according to this disclosure (pharmaceutical or otherwise) may comprise one or more NCDN modulators in combination with one or more other active agents, which one or more other active agents treat or prevent SMA or one or more other diseases or conditions.

25 A composition of this disclosure may be formulated for parenteral (that is administration by injection and including subcutaneous, intradermal, intramuscular and/or intravenous administration).

Where the composition is formulated for parenteral administration, the composition may be formulated for administration to the spinal canal in the back (into the lumbar area). For 30 example, a composition described herein may be administered to the spinal canal during a lumbar puncture type procedure.

Compositions formulated for parenteral administration (for example compositions comprising NCDN modulator compounds) may include sterile solutions or suspensions of the active compounds (for example one or more NCDN modulators) in aqueous or oleaginous vehicles. Compositions disclosed herein may comprise, or further comprise cryoprotectant compounds or compositions, preservative(s), antibiotics, other active agents, adjuvants and the like.

Injectable compositions and vaccines may be adapted for bolus injection or continuous infusion. Such preparations are conveniently presented in unit dose or multi-dose containers, which are sealed after introduction of the formulation until required for use. Alternatively, an active compound (for example one or more NCDN modulators) may be in powder form and re-constituted with a suitable vehicle, such as sterile, pyrogen-free water or phosphate buffered saline PBS before use.

DETAILED DESCRIPTION

The present invention will now be described in detail with reference to the following figures which show:

Figure 1: SmN exhibits similar behaviour to SmB in SH-SY5Y cells. A) SH-SY5Y cells were transfected pEYGP-SmN and fixed after 24 and 48 hours. Cells were immunostained using Y12 and 204-10 to stain for Sm protein containing speckles and Cajal bodies respectively. Arrowheads identify speckles and triangles identify Cajal bodies. B) The localisation of SmN from 300 cells was analysed and grouped into phenotypes, revealing that SmN initially localises diffusely in the cytoplasm, before being incorporated into snRNPS and localising to speckles. mCherry-SmN exhibited similar localisation (not shown). C) Both YFP-SmN and mCherry-SmN are incorporated into snRNPs in SH-SY5Y cell lines constitutively expressing the tagged proteins, revealing that the fluorescent tagged proteins are able to function similarly to endogenous protein. D) mCherry-SmN partially co-localises with BODIPY 493 in mobile lipid rich vesicles in the cytoplasm of SH-SY5Y cells. Arrows identify co-localisation whereas triangles identify BODIPY 493 stained vesicles not containing mCherry-SmN. Cells were imaged approximately every 4 seconds for 9 minutes. E) mCherry-SmN highly co-localises with GFP-SMN similarly to that previously observed with SmB (Prescott et al, 2014), but YFP has minimal co-localisation with SmN. Arrowhead identify foci showing co-localisation, Triangles identify foci without co-localisation. F) mCherry-SmN co-localises with GFP-SMN but not YFP in cytoplasmic vesicles, Counts taken from 5 cells. Bar=7µm Images in A) are deconvolved z-stacks taken with 0.2µm spacing. D) and E) are single deconvolved z-sections of cells.

Figure 2: The interactomes of SmB and SmN are similar, but there are differences present at the individual protein level. A) Immunoblot analysis of affinity purified YFP-SmN, YFP-SmB or YFP alongside 80µg of precleared lysate and the unbound fraction confirms that GFP-Trap effectively immunoprecipitated all three bait proteins. B) After processing the mass spectrometry data, and sorting identified proteins into groups based on function, the interactomes of SmN and SmB are very similar (also see Table 1 for data). NCDN was identified in the interactome of YFP-SmN, with 5 unique peptide hits, all with high individual peptide scores.

%of total proteins identified	
SmN	SmB
12.1%	9.0%
0.3%	0.2%
6.4%	7.4%
11.7%	11.3%
2.2%	1.4%
1.9%	1.8%
1.0%	0.7%
2.8%	1.9%
3.4%	3.5%
12.6%	16.2%
10.8%	10.0%
6.5%	6.0%
1.9%	3.8%
7.4%	6.9%
1.6%	1.5%
5.0%	5.2%
7.8%	5.9%
4.4%	7.3%

C) A comparison between the amino acid sequences of SmN and SmB reveals their similarity. Differences in amino acid sequence are noted in italicised/underlined text. Sequences were from uniprot (entries P63162 (SmN) and P14678-2 (SmB)). D) Neurochondrin (NCDN) was identified in the interactome of YFP-SmN, with 5 unique peptide hits encompassing 9% sequence coverage. Each Ion Score (Mascot Ion Score) was above

the threshold for peptide identity (Mascot identity score), with 2 out of the 5 identified peptides having a score of above double the threshold score.

Figure 3: NCDN co-localises with SmN, SmB and SMN in vesicles. A) In transiently co-transfected SH-SY5Y cells, mCherry-SmB is co-immunoprecipitated with NCDN-GFP, indicating a potential direct interaction between the two proteins. B) In an SH-SY5Y cell line constitutively expressing NCDN-GFP, SmB and SmN, SMN and the coatomer protein β COP confirming that NCDN is able to interact with endogenous proteins previously observed to associate with SmB/SMN vesicles. C) SMN, SMB or Coatomer proteins are not co-immunoprecipitated with YFP alone in an SH-SY5Y cell line constitutively expressing YFP, indicating that the fluorescent protein tag is not responsible for the interactions. D) NCDN and SmN exhibit co-localisation in vesicle-like structures in neurites of SH-SY5Y cells constitutively expressing mCherry-SmN, and transiently expressing NCDN-GFP. Arrows identify SmN containing vesicles with NCDN co-localisation. Similar results were obtained in cells constitutively expressing mCherry-SmB and transiently expressing NCDN-GFP. E) Little co-localisation is observed between mCherry-SmN and YFP in vesicles, indicating the mCherry-SmN and NCDN co-localisation is unlikely to be an artefact of cell shape, imaging or fixation. Triangles identify SmN containing vesicles without YFP co-localisation. F) GFP-SMN, from SHY10 GFP-SMN expressing SH-Sy5Y cells co-immunoprecipitates NCDN, confirming the SMN-NCDN interaction. G) mCherry-SMN and NCDN-GFP, but not YFP co-localise in vesicles within the cytoplasm of SH-SY5Y cells transiently expressing the proteins. Arrowheads identify co-localisation between mCherry-SMN and GFP-NCDN in vesicles, triangles identify SMN vesicles without YFP co-localisation. Bar= 7 μ m, and images are single deconvolved z-sections. H) Panel A: Immunoprecipitation (IP) of endogenous SMN using anti-SMN antibodies co-enriches endogenous NCDN in SH-SY5Y cells. Panel B: Immunoprecipitation of endogenous SMN from murine P8 brain lysate co-enriches NCDN. Control IPs using anti-FLAG antibodies do not enrich NCDN.

Figure 4: Detergent free fractionation of SH-SY5Y cells reveals that SMN, coatomer proteins, NCDN, SmB and SmN are all enriched in the 100,000 RCF vesicle pellet. A) SH-SY5Y cells were fractionated using Dounce Homogenising before differential centrifugation to pellet different compartments of cells. 40 μ g of total protein from each fraction was analysed through Western Blotting. Antibodies to Tubulin and Histone H3 were used to confirm minimal Histone (nuclear contamination) in cytoplasmic fractions, and minimal tubulin (cytoplasmic contamination) in the nuclear pellet. SMN was highly enriched in the 100,000 RCF pellet indicating that this is likely the pellet containing SMN vesicles previously identified. γ COP was also present in this fraction indicating the likely vesicular/membranous

nature of this fraction. B) SH-SY5Y cells constitutively expressing NCDN-GFP, YFP-SmB, YFP-SmN or YFP were also fractionated. Cells constitutively expressing YFP-SmB or YFP-SmN have both present in the same fraction as SMN. Endogenous SmN and SmB also behave similarly (not shown). NCDN-GFP is also present in the 100,000 RCF, but unlike SMN, SmB and SmN, is completely absent from the nuclear pellet indicating that the interaction between NCDN and the other proteins is likely to be restricted to the cytoplasm, and may be specific to vesicles/complexes brought down in the 100,000 RCF centrifugation. YFP is not found the 100,000 RCF fraction. C) Quantification of immunoblot analysis confirms that SMN is highly enriched in the 100,000 g pellet, with enrichment of γ COP also seen. Histone H3 and tubulin are highly enriched in the nucleus and cytoplasm, respectively. Quantification (mean $\pm$ s.d.) of tubulin and histone H3 band density was from seven immunoblots, with values from SMN and γ COP from five and four immunoblots, respectively. D) Quantification of the band densities for the immunoblot shown in C confirms the presence of NCDN-GFP, YFP-SNRPB and YFP-SNRPN in the 100,000 g pellet, together with the restriction of YFP alone to the residual cytosolic supernatant.

Figure 5: Reduction of endogenous NCDN using siRNA in SH-SY5Y cells constitutively expressing GFP-SMN increases localisation of SMN to nuclear gems. A) siRNAs targeting NCDN, SMN, SmB and control siRNAs were transfected into SH-SY5Y cells constitutively expressing GFP-SMN (Green on images), and were fixed after 72 hours, and stained with Hoescht (blue on images). Arrows identify nuclear gems (SMN-positive nuclear foci) containing SMN. SH-SY5Y cells were also transfected with GFP-SMN Δ 7, a mutant known to increase the number of nuclear gems (SMN-positive nuclear foci). Transfection efficiency with siRNAs was greater than 90%, measured by transfection with siGlo Cyclophilin B (not shown). Bar=7 μ m. Images are deconvolved z-stacks taken with 0.2 μ m spacing B) reduction of NCDN, SmB or expression of GFP-SMN Δ 7 increased the number of nuclear SMN foci from 4.353 ± 2.458 (Mean $\pm$ Standard Deviation) in cells treated with siControl to 10.190 ± 4.061 , 10.410 ± 4.918 , 9.873 ± 4.080 , 9.453 ± 3.576 and 10.730 ± 4.608 for siNCDN 18-21 and pooled respectively, 16.710 ± 6.797 for siSmB, and 18.150 ± 5.253 for cells transfected with GFP-SMN Δ 7. The number of nuclear SM foci was 4.230 ± 2.313 for siLamin A/C and 0.733 ± 1.354 for siSMN. The difference between each siNCDN and controls is statistically significant (AVOVA; $P < 0.0001$, n=150 from 3 replicates). A Tukey post-test identified outliers (individual points marked on graph). C) In parallel with these analyses, whole-cell lysates used for immunoblot analysis using antibodies to the endogenous proteins. A reduction of 40-60% compared to siControl cells was typically observed in each knockdown, after signals were normalised to tubulin. D) Immunoblot analysis using antibodies to endogenous NCDN, SMN and SNRPB shows a reduction in expression of each of 40-60% compared to

siControl cells, after signals were normalised to tubulin. Reductions in protein expression (mean $\pm$ s.d.) compared to siControl siRNA are statistically significant (ANOVA; $P<0.0001$ for NCDN, Lamin A/C and endogenous SMN; $P<0.001$ for SNRPB; $P<0.01$ for GFP-SMN, $n=3$). A Dunnett post-test identified the significance of the reduction compared to siControl.

5 * $P\leq 0.05$, ** $P\leq 0.01$, *** $P\leq 0.001$, **** $P\leq 0.0001$.

Figure 6: Reduction of endogenous SMN causes a reduction in cytoplasmic NCDN foci in SH-SY5Y cells. A) SH-SY5Y cells were transfected with plasmids to express shRNAs targeting SMN (shSMN), Cyclophilin B shCyclophilin or with the empty pSuper GFP vector (not shown), fixed after 72 hours, and immunostained for endogenous NCDN and SMN, allowing detection of NCDN foci within the cytoplasm of the cells (identified with Arrowheads), as well as SMN Gems (identified with Triangles). Bar= 7 μ m, images are single deconvolved z-sections B) The number of NCDN foci were counted in 64 cells from 3 replicates, showing a reduction in the number of NCDN foci present in the cytoplasm to 15.340 $\pm$ 7.201 (Mean $\pm$ Standard Deviation) from 20.630 $\pm$ 12.03 and 19.480 $\pm$ 7.618 in shSMN transfected cells compared to cells either transfected with shCyclophilin B or the empty pSuper GFP vector respectively. This is statistically significant (ANOVA $P<0.0005$, $n=64$ from 3 replicates). C) the shSMN caused a reduction of nuclear gems from 2.300 $\pm$ 0.837 or 3.033 $\pm$ 1.033 to 0.1667 $\pm$ 0.379, in cells transfected with shCyclophilin B, empty vector and shSMN respectively. This is statistically significant (ANOVA $P<0.0001$, $n=30$ from 3 replicates). D) SH-SY5Y cells were transfected with plasmids to express shRNAs targeting SMN (shSMN), Cyclophilin B (shCyclophilin) or with the empty pSuper GFP vector (data not shown), fixed after 72 h, and immunostained for endogenous NCDN and SMN allowing detection of NCDN foci within the cytoplasm (chevron arrowheads), as well as SMN-positive nuclear gems (triangular arrowheads). Images are single deconvolved z-sections. Scales bars: 7 μ m.

Figure 7: NCDN does not immunoprecipitate with snRNPs in SH-SY5Y cells constitutively expressing NCDN-GFP. After pre-clearing the lysate with Sepharose 4B beads, the lysate was incubated with either TMG beads or further sepharose to determine whether NCDN could interact with snRNPs. No NCDN was observed to be immunoprecipitated by TMG beads. This suggests that the SMN-NCDN interaction may be independent of snRNP assembly, as endogenous SMN was immunoprecipitated

Figure 8: The interaction between NCDN and SMN may occur in Rab5 vesicles within neurites in neural cells. A) Lysates from SH-SY5Y cells were co-transfected with mRFP-Rab5 and either YFP, GFP-SMN or NCDN-GFP, were incubated with RFP-Trap overnight. The immunoprecipitated was immunodetected for both mRFP and YFP/GFP tags to confirm

that mRFP-Rab5 was immunoprecipitated, and to determine whether the GFP/YFP tagged proteins were co-immunoprecipitated. NCDN-GFP and GFP-SMN were both co-immunoprecipitated in small amounts, whereas YFP was absent in the RFP-Trap immunoprecipitated. Endogenous SMN was also observed to be co-immunoprecipitated, confirming a Rab5-SMN interaction. B) Cells were also imaged from this co-transfection and imaged, revealing co-localisation in punctate structures within neurites between mRFP-Rab5 and both NCDN-GFP and GFP-SMN, but not YFP. Bar= 7 μ m, images are single deconvolved z-sections.

Figure 9: NCDN is expressed throughout the spinal cord of mice, with clear enrichment seen in motor neurons, placing it in the cells most severely affected in SMA. Panel A: NCDN (green: middle panel) is expressed throughout the spinal cord, with increased expression in motor neurons (arrows), as identified with anti-ChAT antibody (magenta). Panel B: Higher magnification imaging confirms the presence of NCDN in ChAT-positive motor neurons (single deconvolved z-section). Scale bars: 500 μ m (panel A); 10 μ m (panel B).

Figure 10: NCDN is enriched in synapse preparations from mice, placing it in the sub-cellular region of neurones that shows the earliest signs of damage in SMA model mice. Immunoblot detection of neurochondrin in equal amount of whole brain lysate and purified synaptosomes from mice shows the presence and clear enrichment of NCDN in synaptosomes.

MATERIALS & METHODS

Plasmid constructs

pEGFP-SMN, pEYFP-SmB and mCherry-SmB have been described previously (Sleeman et al., 1999; 2001; Clelland et al, 2009). pEYFP-SmN and pmCherry-SmN were generated by sub-cloning cDNA of Human SmN from SH-SY5Y cells into pEYFP-C1 and pmCherry-C1 respectively, using SNRPNEcoRI forward primer: TAGAATTCCATGACTGTTGGCAAGAGTAGC (SEQ ID NO: 7), and SNRPNBamHI reverse primer: TAGGATCCCTGAGATGGATCAACAGTATG (SEQ ID NO: 8). pmCherry-SMN was generated by subcloning SMN DNA from the pEGFP-SMN plasmid into pmCherry-C1 using an SMNEcoRI Forward primer: GCGGAATTCTATGGCGATGAGC (SEQ ID NO: 9) and SMNBamHI Reverse Primer: GCAGGATCCTTAATTTAAGGAATGTGA (SEQ ID NO: 10). To generate pEGFP-NCDN, NCDN cDNA from SH-SY5Y cells was subcloned into a pEGFP-N3 plasmid using NCDNEcoRI forward primer: GCGGAATTCATGGCCTCGGATTGCG (SEQ ID NO: 11) and NCDNSall reverse primer: GCTGCTGACGGGCTCTGACAGGC (SEQ ID NO: 12). All cDNAs were amplified using GoTaq G2 (Promega), restriction digested using EcoRI and either BamHI or Sall (Promega),

before ligation with T4 DNA ligase (Thermo Scientific). mRFP-Rab5 (Vonderheit et al, 2005) was a gift from Ari Helenius (Vonderheit et al, 2005).

Cell lines and cell culture

SH-SY5Y cells were from ATCC. Cells were cultured in DMEM with 10% FBS at 37°C, 5% CO₂. Transfections were carried out using Effectene (Qiagen) according to the manufacturer's instructions. Stable SH-SY5Y cell lines expressing mCherry-SmB and GFP-SMN have been described previously (Clelland et al, 2009; Prescott et al, 2014). SH-SY5Y cell lines stably expressing YFP-SmN, YFP-SmB, YFP, mCherry-SmN, NCDN-GFP were derived by selection with 200 µg/ml G418 (Roche) following transfection.

Immunostaining, Microscopy and image analysis

Immunostaining and cell fixing were both carried out as described previously (Sleeman et al., 2003). Live cell and fixed cell microscopy, and image processing were both carried out as described previously (Prescott et al, 2014). BODIPY-488 (Life Technologies) was added to culture medium at 2 µg/ml overnight. Antibodies used for immunostaining were Mouse monoclonal Y12 anti-Smith (SmB) (Abcam, 1:20), 204-10 (anti-Coilin) (1:500), Mouse monoclonal anti-SMN (BD Transduction, 1:50) and Rabbit polyclonal anti-NCDN (Proteintech, 1:50). Overlays of images were made using Adobe Photoshop CS5. Bar charts, Box and Whisker plots, and statistical analyses were generated using Prism4 (GraphPad).

Preparation of cell lysates, immunoblotting and immunoprecipitation

Cells were grown in 10cm dishes, before being detached and collected by centrifugation at 180 RCF for 5 minutes (centrifuge). The cell pellet was washed 3 times in PBS before being lysed in 100 µl of ice cold lysis buffer per dish (50 mM Tris-HCl pH 7.5; 0.5 M NaCl; 1% (v/v) Nonidet P-40; 1% (w/v) sodium deoxycholate; 0.1% (w/v) SDS; 2 mM EDTA plus Complete mini EDTA-free protease inhibitor cocktail (Roche, one tablet per 10 ml), before being homogenised by sonication. Isolation of YFP/GFP and mCherry/mRFP- tagged proteins was carried out as described previously with GFP or RFP-Trap (Chromotek)(Prescott et al, 2014). Lysates were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to nitro-cellulose (Hybond-C+ or Protran premium 0.2 µm, both GE Healthcare) membranes for immunoblotting. Antibodies used were rat mAb anti-RFP (Chromotek, 1 : 500); goat polyclonal anti-γCOP (Santa Cruz, 1 : 250), Rabbit polyclonal anti-GFP (Abcam, 1:2000), Rabbit polyclonal anti-SNRPN (SmN) (Proteintech,1:800), Mouse monoclonal Y12 anti-Smith (SmB) (Abcam, 1:100), Rabbit polyclonal anti-SMN (Santa Cruz, 1:500), Mouse

monoclonal anti-SMN (BD Transduction labs, 1:500), Rabbit polyclonal anti-COPB1 (CUSAB, 1:500), Mouse monoclonal anti- Lamin A/C (Santa Cruz, 1:500) and Rabbit polyclonal anti-NCDN (Proteintech, 1:500).

5 Secondary antibodies were conjugated either goat anti-mouse IRDye 800CW and goat anti-rabbit IRDye 680RD (Li-cor, 1:25,000); goat anti-rabbit Dylight700 or goat anti-mouse Dylight 800CW (Pierce, 1:15,000) or secondary antibodies conjugated to horseradish peroxidase (HRP) (Pierce, 1 : 20,000). Detection was carried out with either an Odessey CLx (Li-cor) or ECL Plus (GE Healthcare) and imaged using a Fujifilm LAS-3000 imaging system. Quantification of protein bands was performed using the Odessey CLx.

10 Immunoprecipitation of intact snRNPs

To immunoprecipitate intact snRNPs, lysates from SH-SY5Y stable cell lines were incubated with anti-2,2,7-trimethylguanosine conjugated to agarose beads (Millipore NA02A), with Sepharose 4B (Sigma Aldrich) as a control. 40 ng of pre-cleared lysate and unbound protein were separated by SDS-PAGE together with the agarose control beads and TMG antibody
15 beads. Subsequent detection was carried out using Rabbit anti-GFP (1:2000, Abcam) or Rat mAb anti-RFP (1:500, Chromotek).

Mass spectrometry and data analysis

SH-SY5Y cells constitutively expressing either YFP, YFP-SmN or YFP-SmB were lysed in coimmunoprecipitation lysis buffer (10mM Tris pH7.5, 150mM NaCl, 0.5mM EDTA, 0.5% NP40, 1 complete EDTA-free protease inhibitor tablet (Roche) per 10ml), before the YFP tag
20 was immunoprecipitated with GFP-Trap as above. 5µl of the precipitate, alongside precleared lysate and unbound lysate was immunoblotted (as above) and immunodetected using Rabbit anti-GFP (Abcam) to confirm efficient immunoprecipitation. Samples were then electrophoresed on a Novex NuPAGE pre-cast gel, Coomassie stained using SimplyBlue SafeStain (Invitrogen), gel chunks excised and analysed by the Mass Spectrometry facility at
25 the University of St Andrews Tandem mass spectra were extracted by [unknown] version [unknown]. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analysed using Mascot (Matrix Science, London, UK; version 2.5.0). Mascot was set up to search the NCBI nr_20141031 database (selected for Homo sapiens, unknown
30 version, 284317 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.100 Da and a parent ion tolerance of 20 PPM. O+18 of pyrrolysine and iodoacetamide derivative of cysteine were specified in Mascot as fixed modifications. Oxidation of methionine was specified in Mascot as a variable modification.

Scaffold (version Scaffold_4.5.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm (Keller, A et al Anal. Chem. 2002;74(20):5383-92). Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, Al et al Anal. Chem. 2003;75(17):4646-58). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

10 RNAi assays

Reduction of protein expression using siRNA was achieved by transfecting the appropriate cell lines with siRNAs (Dharmacon) using viromer green (Lipocalyx GmbH) according to the manufacturer's instructions. Cells were lysed for assay by immunoblotting or fixed with paraformaldehyde for fluorescence microscopy 48 hours after transfection. Sequences used were SMN: CAGUGGAAAGUUGGGGACA (SEQ ID NO: 13); SmB, a mixture of CCCACAAGGAAGAGGUACU (SEQ ID NO: 14), GCAUAUUGAUUACAGGAUG (SEQ ID NO: 15), CCGUAAGGCUGUACAUAGU (SEQ ID NO: 16), CAAUGACAGUAGAGGGACC (SEQ ID NO: 17); NCDN, individually and a mixture of NCDN 18 GUUCAUUGGUGACGAGAAA (SEQ ID NO: 18), NCDN 19 AGACCUCAUCCUUGCGUAA (SEQ ID NO: 19), NCDN 20 AGGCCAAGAAUGACAGCGA (SEQ ID NO: 20), NCDN 21 GGCCAUUGAUUACGCAGUU (SEQ ID NO: 21); negative control (siControl) targeting luciferase, UAAGGCUAUGAAGAGAUAC (SEQ ID NO: 22); positive control targeting Lamin A/C (To be completed); SiGlo Cyclophilin B to determine transfection efficiency GGAAAGACUGUUCGAAAAA (SEQ ID NO: 23) Reduction of protein expression using shRNA was achieved by transfecting SH-SH5Y cell lines with pSUPER-GFP.Neo plasmids (oligoengine) expressing shRNA to SMN and Cyclophilin B, which have been described previously (Clelland et al, 2012).

Fractionation

Cells were pelleted from the appropriate cell line, and incubated in Buffer A (10mM HEPES pH7.9, 1.5mM MgCl₂, 10mM KCl, 0.5mM DTT, 1 cOmplete EDTA-free protease inhibitor tablet per 10ml) for 5 minutes, before being Dounce Homogenised 25 times to disrupt the cell membrane. This was then centrifuged at 300 RCF for 5 minutes to pellet the nuclei. The supernatant was removed, re-centrifuged at 300 RCF to further remove nuclei, before the supernatant was centrifuged at 16,100 RCF for 30 minutes. The nuclei were washed in Buffer S1 (250mM Sucrose, 10mM MgCl₂), before Buffer S3 (880mM Sucrose, 0.5mM

MgCl₂) was layered over, before centrifugation at 2800 RCF for 10 minutes to wash and pellet the nuclei. The supernatant from the 16,100 RCF centrifugation was then further centrifuged at 100,000 RCF for 60 minutes. The supernatant was then removed and kept. The 16,100 and 100,000 RCF pellets were then washed in further Buffer A and re-centrifuged. Each pellet was then resuspended in lysis buffer. To confirm efficient separation of cytoplasmic fractions from the nuclear fractions, Mouse anti-tubulin (Manufacturer, 1:500) and Rabbit polyclonal anti-Histone H3 (Proteintech, 1:300) antibodies were used.

Results

SmN exhibits similar behaviour to SmB, localising to vesicles containing SMN in the cytoplasm.

The Sm proteins SmB, SmD1 and SmE have previously been shown to exhibit a characteristic pathway within the cell, indicative of the snRNP maturation pathway (Sleeman and Lamond 1999). To determine how SmN localised during maturation and incorporation into snRNPs, YFP-SmN was transiently expressed in an SH-SY5Y neuroblastoma cell line constitutively expressing mCherry-SmB. YFP-SmN initially localised diffusely in the cytoplasm of cells, before co-localising with mCherry-SmB in nuclear speckles at 48 hours (Fig 1A, B). Similarly to previous results investigating SmB, SmD1 and SmE in HeLa and MCF-7 cells, some accumulation in nuclear Cajal Bodies (CBs, detected with anti coilin) was seen. However, CBs were not prominent in the majority of SH-SY5Y cells transiently expressing YFP-SmN. Both YFP-SmN and mCherry-SmN are efficiently incorporated into splicing snRNPs, as evidenced by their co-immunoprecipitation using antibodies against the characteristic hypermethylated CAP structure (2,2,7-trimethylguanosine) found on snRNAs –(TMG Beads) (Fig 1C). To determine whether the similarities between SmN and SmB extends to its localisation in vesicles in the cytoplasm of neural cells, (Prescott et al, 2014), live cell imaging of cells constitutively expressing mCherry-SmN was performed. Mobile mCherry-SmN foci were observed. In common with the SmB vesicles, these stained positive with the lipophilic dye, BODIPY 493, indicating that they are vesicular in nature, (Fig 1D). Finally, to confirm that the mCherry-SmN containing vesicles were similar to those previously identified with SmB, SH-SY5Y cells constitutively expressing mCherry-SmN were transfected with either GFP-SMN or YFP alone. GFP-SMN co-localised with mCherry-SmN in 82.90±11.12% (Mean ± Standard Deviation) of SmN-positive vesicles, which is statistically significant when compared to 8.37±4.52% of mCherry-SmN vesicles that co-localised with YFP signal (Fig 1E, F).

Mass Spectrometry reveals the similarities between the interactomes of SmN and SmB

As SmN appeared to behave very similarly to SmB in neural cells, it was unclear why neural cells express two almost identical proteins. It was decided to investigate whether SmN and SmB may have differences at the interactome level. Proteins interacting with YFP-SmB and YFP-SmN were affinity purified from whole-cell lysates of SH-SY5Y cell lines constitutively expressing the tagged protein with a cell line expressing YFP alone as a control for non-specific binding to the tag or bead matrix. Immunoblot analysis using antibodies to YFP demonstrated that the enrichment of the tagged proteins was 20X, 23X and 36.5 for YFP-SmN, YFP-SmB and YFP alone respectively (Fig 2A). The affinity purified material was size separated using SDS-PAGE and analysed by Mass Spectrometry (use whatever the official name is here facility at St Andrews) to identify proteins interacting with YFP-SmB and YFP-SmN. Following removal of likely contaminants identified by their interaction with YFP alone, or their previous identification as common interactors of the sepharose beads (Trinkle-Mulcahy et al, 2008), the interacting proteins were categorised using Uniprot Genome Ontology annotations. The data set is available in Table S1). The overall proportions of proteins in each category were similar when comparing the interactomes of SmN to SmB, though there were differences identified at the level of individual proteins. Of particular interest were a number of proteins with potential neural specific roles, which were identified in one or both samples. One of these was Neurochondrin (NCDN), a relatively poorly characterised neural protein, which was identified in the YFP-SmN interactome.

Neurochondrin interacts with SmB, SmN and SMN

To verify the interaction between Sm proteins and Neurochondrin, a construct expressing NCDN-GFP was generated. Affinity purification of NCDN-GFP from whole Cell Lysates of SH-SY5Y cells co-expressing NCDN-GFP and mCherry-SmB demonstrated interaction between NCDN-GFP and mCherry-SmB (Fig 3A). To further investigate interactions between NCDN and the Sm proteins in the context of SMA pathology, an SH-SY5Y cell line constitutively expressing NCDN-GFP was established. Affinity purification of NCDN-GFP from whole-cell lysates followed by immunoblot analysis using antibodies against endogenous SmN, SmB (Fig 3B) revealed that NCDN interacts with both SmN and SmB. Furthermore, both endogenous SMN and endogenous β COP (a coatamer vesicle protein) were also revealed to interact with NCDN, suggesting that NCDN interacts with the Sm proteins and SMN in the context of cytoplasmic vesicles. Affinity purification of YFP alone from whole cell lysates of an SH-SY5Y cell line constitutively expressing YFP does not result in co-purification of endogenous SMN, SmB or β COP (Fig 3B, C). To further investigate the interaction between SMN and NCDN, a reciprocal experiment was performed using GFP-Trap to affinity purify GFP-SMN from an SH-SY5Y cell line constitutively expressing GFP-SMN (Clelland et al, 2009). Subsequent immunoblot analysis using antibodies to endogenous NCDN confirmed the interaction between SMN and NCDN (Fig 3F). To verify

that SMN and NCDN co-localise in vesicles, SH-SY5Y cells were co-transfected with mCherry-SMN and NCDN-GFP or YFP, where it was observed that, similar to with SmN and SmB, SMN and NCDN co-localised in vesicles (Fig 3G).

NCDN, SMN and Sm protein co-fractionate with coatomer proteins.

5 To further investigate the possibility that the documented interaction of NCDN with SMN and the Sm proteins occurs within the cytoplasmic vesicles, fractionations were performed on both parental SHSY5Y cells and SH-SY5Y cell lines constitutively expressing GFP-NCDN, YFP-SmB, YFP-SmN or YFP. Sequential centrifugation was used to separate the cells into a nuclear fraction, 16,000 RCF and 100,000 RCF cytoplasmic pellets and cytosolic
10 supernatant. Immunoblotting of these sub-cellular fractions revealed that GFP-NCDN, YFP-SmB and YFP-SmN, are all enriched in 100,000 RCF pellet, along with endogenous SMN and Coatomer proteins (Fig 4). This fraction would be expected to contain small cytoplasmic vesicles including small coatomer type endocytic vesicles. Together with the co-localisations seen between GFP- and mCherry-tagged proteins (Fig, 3), this suggests that the interactions
15 between NCDN, SMN and the Sm proteins likely take place in small cytoplasmic vesicles. The reduction in gene expression, documented by immunoblotting, for each siRNA was typically 40-60% (Fig 5C). This suggests that NCDN is required for the correct sub-localisation of SMN. Of potential relevance for SMA pathology, depletion of either NCDN or SmB causes GFP-SMN to adopt a sub-cellular localisation reminiscent of that shown by
20 GFP-SMN Δ 7, a truncated version of SMN that mimics the product of the SMN2 gene and is unable to substitute for full-length SMN in models of SMA (Monani et al, 1999).

NCDN is required for the correct sub-cellular localisation of SMN

We have previously documented that reduction of SmB expression results in re-localisation of SMN into numerous nuclear structures, probably analogous to gems (Gemini of Cajal
25 Bodies), and its loss from cytoplasmic structures (Prescott et al, 2014). To investigate the requirement for NCDN in cytoplasmic SMN localisation, an SH-SY5Y cell line constitutively expressing GFP-SMN was transfected with siRNAs targeting either NCDN (4 different siRNAs (Dharmacon) and a pooled sample). A reduction in NCDN expression caused an increase in the number of SMN-positive gems present in the cell nucleus, as did a reduction
30 of SmB expression (Fig 5A, B). Conversely, reduction in SMN expression reduced the number of nuclear gems. The use of non-targeting control (siControl) sequences or siRNAs targeting Lamin A/C had no effect on the number of nuclear gems. The reduction in gene expression, documented by immunoblotting, for each siRNA was typically 40-60% (Fig 5C). This suggests that NCDN is required for the correct sub-localisation of SMN. Of potential
35 relevance for SMA pathology, depletion of either NCDN or SmB causes GFP-SMN to adopt a sub-cellular localisation reminiscent of that shown by GFP-SMN Δ 7, a truncated version of

SMN that mimics the product of the SMN2 gene and is unable to substitute for full-length SMN in models of SMA (Monani et al. 1999; Monani et al. 2000).

SMN is required for the correct sub-cellular localisation of NCDN

To investigate whether NCDN requires the SMN protein for its localisation to vesicles in neural cells, SH-SY5Y cells were transfected with shRNA constructs previously validated to reduce the expression of SMN and carrying a GFP marker (Clelland et al, 2012). Reduction of SMN reduced the number of cytoplasmic foci containing endogenous NCDN (Fig 6 A-C). This, together with data in figure 5, suggests that NCDN and SMN are both required for the formation of these structures.

NCDN does not co-purify with splicing snRNPs, suggesting it is not involved in snRNP assembly

To investigate whether the interaction between NCDN and SMN could reflect a previously unidentified role for NCDN in snRNP assembly, splicing snRNPs were affinity purified from whole cells lysates of SH-SY5Y cells constitutively expressing NCDN-GFP using agarose beads couple to antibodies against the characteristic tri-methyl guanosine CAP of snRNAs (TMG beads) (Fig 7A). NCDN-GFP did not co-purify with splicing snRNPs, suggesting that NCDN is not involved in snRNP assembly or processing. This raises the intriguing possibility that the interaction between SMN and NCDN reflects a novel, snRNP-independent role for SMN.

SMN interacts with Rab5 in SH-SY5Y cells and co-localises with a sub-set of Rab5 vesicles.

Rab5 is a marker of early endosomes and endocytic vesicles, as well as being a regulator of these trafficking pathways (Bucci et al. 1992). As NCDN and Rab5 have previously been shown to interact, and Rab5 to have a role in dendrite morphogenesis and cell polarity (Sato et al. 2008; Oku et al. 2013; Guo et al. 2016), we next sought to investigate the possibility that some of the SMN-rich vesicles are Rab5 vesicles. SH-SY5Y cells were co-transfected with plasmids to express mRFP-Rab5 (Vonderheit and Helenius. 2005) together with GFP-SMN, NCDN-GFP or YFP. mRFP-Rab5 was affinity-purified from whole-cell lysates from each co-transfection to determine whether GFP-SMN, NCDN-GFP or YFP alone interacted with mRFP-Rab5 (Fig 7B). Subsequent immuno-blotting revealed co-purification of GFP-SMN and NCDN-GFP, but not YFP alone, with mRFP-Rab5. Furthermore, endogenous SMN and NCDN also co-purified with mRFP-Rab5. In parallel experiments, co-localisation of mRFP-Rab5 with GFP-SMN and NCDN was investigated (Fig 6C). In accordance with previous publications, Rab5 was found to partially co-localise with NCDN-GFP (Oku et al. 2013). GFP-SMN showed a similar degree of co-localisation with mRFP-Rab5, while there was minimal co-localisation between YFP and mRFP-Rab5. Taken

together with the data in fig 7 above this suggests that NCDN and SMN co-localise in the context of Rab5 vesicles.

DISCUSSION

The genetic cause of SMA has been known since 1995, but there is still little available in the way of treatment. A significant reason for this is uncertainty about the cellular roles of SMN, which appear to be numerous. In particular, it is not clear why motor neurons are so exquisitely sensitive to reduced levels of SMN when the key roles of the protein appear to be in pathways required in all cell types. By comparing the interactomes of two very similar members of Sm protein family, SmB and the neural-specific SmN, we have uncovered an interaction between SMN and the essential neural protein NCDN, which may open novel avenues for therapy development.

Neurally expressed SmN behaves similarly to SmB, but individual differences in interactomes may indicate alternative roles and specific interactions

Differences between members of the Sm protein family have not been systematically investigated, although non-splicing roles have been proposed for SmB in mRNA localisation in *Drosophila* and for SmD1 in miRNA biogenesis. As SmB and SmN perform the same primary function in snRNPs, it is currently unknown why SmN is expressed in neural tissues as well as or instead of SmB. Current research has suggested that the expression of SmN may cause tissue specific alternative splicing of pre-mRNA transcripts (Lee et al, 2014). However, an alternative, but complimentary hypothesis is that SmN may be adapted for secondary, neural specific roles. We demonstrate here that SmN localises identically to SmB, both during snRNP maturation and at steady-state where both localise to vesicles containing SMN in the cytoplasm and neurites of SH-SY5Y cells. Our parallel proteomic study used SH-SY5Y neural cell lines constitutively expressing YFP-SmN and YFP-SmB to investigate difference between the interactomes of these two, very similar, proteins. YFP-SmN has a proline rich C-terminal tail that YFP-SmB lacks. Several proteins were identified in the SmN interactome but not the SmB interactome. This suggests that some of these proteins may interact preferentially with SmN, perhaps mediated by the proline-rich tail. However, further validation and additional experimentation would be required to confirm these differences in interactome between SmN and SmB and to investigate specific functions for distinct Sm protein family members.

NCDN interacts with SMN, SmB and SmN, and co-localises with them in vesicles, suggesting a novel cellular role for SMN.

Previous research into neural-specific functions for SMN has identified several new protein-protein interaction involving SMN. These novel SMN partners have, in the main, been RNA

interacting proteins. There is growing appreciation that SMN-mediated transport may be of particular importance in neural cells and involve COP1-type vesicles containing SmB and transported by Dynein (Prescott et al, 2014). The nature and content of these vesicles is not clear and is likely to be of significance for the cell-type bias of SMA symptoms. The SmN/SmB interactome screen presented here suggested a large number of non-snRNP proteins as potential cellular partners for the Sm proteins.

We chose to investigate to neural protein NCDN further, as it has characteristics that may be of relevance for SMA. NCDN is predominantly expressed in neural tissue, and little is known about its structure or function, as it shares little sequence homology with other eukaryotic proteins (Shinozaki et al, 1997). Though characterised relatively poorly, it is associated with dendrite morphogenesis and maintenance of cell polarity (Oku et al, 2013) as well as being a modulator of signal transduction pathways (Dateki et al, 2005; Franke et al, 2006; Wang et al, 2009; 2012; Ward et al, 2009; Matosin et al, 2015; Pan et al, 2016). These neural-specific roles suggest that the interaction between NCDN and the Sm proteins may help to better understand the exact mechanism of pathogenesis of SMA.

Reciprocal affinity-purification of GFP-tagged and endogenous NCDN and Sm proteins (fig.) validated the interaction detected in the interactome analysis. Although originally identified as a protein interacting with SmN but not SmB, further investigation indicates that NCDN is, in fact, capable of interacting with both of these Sm proteins. Of much greater interest, however, is the interaction documented between NCDN and SMN, which appears more robust than that between NCDN and the Sm proteins (fig). Furthermore, NCDN is excluded from the cell nucleus and localises with SMN and the Sm proteins in mobile vesicles in the neurites of SH-SY5Y cells suggesting that it shares cytoplasmic, rather than nuclear, roles with SMN.

Reducing expression of NCDN or SMN causes a reciprocal mislocalisation, suggesting that the localisation of these two proteins to cytoplasmic vesicles is co-dependant.

Reduction of NCDN expression caused SMN to mislocalise to an increased number of nuclear foci reminiscent of those formed by the truncated product of the SMN2 gene, SMN Δ 7. In parallel with this, reductions of SMN expression caused a reduction in cytoplasmic foci containing NCDN in SH-SY5Y cells. This suggests that both proteins promote each other's sub-cellular localisation in neural cells. This places NCDN as a potential therapeutic target for SMA. Therapies directed to increase the expression or function of NCDN could be complimentary to therapies currently available or under development that target the SMN2 gene to increase SMN expression. If the localisation of

SMN to cytoplasmic vesicles is indeed required for mRNA transport to growth cones, enhancing this localisation by modulating NCDN expression or function could promote the development and survival of motor neurons.

Potential consequences of NCDN mislocalisation associated with SMN reduction

5 The interaction between SMN and NCDN appears to be important for the correct localisation of NCDN. In SMA, reduction of SMN and consequent mislocalisation of NCDN could potentially disrupt downstream neural signalling pathways including.. We have also demonstrated an interaction and partial co-localisation between SMN and Rab5 vesicles. Rab5 positive vesicles are linked to dendrite morphogenesis and somatodendritic polarity
 10 (Sato et al, 2008; Guo et al, 2015). If SMN is required for the function of a sub-set of these vesicles, an insufficiency in SMN within them could potentially cause problems with both morphogenesis and maintenance of polarity, the latter particularly vital in such elongated cells as motor neurons. NCDN is also associated with Rab5 ((Oku et al, 2013) and fig. 8), which, alongside dynein, is associated with maintenance of cell polarity (Sato et al, 2008,
 15 Guo et al, 2016). Whether SMN, NCDN and Rab5 are present in the same vesicles is yet to be determined. Further work will be required to investigate the potential involvement of NCDN-associated signalling pathways and cell polarity in the cellular pathology of SMA.

SMN has now been linked to several functions other than its canonical role in snRNP assembly. While reduction in snRNP assembly cause by lowered SMN may cause splicing
 20 defects, the key transcripts preferentially affecting motor neurones are still to be identified. SMN has an established role in the trafficking of mature mRNAs destined for localised translation. The nature of the structures involved in this role is not completely clear, however, with different authors describing the structures as vesicular or granular (Prescott et al, 2014). Reduction of SMN has also been linked with endosomal defects, suggestive of the
 25 importance of SMN for vesicular transport. Here we provide further evidence for the presence of SMN in vesicles and identify the essential neural protein, NCDN as a potential target for therapy development in SMA.

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Claims

1. A neurochondrin (NCDN) modulator for use in the treatment and/or prevention of a neurodegenerative disorder.
2. The neurochondrin (NCDN) modulator of claim 1 for use of claim 1, wherein the
5 neurodegenerative disorder is a neuromuscular disorder or spinal muscular atrophy (SMA).
3. A neurochondrin (NCDN) modulator for use in the treatment and/or prevention of a spinal muscular atrophy.
4. The neurochondrin (NCDN) modulator of any preceding claim 1 for use of any
preceding claim, wherein the NCDN modulator is any substance, compound or composition
10 comprising the same, which increases or decreases the expression, function and/or activity
of the NCDN protein.
5. The neurochondrin (NCDN) modulator of any preceding claim for use of any
preceding claim, wherein the NCDN modulator is any substance, compound or composition
comprising the same, which increases the expression, function and/or activity of the NCDN
15 protein.
6. The neurochondrin (NCDN) modulator of any preceding claim for use of any
preceding claim, wherein the NCDN modulator comprises:
 - (i) all or part of the sequence provided by SEQ ID NO: 1;
 - (ii) a functional fragment of SEQ ID NO: 1;
 - 20 (iii) a sequence at least 65% identical to SEQ ID NO: 1; and
 - (iv) a functional fragment of the sequence provided by claim 6 part (iii).
7. The neurochondrin (NCDN) modulator of claim 6, for use of claim 6, wherein a
functional fragment is a fragment which modulates one or more of the following:
 - (i) signaling pathways (in neurons);
 - 25 (ii) signal transduction events (in neurons);
 - (iii) neural outgrowth;
 - (iv) synaptic plasticity;

(v) dendrite morphogenesis; and

(vi) cell polarity (of neurons).

8. A method of determining whether or not a test agent possesses NCDN activity, said method comprising;

5 providing a cell with reduced levels of NCDN and/or SMN gene and/or protein expression;

contacting the cell with a test agent; and

determining whether or not the test agent replicates or replaces any aspect of NCDN or SMN function or activity.

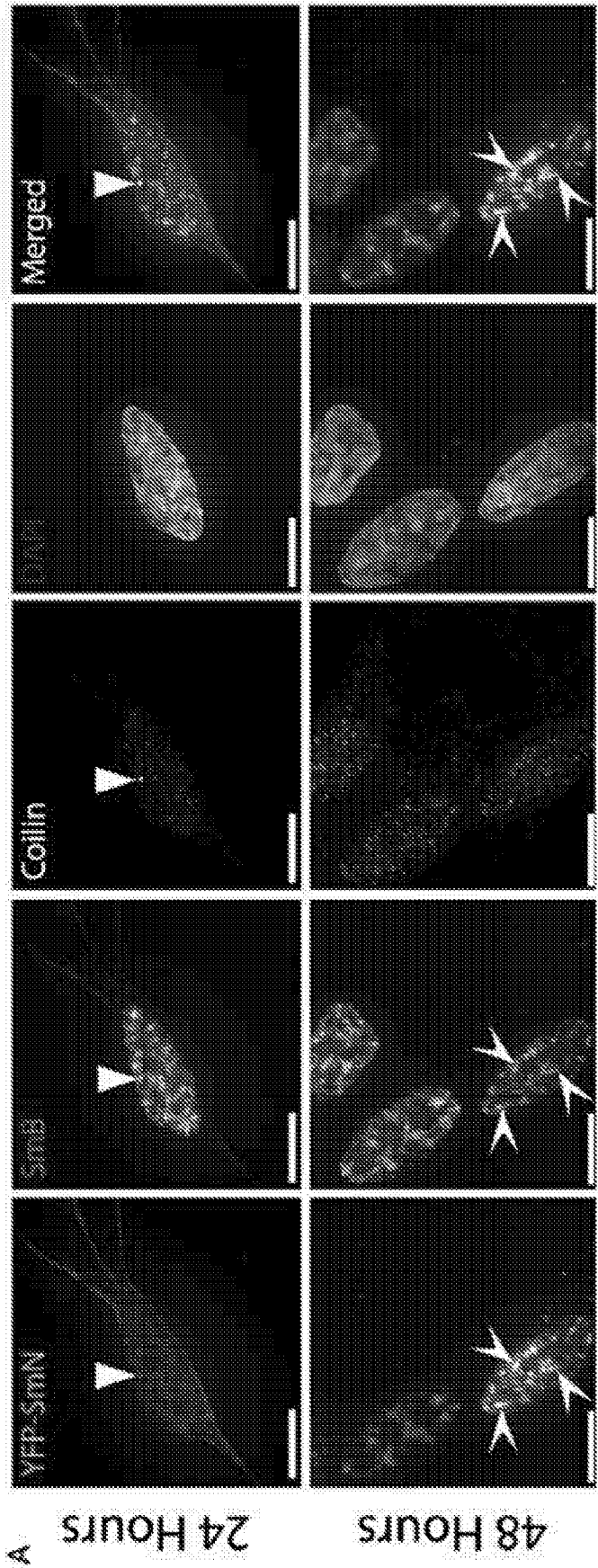


Figure 1 A

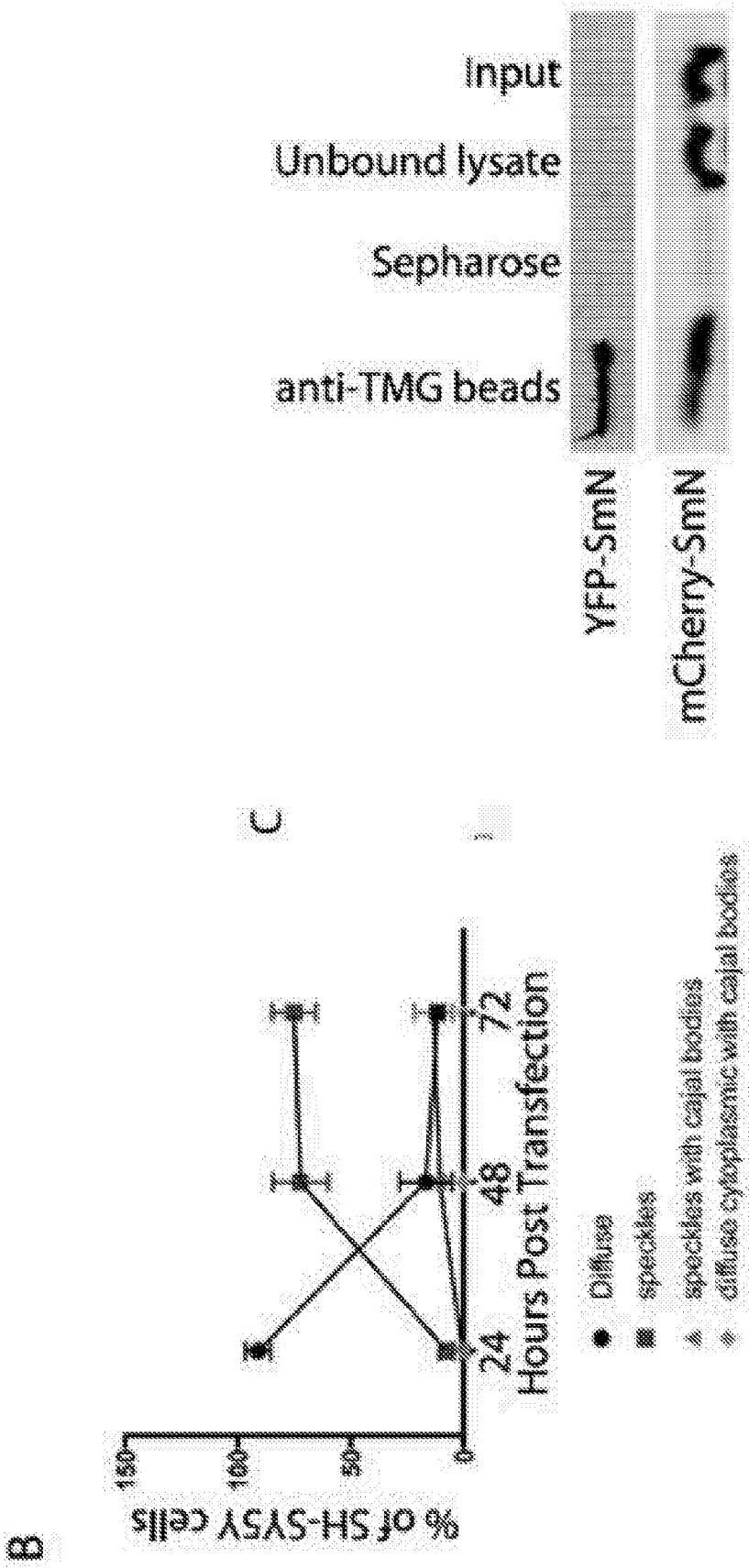


Figure 1B & C

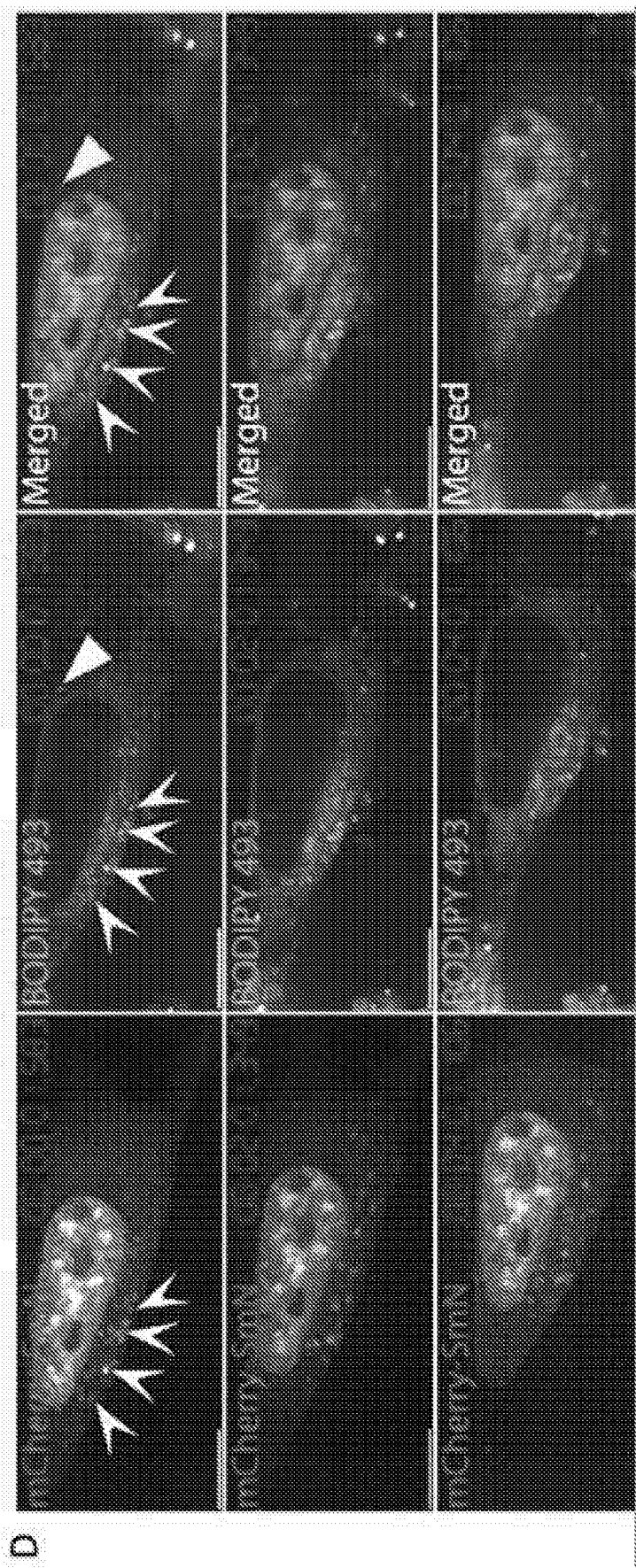


Figure 1 D

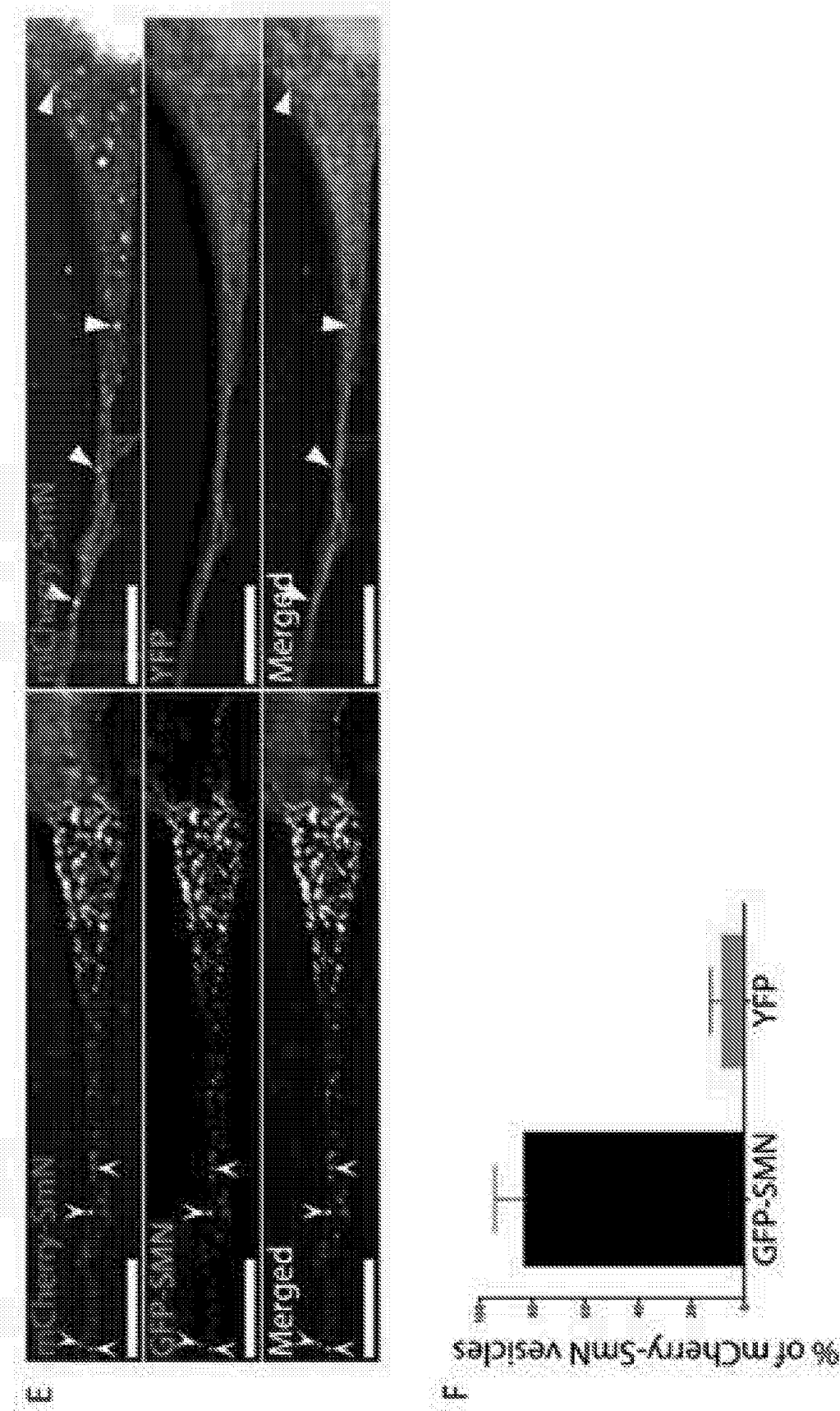


Figure 1 E & F

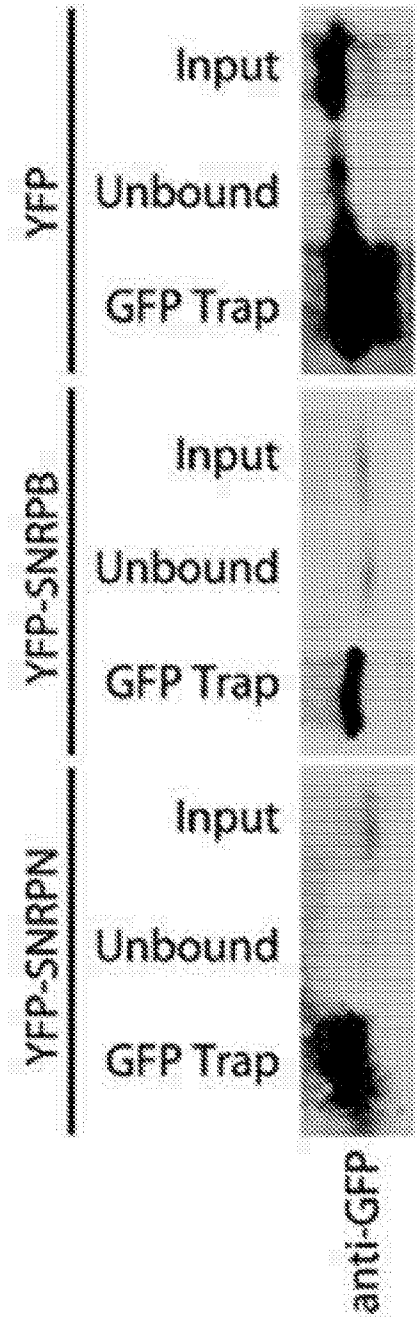


Figure 2A

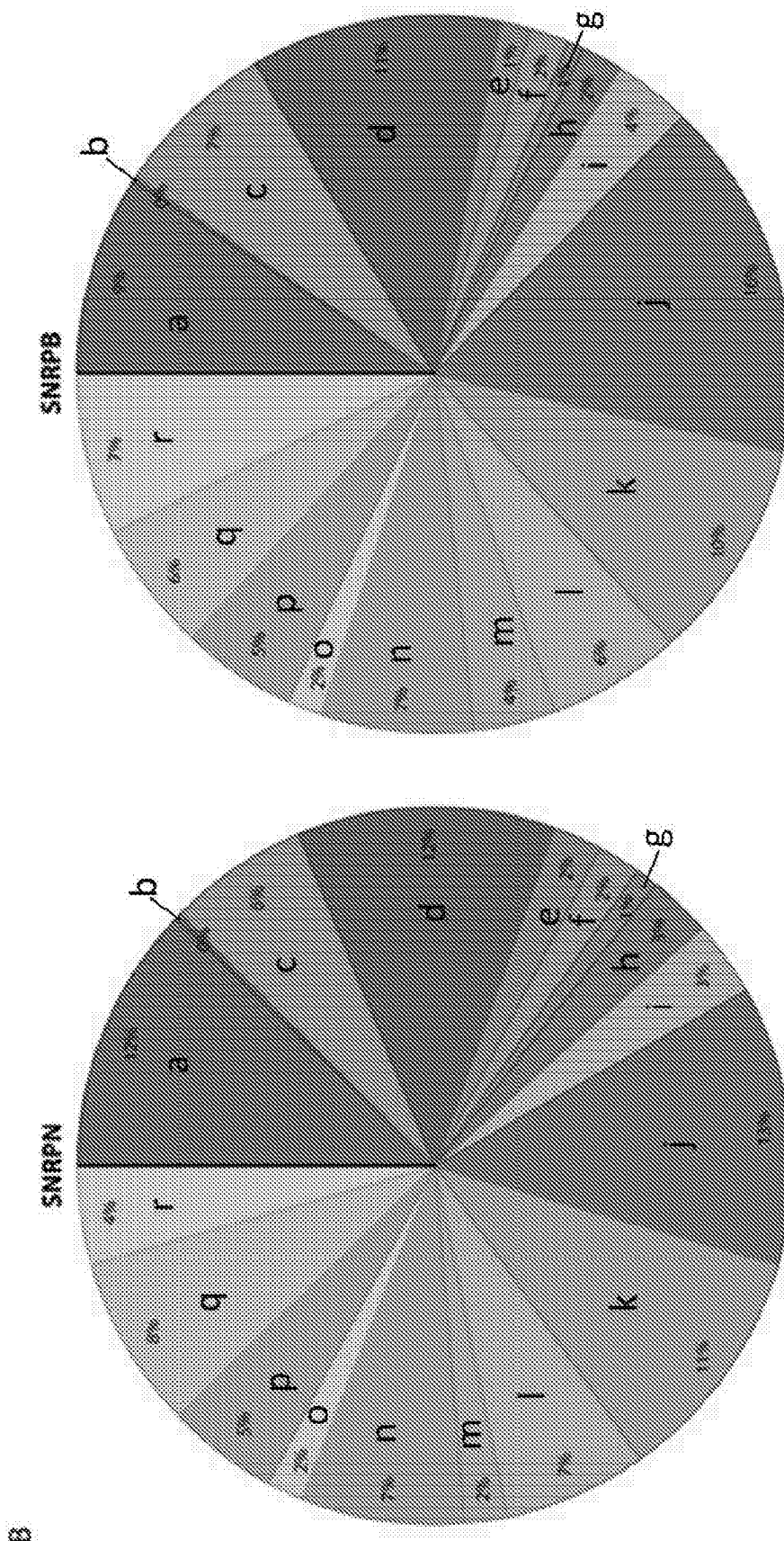


Figure 2B

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B (legend)

		% of total proteins identified	
		SNRPN	SNRPB
a	Splicing and snRNP assembly	12.1%	9.0%
b	Methylosome	0.3%	0.2%
c	Vesicle transport	6.4%	7.4%
d	Signal transduction	11.7%	11.3%
e	Neural proteins	2.2%	1.4%
f	Apoptosis	1.9%	1.8%
g	Motor proteins	1.0%	0.7%
h	Cytoskeletal related proteins	2.8%	1.9%
i	Mitochondria	3.4%	3.5%
j	Metabolism and synthesis	12.6%	16.2%
k	DNA replication and repair	10.8%	10.0%
l	Transcription	6.5%	6.0%
m	Post translational modification	1.9%	3.8%
n	RNA interacting/processing	7.4%	6.9%
o	RNA degradation	1.6%	1.5%
p	Proteolysis	5.0%	5.2%
q	Unknown	7.8%	5.9%
r	Other	4.4%	7.3%

Figure 2B (continued)

SmB: MTVGKSSKMLQHIFYRMCILQDGRIFIGTFKAFDKHMNLILCDCDEFKIKPKN
SmN: MTVGKSSKMLQHIFYRMCILQDGRIFIGTFKAFDKHMNLILCDCDEFKIKPKN

SmB: SKQAEEREKRVLGLVLLRGENLVSMTVEGPPPKDTGIARVPLAGAAGGPGIGRAA
SmN: AKQPEREEKRVLGLVLLRGENLVSMTVEGPPPKDTGIARVPLAGAAGGPGVGRAA

SmB: GRGIPAGVMPQAPAGLAGPVRGVGGPSQQVMT PQGRGTVAATAAATAIAGAP
SmN: GRGVPAGVIPQAPAGLAGPVRGVGGPSQQVMT PQGRGTVAATAAATAIAGAP

SmB: TQYPPGRGGPPPPMGRGAPPPGMMGPPPPGMRPPPMGPPMGIPPGRGTPMGMPPPGM
SmN: TQYPPGRGTPPPPVGRATPPPGIMAPPPGMRPPMGPPIGLPPARGTPIGMPPPGM

SmB: RPPPPGMRGLL (SEQ ID NO: 24)
SmN: RPPPPGIRGPPPPGMRPPRP (SEQ ID NO: 25)

Figure 2C

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Sequence	Scaffold peptide identification probability	Mascot ion score	Mascot Identity Score	Mascot Delta Ion Score	Observed Mass	Actual Mass	Charge	Δ Dalton	Δ PPM
(R) IFDAVGFTFFNR(L) (SEQ ID NO: 26)	100%	45.4	35.1	38.3	692.35	1382.68	2	-0.0140	-10.00
(K) IPILSTFLTAR(G) (SEQ ID NO: 27)	100%	56.5	31.7	50.4	616.37	1230.72	2	-0.0150	-12.00
(R) LALLETGTEVK(E) (SEQ ID NO: 28)	100%	47.1	36.6	47.1	595.32	1188.62	2	-0.0079	-6.60
(R) LLSTSPALQGTASR(G) (SEQ ID NO: 29)	100%	111.1	34.8	98.9	749.90	1497.79	2	-0.0200	-14.00
(R) LQAGEETASHYR(M) (SEQ ID NO: 30)	100%	75.1	34.3	75.1	454.55	1360.63	2	-0.0049	-3.60

Figure 2D

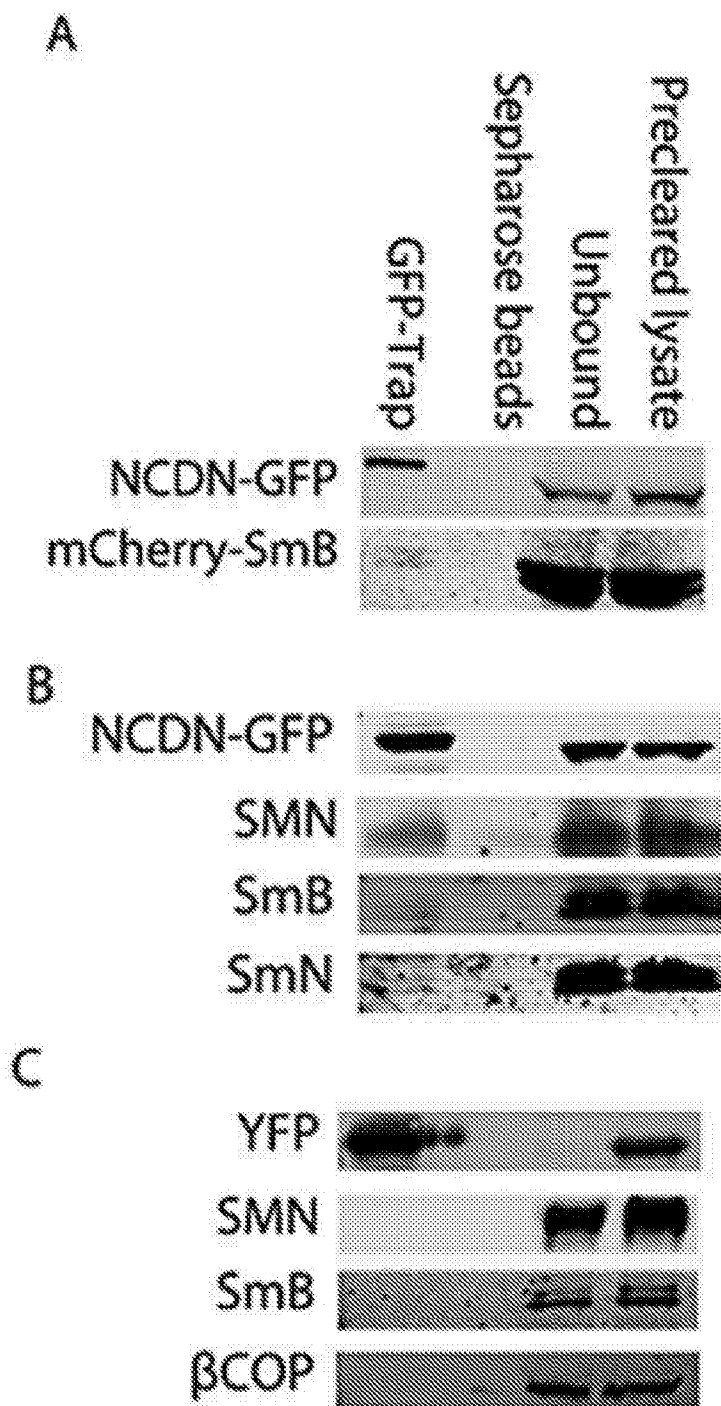
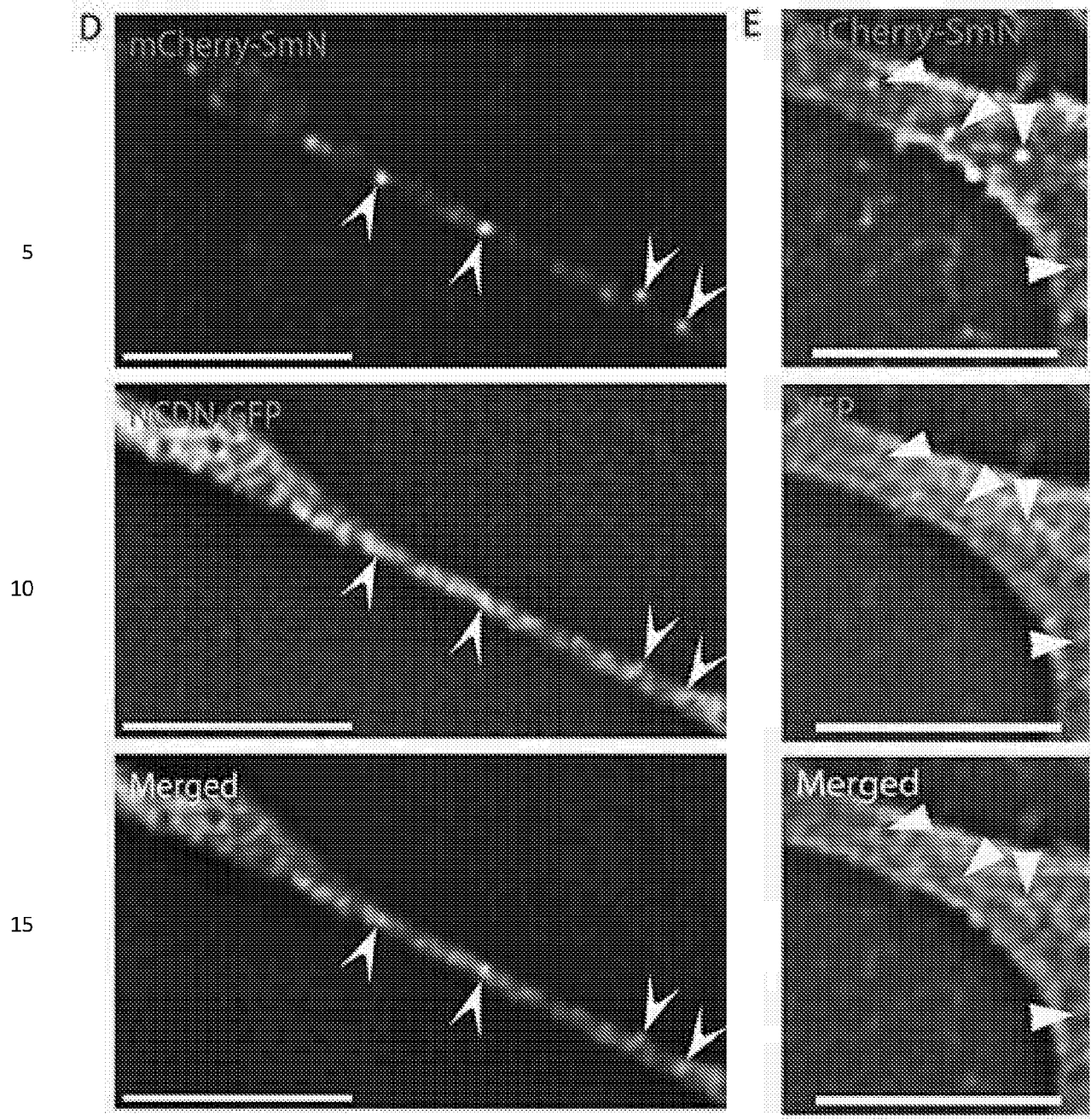


Figure 3 A, B & C



20 Figure 3 D & E

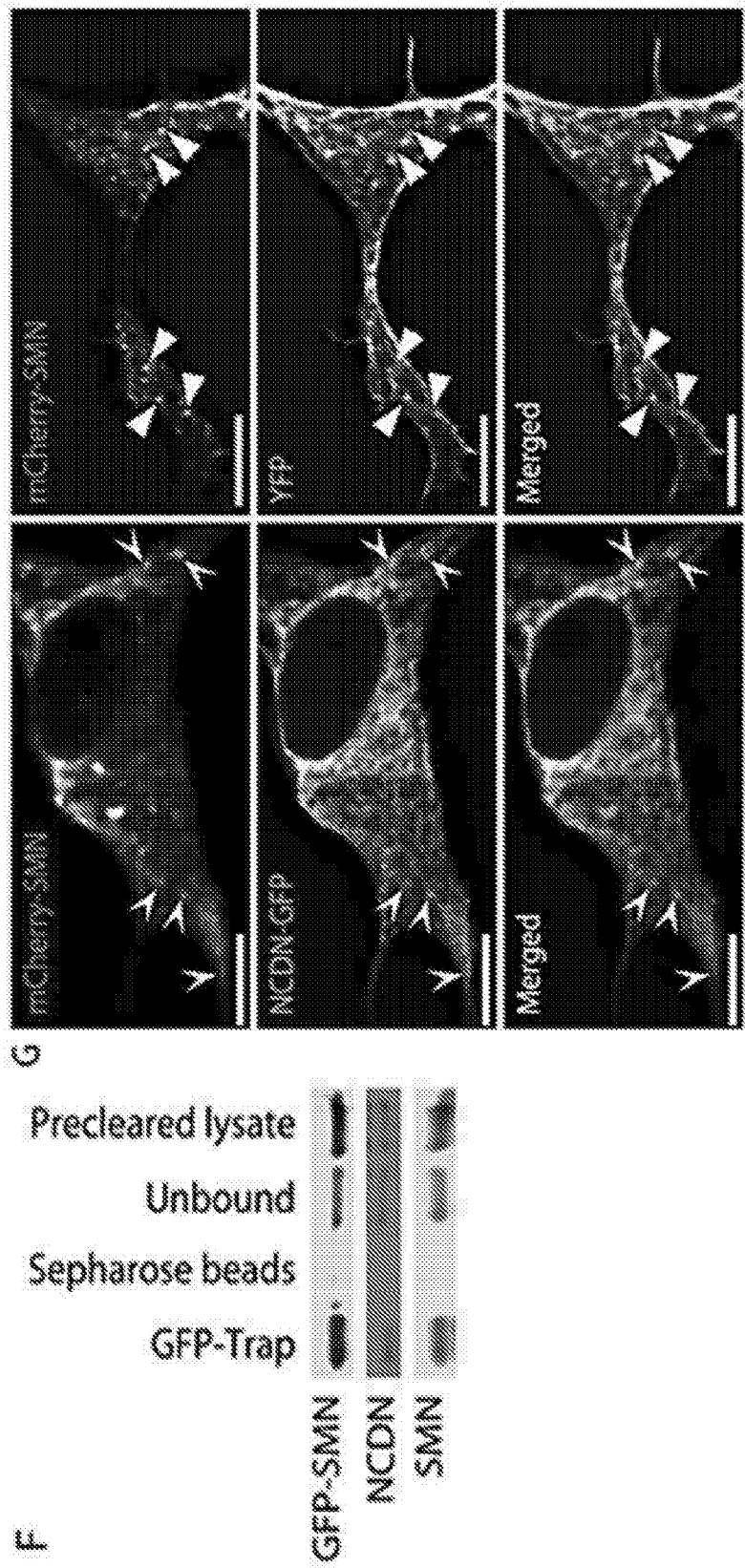


Figure 3 F & G

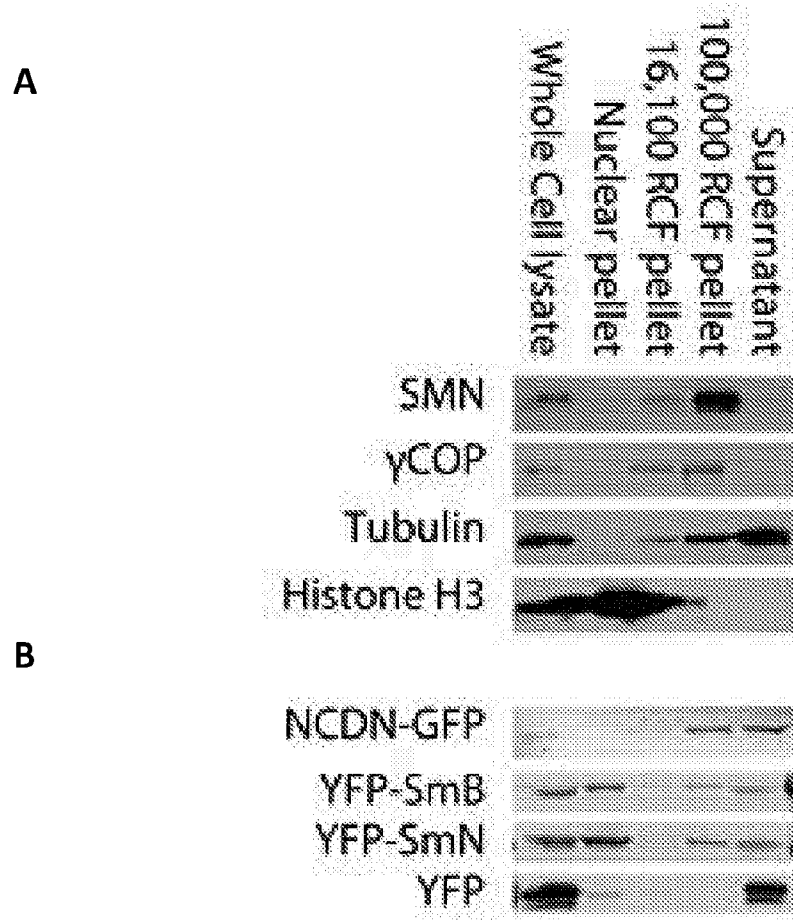


Figure 4 A&B

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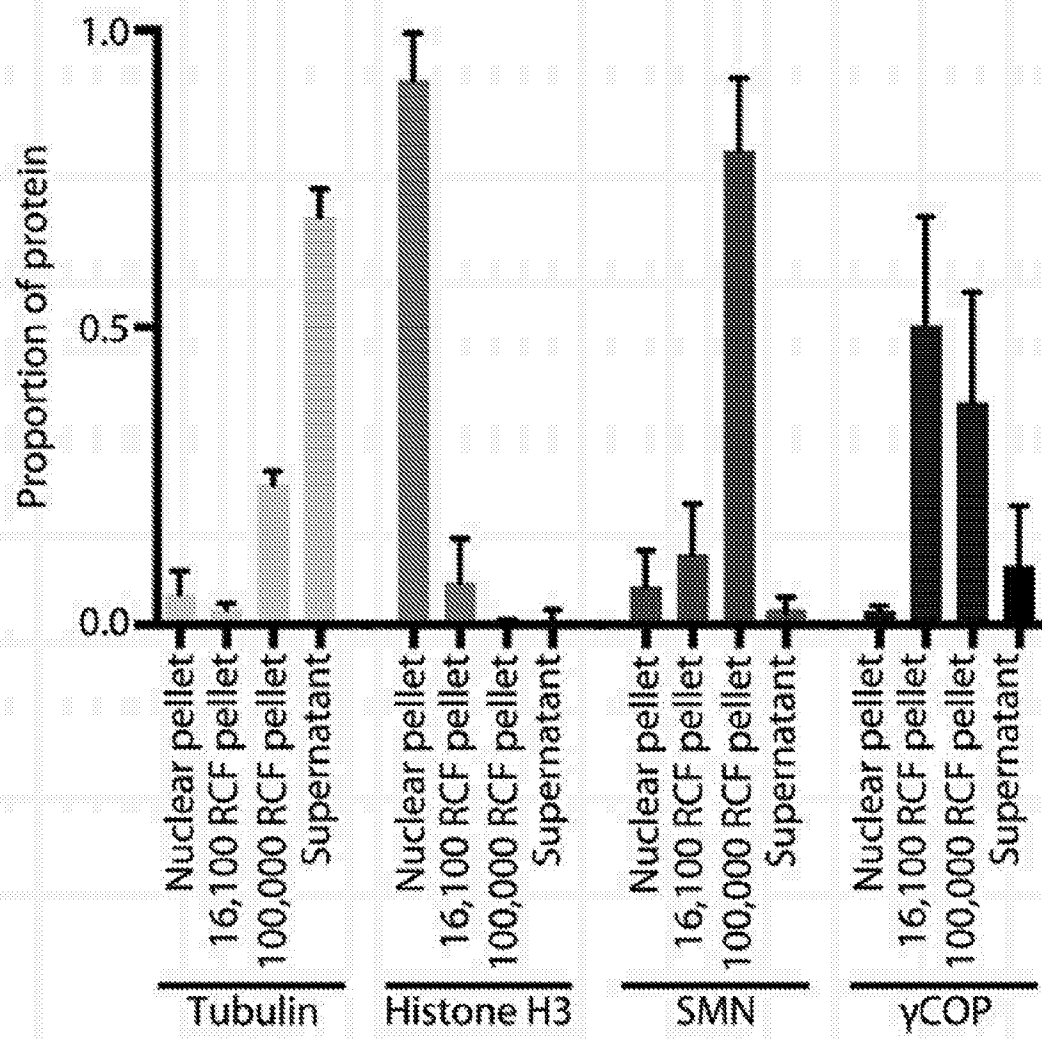


Figure 4C

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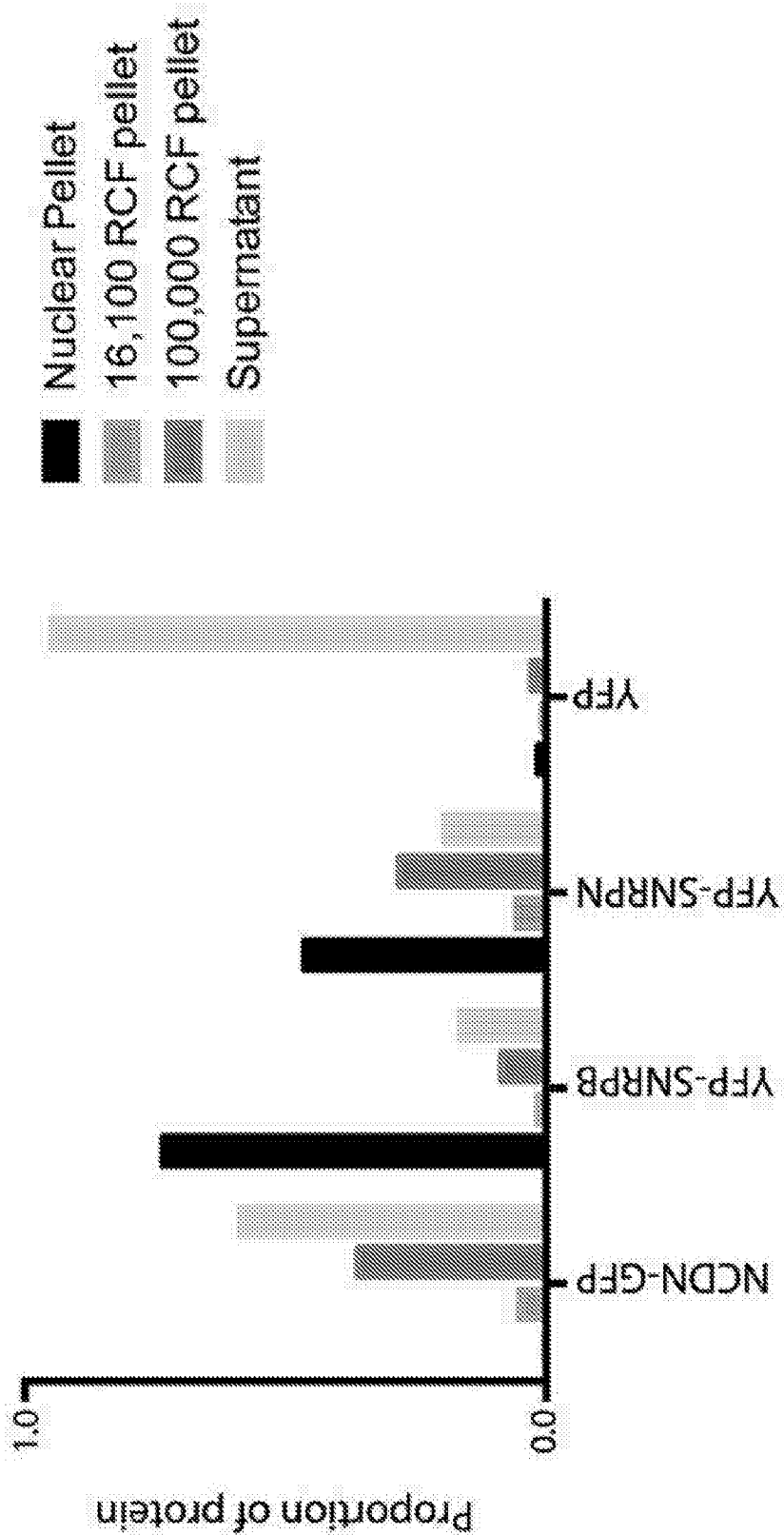


Figure 4D

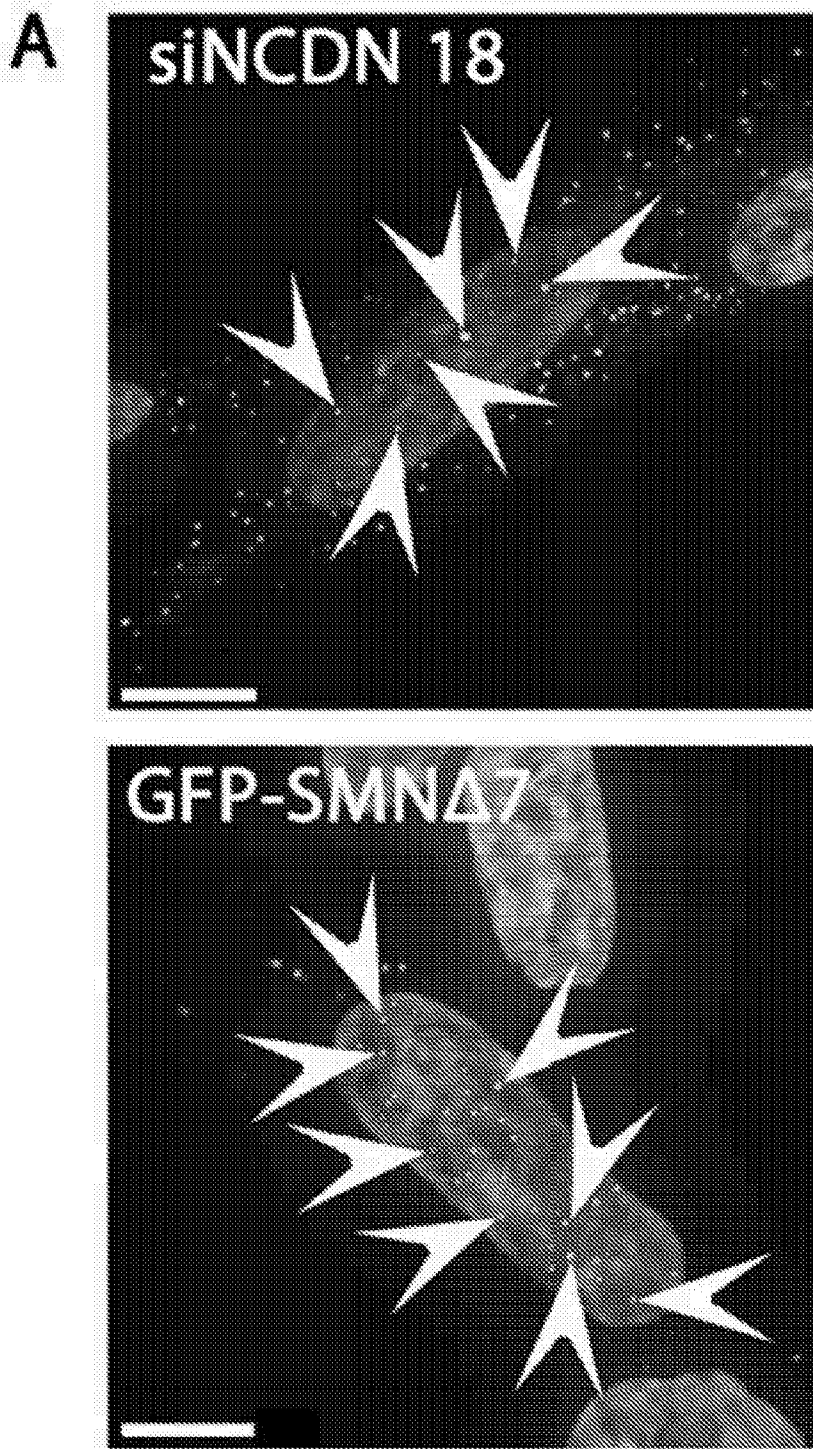


Figure 5 A

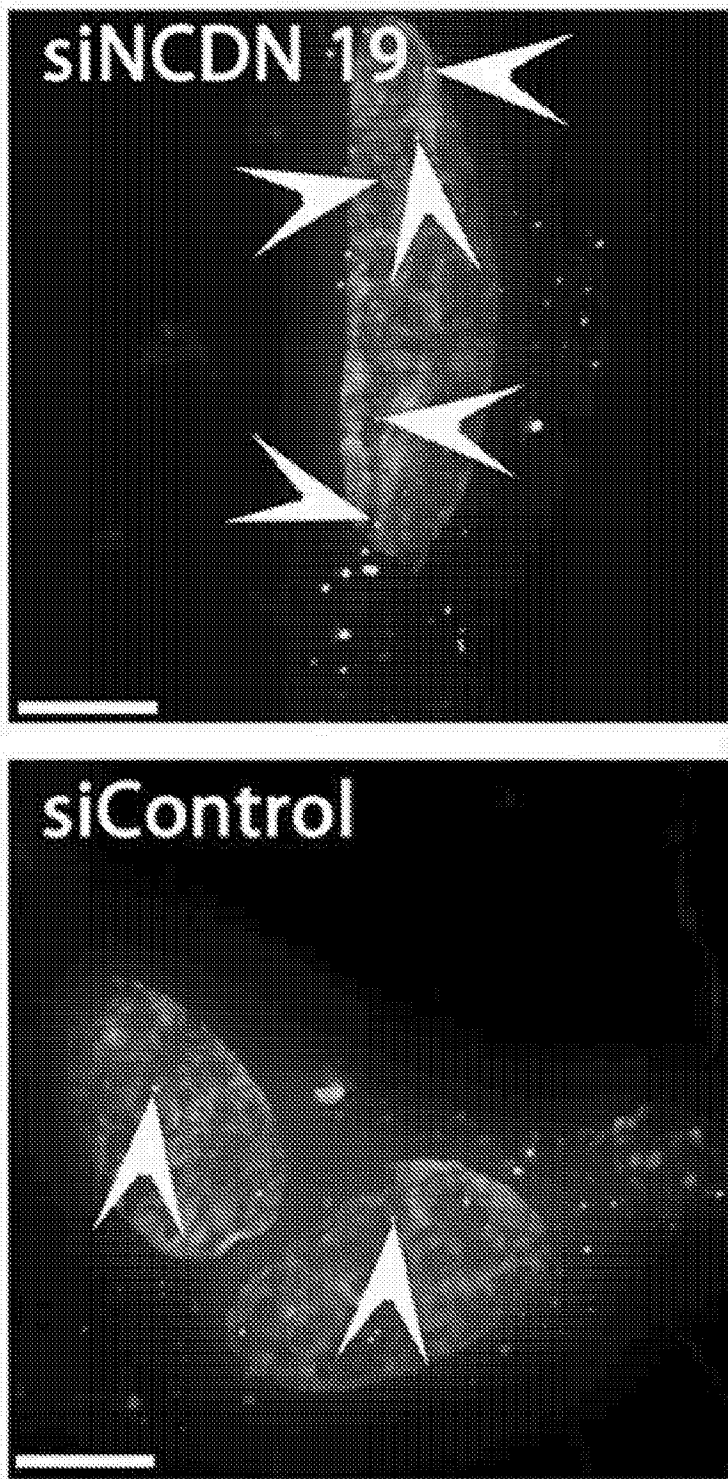


Figure 5 A continued

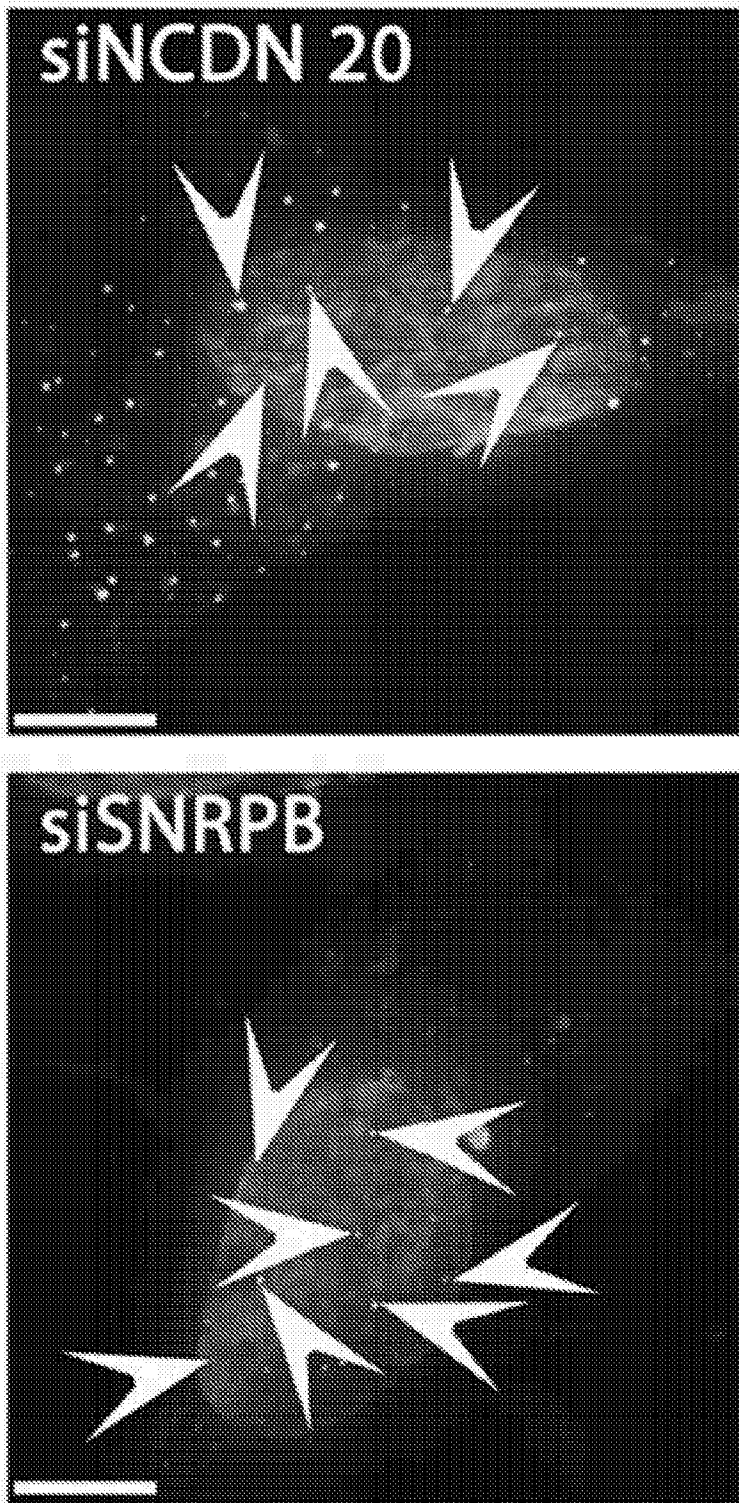


Figure 5 A continued

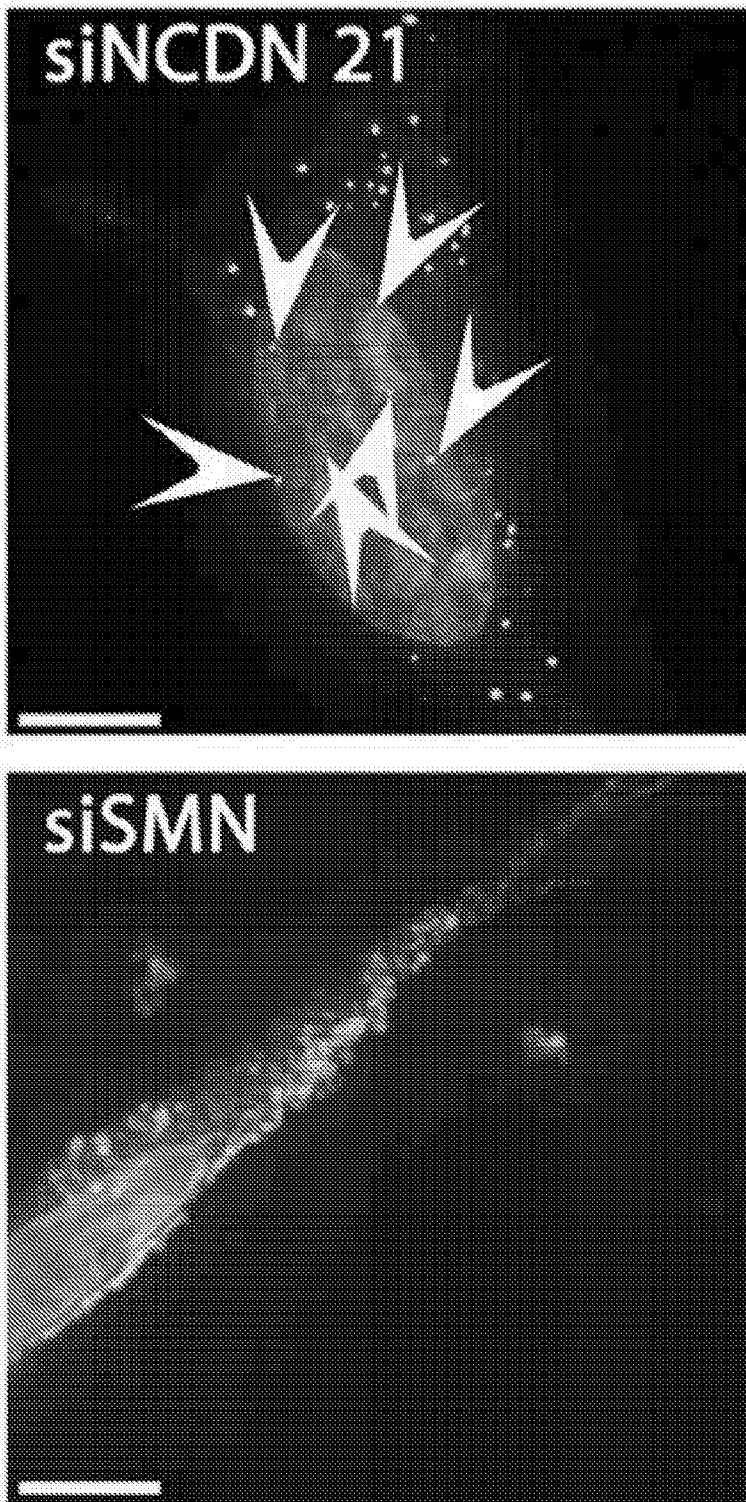


Figure 5 A continued

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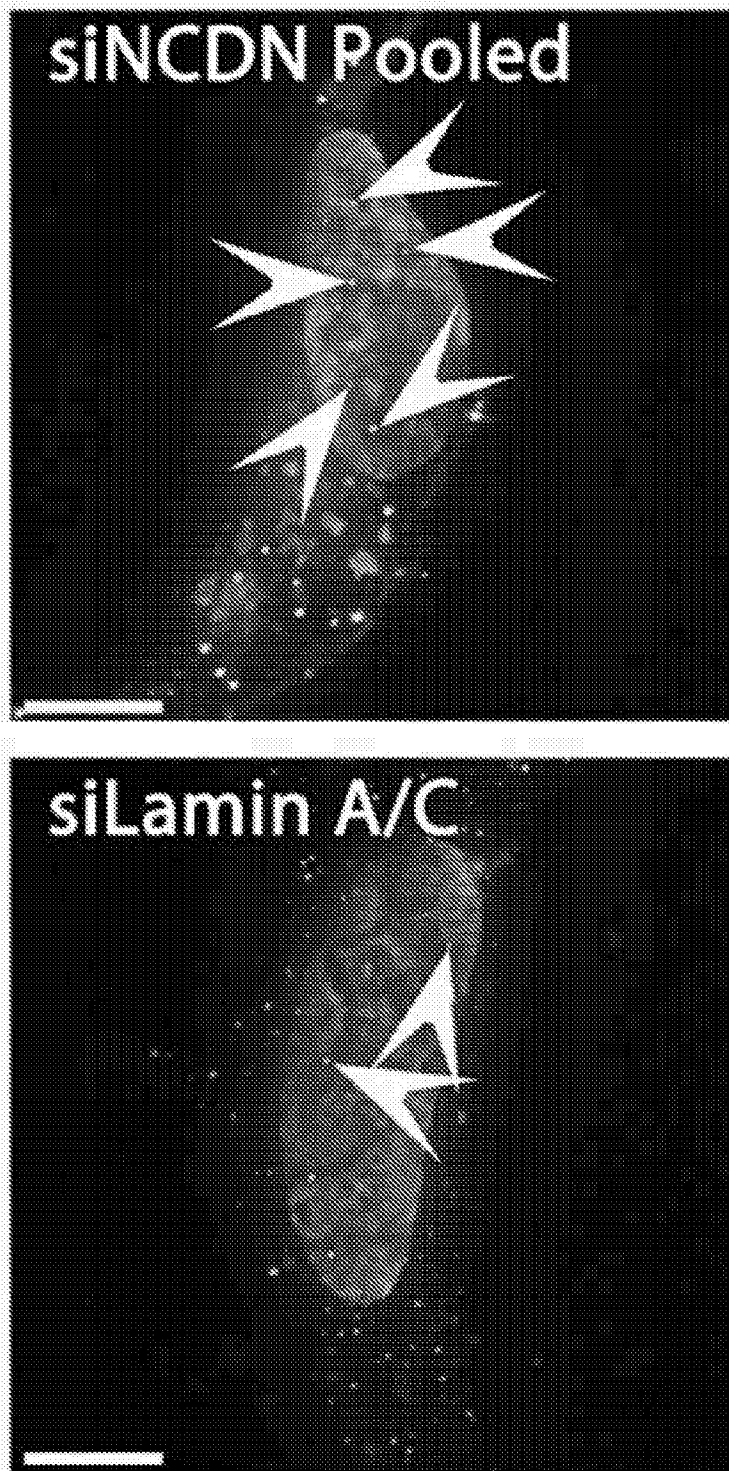


Figure 5 A continued

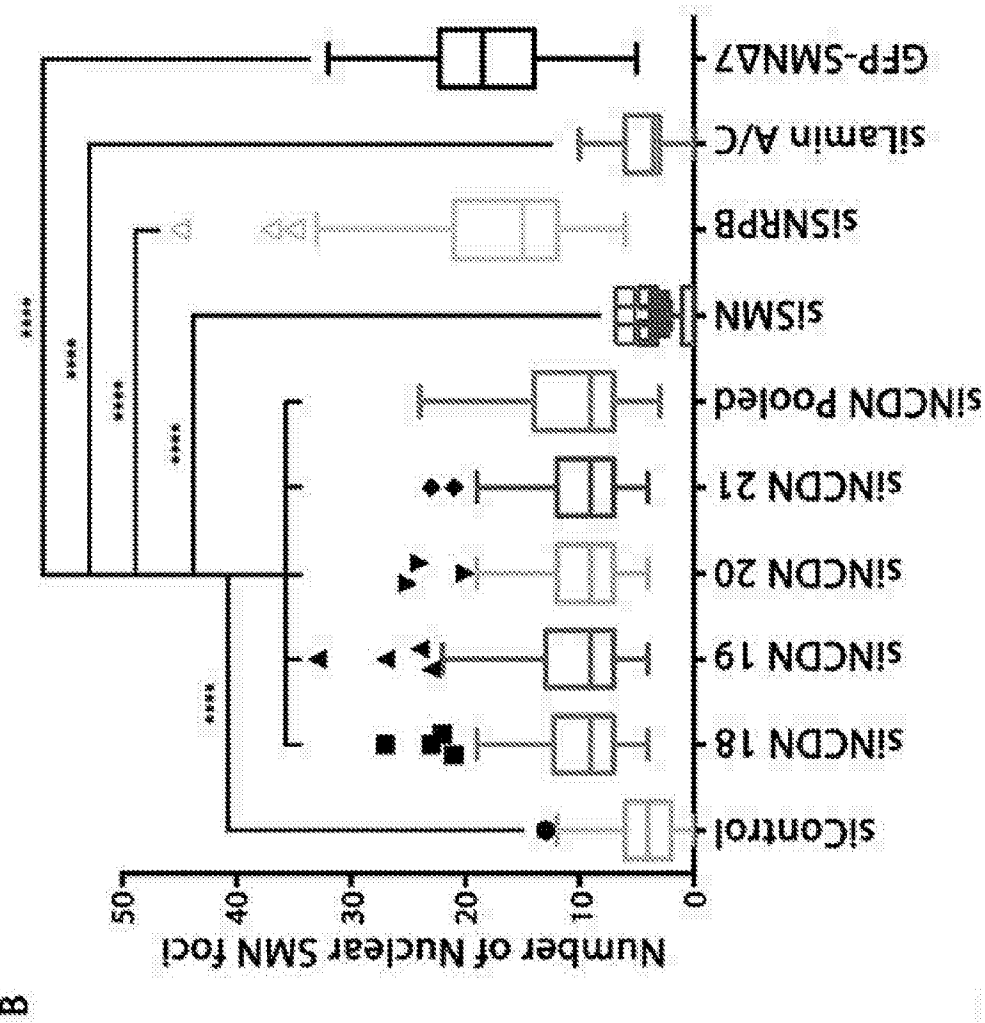


Figure 5B

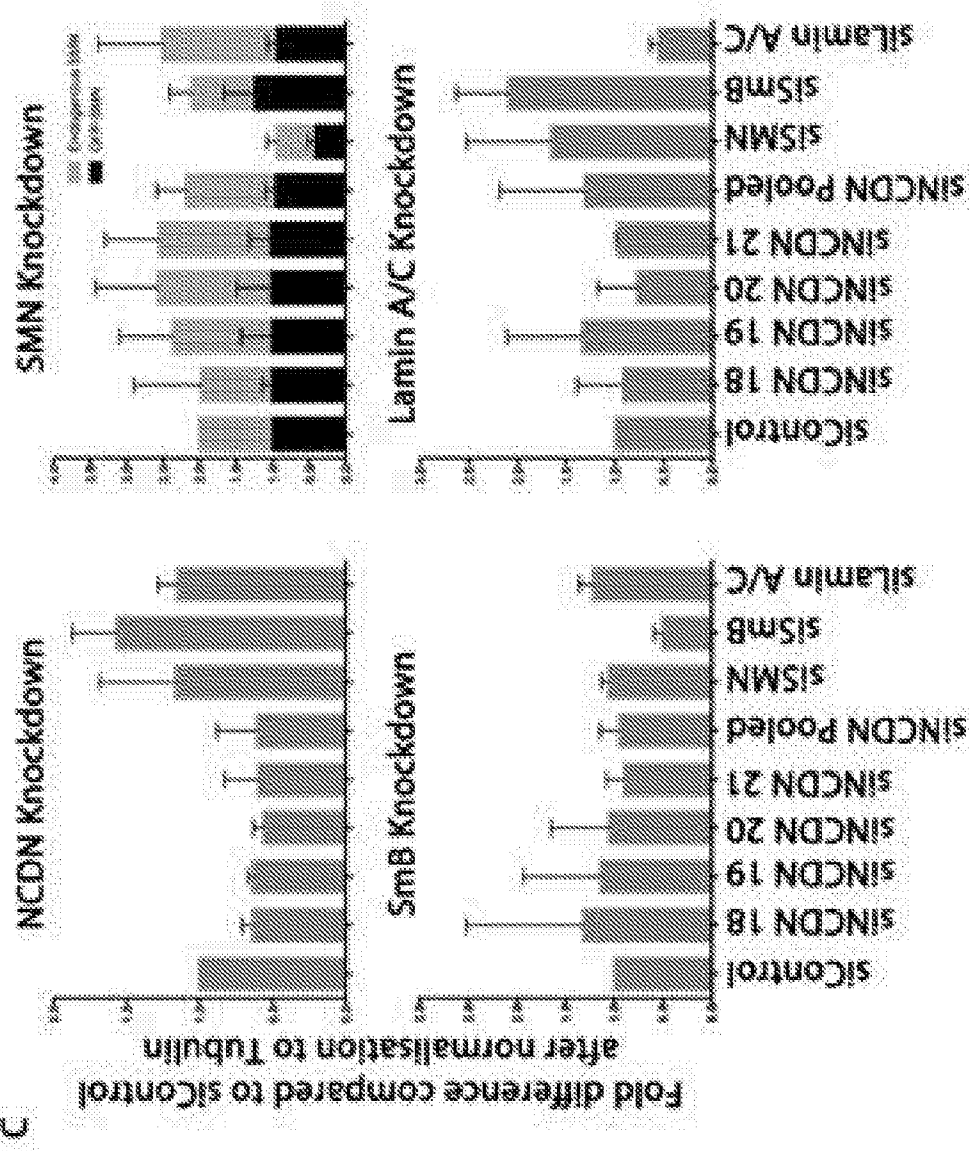


Figure 5C

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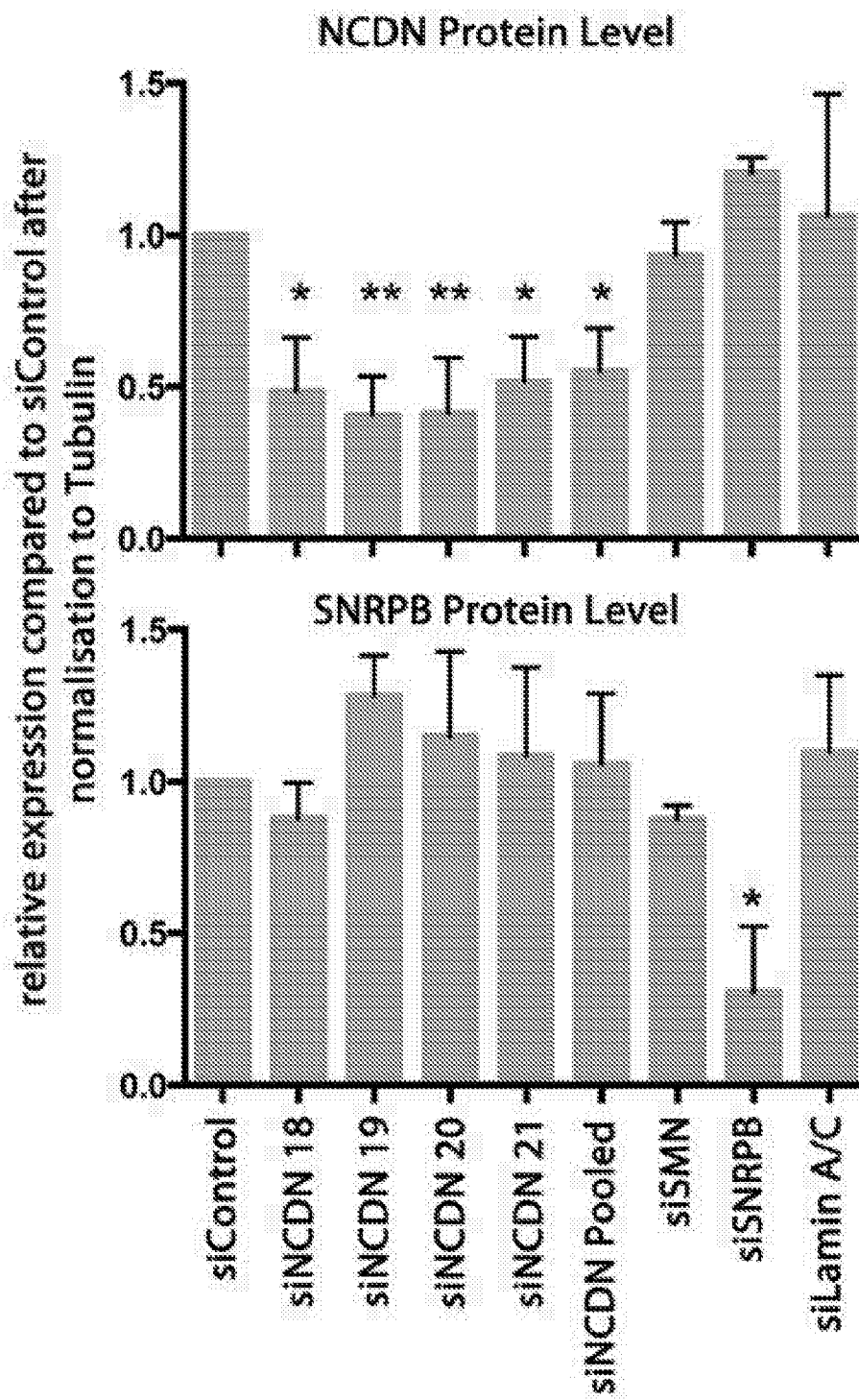


Figure 5D

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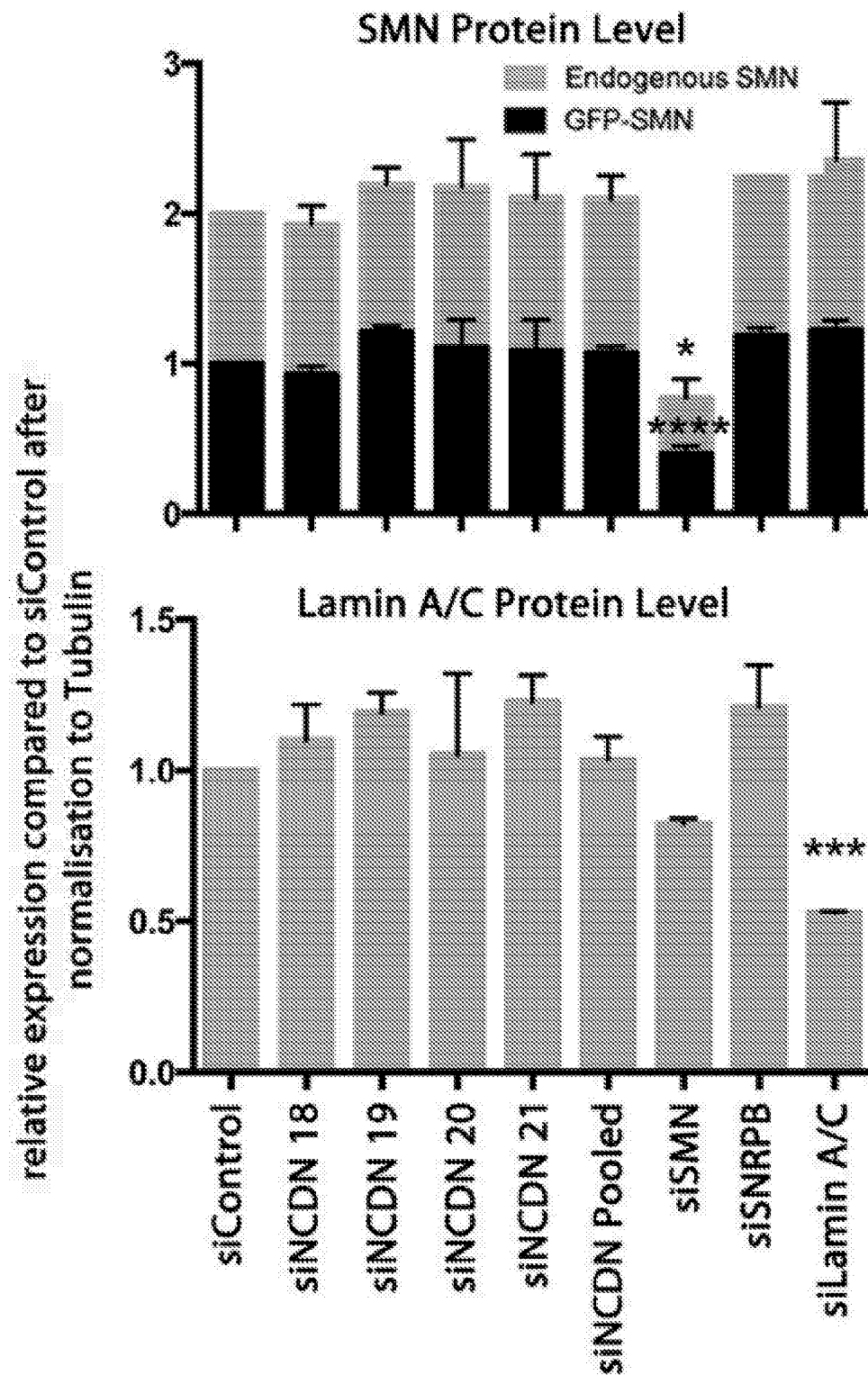


Figure 5D continued

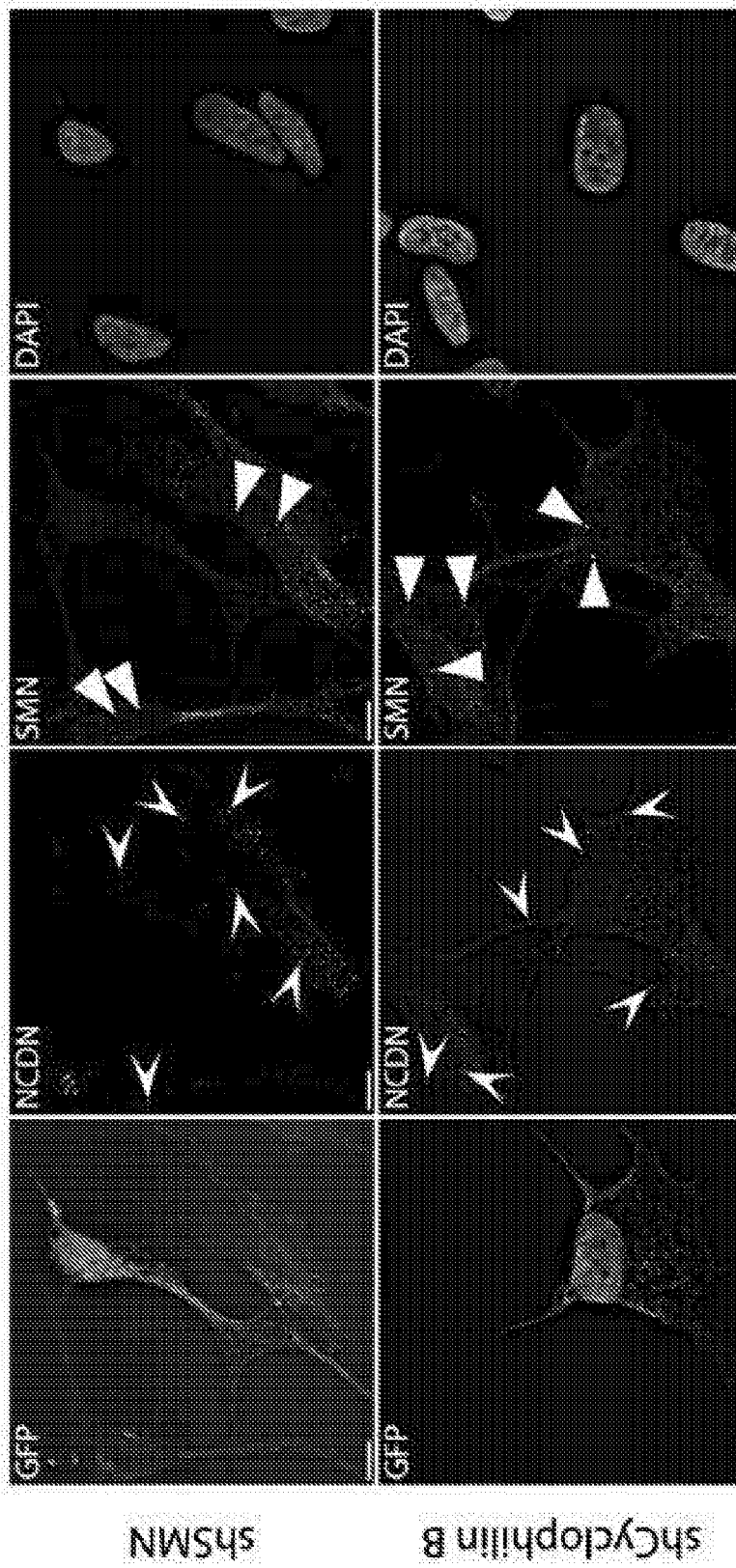


Figure 6A

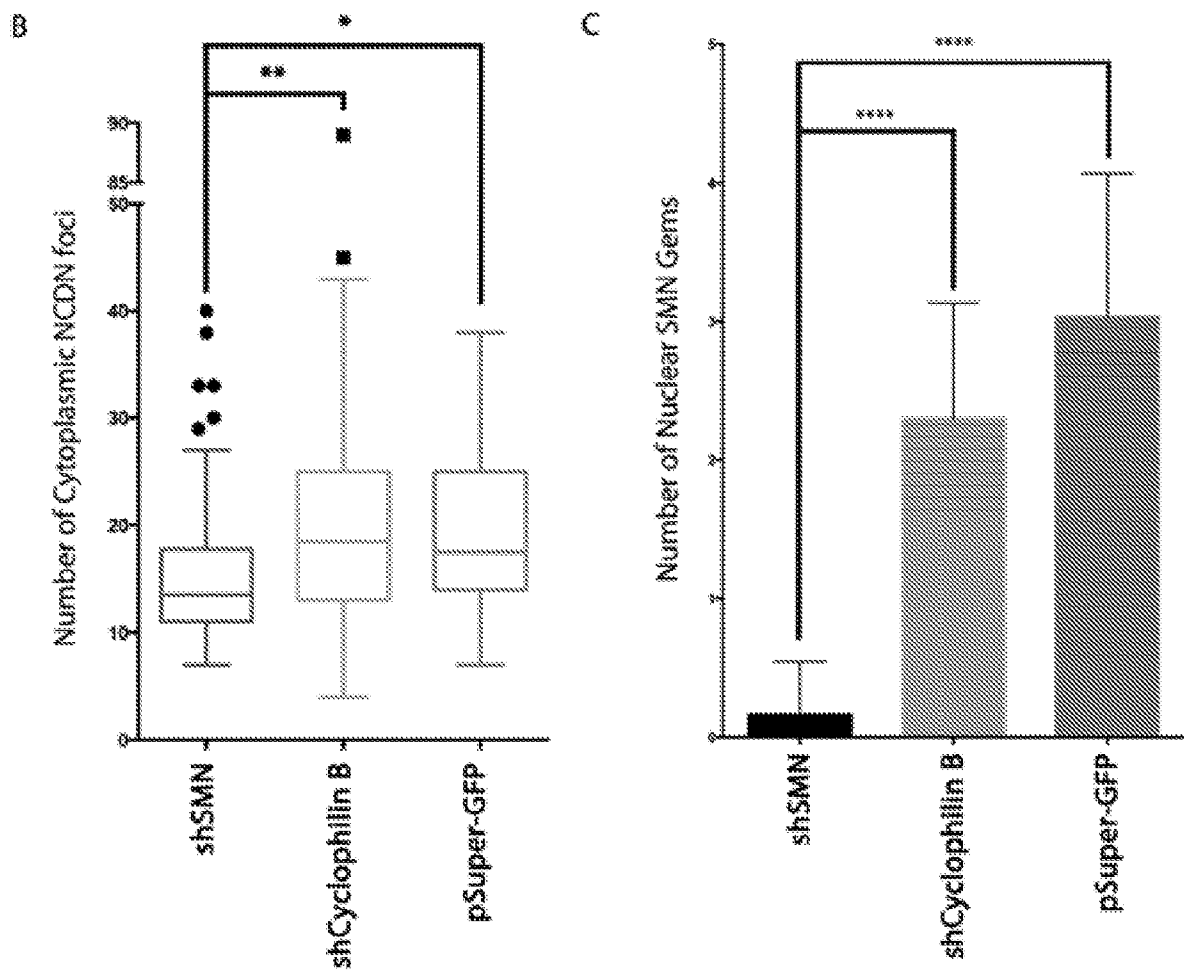


Figure 6 B & C

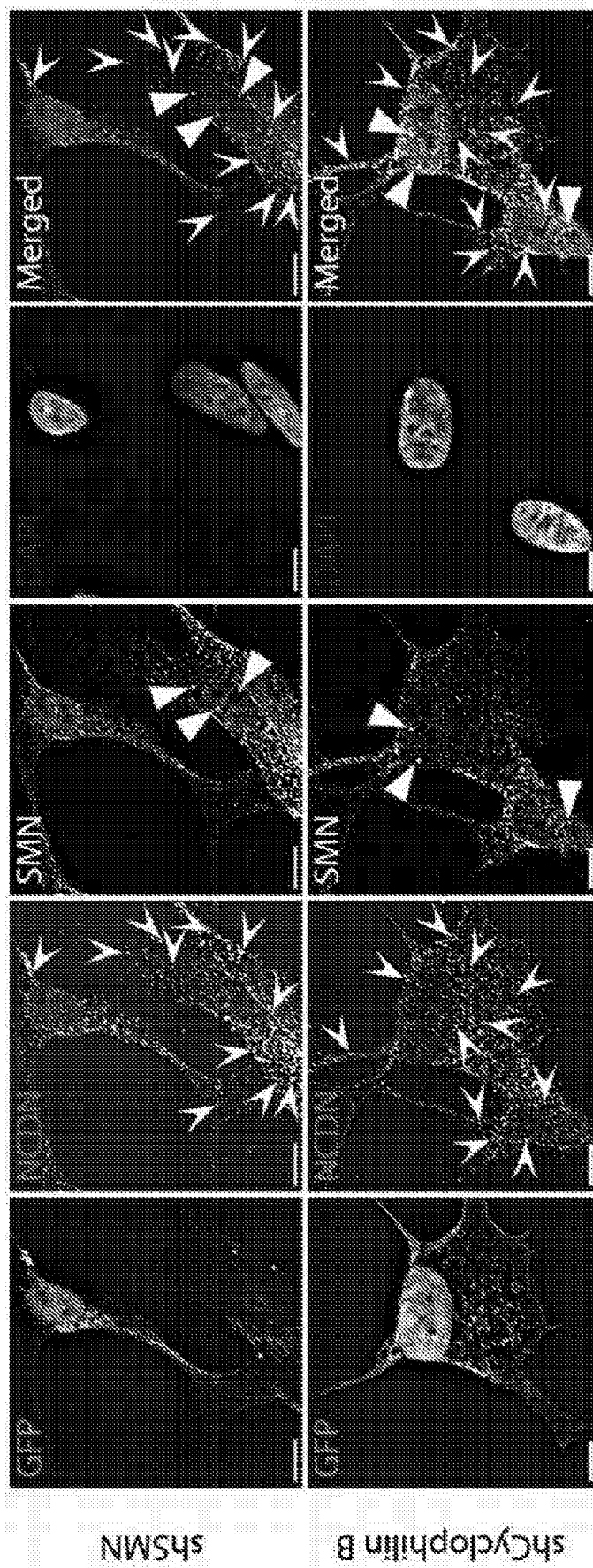


Figure 6 D

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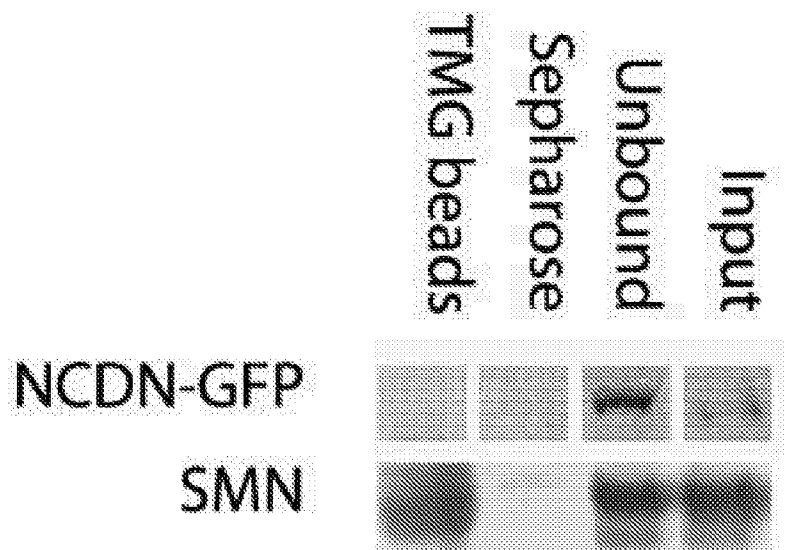


Figure 7

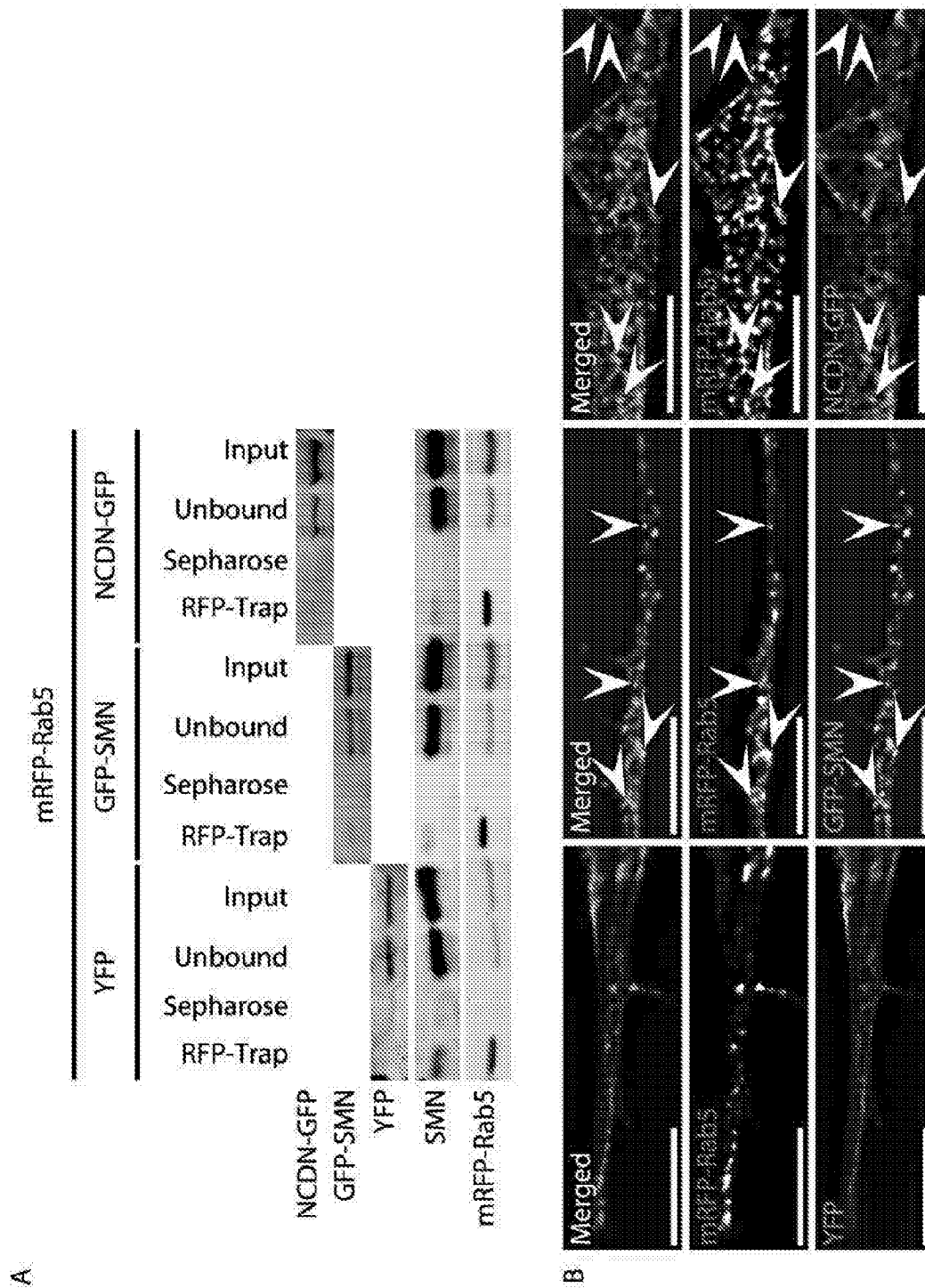


Figure 8

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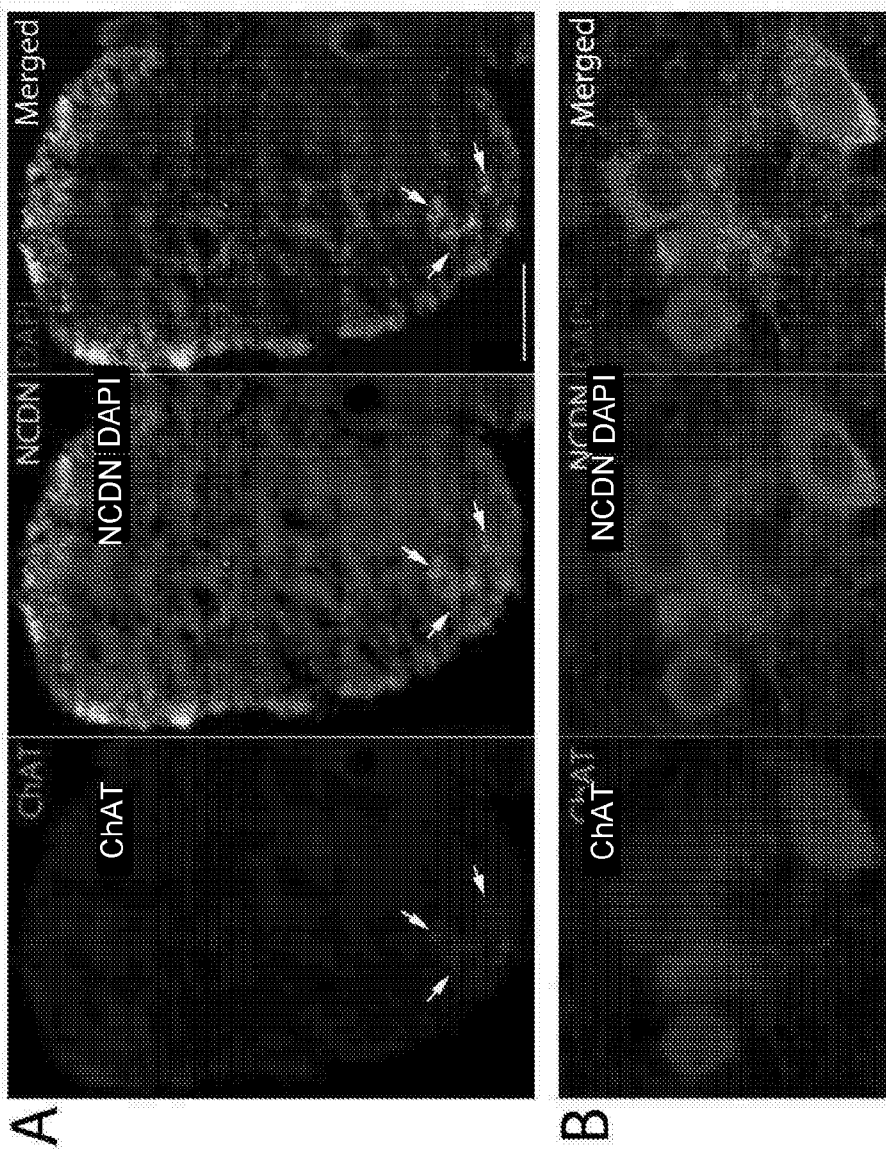


Figure 9

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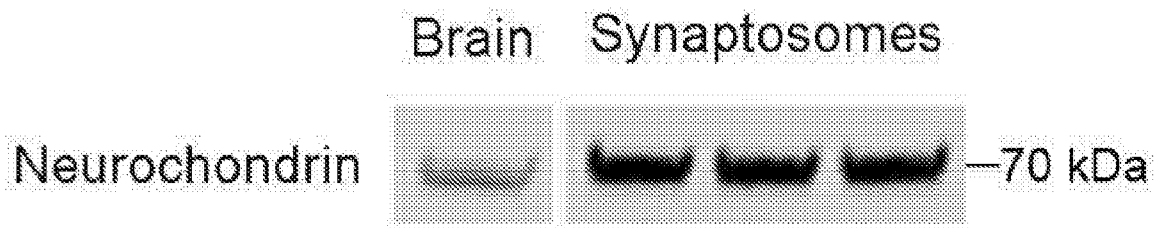


Figure 10

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2018/051842

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K38/17 G01N33/00 A61P25/16 A61P25/28
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K G01N A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 3 086 120 A1 (EUROIMMUN MEDIZINISCHE LABORDIAGNOSTIKA AG [DE]) 26 October 2016 (2016-10-26)	1,2,4-7
Y	abstract paragraphs [0017], [0035], [0050], [0052]	1-8
X	----- WO 2004/007674 A2 (UNIV JOHNS HOPKINS [US]; ZACK DONALD J [US]; HACKAM ABIGAIL SHOSHANNA) 22 January 2004 (2004-01-22)	1,4
Y	abstract claims 1,3-7	1-8
X	----- WO 2008/148489 A1 (HOFFMANN LA ROCHE [CH]; BERNDT PETER [CH]; BOHRMANN BERND [CH]) 11 December 2008 (2008-12-11)	8
Y	abstract page 9	1-8



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

3 September 2018

Date of mailing of the international search report

25/09/2018

Name and mailing address of the ISA/

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Weisser, Dagmar

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB2018/051842

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2018/051842

Patent document cited in search report		Publication date		Patent family member(s)		Publication date
EP 3086120	A1	26-10-2016	CN	106065028 A		02-11-2016
			EP	3086120 A1		26-10-2016
			US	2016311876 A1		27-10-2016

WO 2004007674	A2	22-01-2004	AU	2003272204 A1		02-02-2004
			US	2004180048 A1		16-09-2004
			WO	2004007674 A2		22-01-2004

WO 2008148489	A1	11-12-2008	NONE			
