Cysteines in the Neuropilin-2 MAM Domain Modulate Receptor Homooligomerization and Signal Transduction

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ABSTRACT:

Neuropilins (NRPs) are transmembrane receptors involved in angiogenesis, lymphangiogenesis, and neuronal development as well as in cancer metastasis. Previous studies suggest that NRPs exist in heteromeric complexes with vascular endothelial growth factors (VEGFs) and VEGF receptors as well as plexins and semaphorins. We determined via site-directed mutagenesis and bioluminescent resonance energy transfer assays that a conserved cysteine (C711) in the Danio rerio NRP2a MAM (meprin, A-5 protein, and protein tyrosine phosphatase μ) domain modulates NRP2a homomeric interactions. Mutation of this residue also disrupts semaphorin-3F binding in NRP2a-transfected COS-7 cells and prevents the NRP2a overexpression effects in a zebrafish vascular model. Collectively, our results indicate the MAM domain plays an important role in defining the NRP2 homodimer structure, which is important for semaphorin-dependent signal transduction via NRP2. © *2015 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 104: 371–378, 2015.*

Keywords: membrane proteins; neuropilin; receptor dimerization; signal transduction

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INTRODUCTION

Neuropilins (NRPs) are type I transmembrane (TM) receptors that form heterodimeric complexes with two key classes of signaling TM receptors: plexins and vascular endothelial growth factor receptors (VEGFRs).[1] There are two main NRP receptors (NRP1 and NRP2), with multiple extracellular and TM isoforms observed for each *in vivo*.[2,3] NRPs are comprised of two extracellular CUB (complement protein C1r/C1s, Uegf, and Bmp1) domains, two coagulation factor V/VIII (FA V/VIII) domains, one MAM (meprin, A-5 protein, and protein tyrosine phosphatase μ, PTPμ) domain, a single-spanning TM region, and a short cytosolic tail (Figure 1).[1,3–10] The short cytosolic domain (CYTO) for NRPs is in contrast to VEGFRs, which contain a cytosolic tyrosine kinase signaling domain, and plexins, which contain a cytosolic guanine nucleotide exchange factor domain.[1] Therefore, NRPs are thought primarily to modulate the affinity and specificity of

Additional Supporting Information may be found in the online version of this article.

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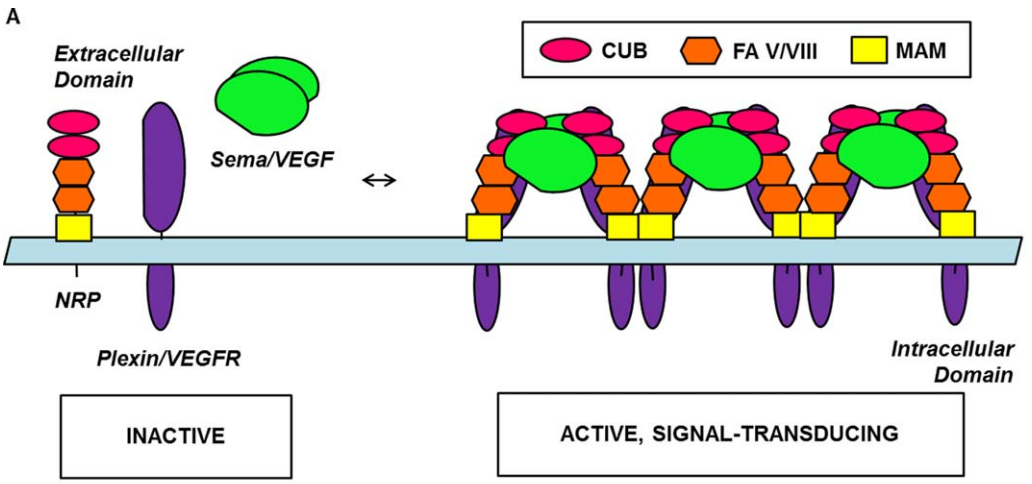

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FQFPEWGTAETPSEPPVTHVIDKDNSWLYSLDPILLTIIVMSSLGVLLGAVCAGLLLYCTCSYGGLTSRS
STTLENYNFELYDGIKHKVKLNQQRCCSEA*

FIGURE 1 Neuropilin 2a (NRP2a) is a transmembrane receptor. (A) NRP2a consists of two complement protein C1r/C1s, Uegf, and Bmp1 domains, two factor V/VIII domains, a meprin, A-5 protein, and protein tyrosine phosphatase μ (MAM) domain, a single-spanning transmembrane region, and a short cytosolic tail. NRP homooligomerization may play a role in NRP-plexin-Sema and NRP- vascular endothelial growth factor receptor (VEGFR)-VEGF signal transduction by promoting aggregation. (B) Primary sequence of the *Danio rerio* NRP2a transmembrane and juxtamembrane domains. The MAM domain (blue, bold) contains four conserved cysteines that impact homooligomerization ofMAM domains in other proteins. Red, bold, italicized font indicates the transmembrane domain and green font indicates the complete cytosolic domain, as determined using the eukaryotic linear motif (ELM) database.

extracellular ligand binding upon co-receptor complex formation.[11] In many instances, the co-receptor complex also includes additional cell adhesion molecules (CAMs) such as L1CAM and NrCAM as well as β1 integrins;[7,12,13] thus, a functional co-receptor complex may involve three or more receptors at the cell surface. Plexin-NRP co-receptor complexes bind semaphorins (Semas), which are a large class of extracellular, dimeric ligands (20 in total) that act as either attractive or repulsive cues during cell migration in a diverse array of processes including axon guidance, vascular patterning, and bone formation.[14–17] Additionally, VEGFR-NRP co-receptor complexes bind vascular endothelial growth factors (VEGFs), which are an equally large family of pro-angiogenic extracellular ligands.[18] Therefore, NRPs act as key regulators of extracellular signaling through imparting specificity in ligand-co-receptor complex formation.

Given the diversity of biological processes in which Sema and VEGF modulate cell migration, dysregulation of NRP-dependent signaling has been linked to a variety of cancers.[6] In particular, Sema3F has been shown to exhibit strong anti-angiogenic activity through binding to NRP2, with forced overexpression of Sema3F in a mouse melanoma model inhibiting tumor angiogenesis.[19] NRP2 activation in response to VEFG-C binding is also linked to enhanced autophagy in cancer cells through an mTOR-dependent pathway, allowing them to survive after chemotherapeutic treatment.[20] Additional studies have demonstrated the up-regulation of numerous other signaling pathways known to positively influence tumor metastasis in response to NRP2 activation, including chemokine receptor CXCR4 in breast cancers and insulin-like growth factor-1 receptor in high-grade, PTEN-null prostate cancer.[21,22] In general, it has been observed that NRP1 and NRP2 are both overexpressed in multiple cancer types, and in the case of NRP1, positively correlate with tumor progression.[4,6,23] Likewise, therapeutics targeted to NRP1 and NRP2 that block signal transduction have shown anti-metastatic potential in multiple cancer types, suggesting NRPs may serve as effective biomarkers that specifically target metastatic tumors.[5,24,25] Thus, NRPs act as key regulators of cell migration, and

dysregulation of NRP-dependent signaling can lead to enhanced pro-angiogenic tumor growth as well as cancer cell survival post-treatment.

In the case of NRP1-PlexinA1 signaling, it has been observed that the receptors cluster upon Sema3A addition and subsequent signal transduction.[26] Forced homodimerization of the PlexinA1, A2, A4, and C1 cytosolic domains also enhances receptor GTPase-activating protein (GAP) activity in an *in vitro* RapGAP assay.[27] Collectively, these results suggest plexin homomeric interactions are important for NRP-plexin-Sema signal transduction (Figure 1A). A low-resolution crystal structure of the CUB and FA V/VIII domains of NRP1 with the four N-terminal PlexinA2 domains bound to Sema3A reveals a 2:2:2 NRP1:PlexinA2:Sema3A stoichiometry, with the CUB domains of NRP1 acting as a linker to stabilize the Sema3A-PlexinA2 complex.[28] No major structural rearrangements in NRP1 or PlexinA2 are observed between the Sema3A-liganded and unliganded states, and both the NRP1 and PlexinA2 extracellular fragments in the crystal structure are monomeric in solution.[28] Similarly, plexin cytosolic domains appear predominately monomeric in solution with only weak homomeric interactions observed,[27,29] implying plexin dimerization and clustering is influenced through the TM domain and proximal regions and/or the NRP co-receptor.

The full-length NRP1 receptor appears dimeric when analyzed via western blots and co-immunoprecipitation (co-IP) experiments, as does the full-length NRP2 receptor.[8,30–32] Studies on the TM domain of NRP1 indicate that mutations to TM glycines contributing to a conserved 'G-x_3-G' motif disrupt dimerization of purified NRP1 TM domain peptides, implying a role for the TM domain in NRP1 homodimeric interactions.[33] Domain-binding and -deletion studies also suggest homomeric interactions may be facilitated by the NRP juxtamembrane MAM domain.[8,30,31] The involvement of the MAM domain in NRP homomeric interactions is further supported by the inability of MAM-deletion constructs to co-IP with the full-length receptor and the ability of an alkaline phosphatase (AP)-tagged NRP2 MAM domain to bind COS cells upon expression of the full-length NRP1 or NRP2 receptors.[8,30] Thus, while the NRP transmembrane domain exhibits a G-x_3-G motif, a driving force for NRP2 MAM homomeric interactions and their role in receptor clustering and activation remains unresolved.

MAM domains are also present in meprins and protein tyrosine phosphatase (PTP) subclass IIB proteins. This domain in both meprin α and PTPμ has been shown to influence homomeric interactions.[34,35] The MAM domains of neuropilins, PTPμ, and meprin α all contain four conserved cysteines, with the MAM domain of meprin α containing one additional cysteine.[34,35] Mutation of this fifth cysteine in a secreted truncated meprin α protein or addition of reducing agent to this same protein results in monomeric meprin α in SDS-PAGE.[34] While the PTPμ MAM domain cross-linked in solution runs as a dimer on SDS-PAGE, addition of reducing agent to the cross-linking reaction results in monomeric PTPμ.[35] Collectively, these results suggest cysteine chemistry influences MAM domain interactions in meprin α and PTPμ.

To determine if cysteine chemistry in the MAM domain also influences homooligomerization of NRP2, we used a series of biochemical and genetic tools to identify the ability of cysteine-mutant constructs to self-interact. Our results indicate cysteines in the *Danio rerio* NRP2a MAM domain, in particular a conserved cysteine (C711), play a significant role in homooligomerization and function. Notably, we predict that a C711-dependent disulfide bond dictates proper formation for the activated, clustered NRP2 (Figure 1A). Mutations to select MAM domain cysteines in the full-length receptor in a bioluminescence resonance energy transfer ($BRET^2$) assay enhance dimerization. When expressed in transiently-transfected COS-7 cells, the NRP2a mutation C711S reduces Sema3F binding, signifying a role for this residue in dictating interactions necessary for the activated, clustered state. Furthermore, while injection of wild-type (WT) NRP2a RNA into zebrafish embryos resulted in branched intersegmental vessels (ISVs), injection of C711S mutant RNA resulted in significantly fewer embryos with this NRP2a overexpression phenotype. Collectively, these results indicate cysteine chemistry in the NRP2a MAM domain contributes to the protein's mechanisms for homooligomerization and provides insight into the structural organization of NRP co-receptor complexes important for signal transduction (Figure 1A).

MATERIALS AND METHODS

Plasmids

Zebrafish are known to express two NRP2 isoforms (a and b).[36] Full-length WT *Danio rerio* NRP2a (NCB Accession # BC162118.1, Thermo Scientific) was generated by PCR for cloning into pcDNA3.1/V5-His-TOPO (Invitrogen) as per manufacturer's instructions with a C-terminal FLAG-tag, $pGFP^2$-N3 (BioSignal Packard) as a NheI/HindIII insert, and pRLuc-N1 (BioSignal Packard) at XhoI/HindIII. Mutations were made using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) as per manufacturer's instructions.

To generate hook-Sema3F, the hook moiety complete with an N-terminal HA-tag and two C-terminal myc-tags was obtained by PCR from the pHook-2 plasmid (Invitrogen; residues 2096–3066) provided by Paul Billings (University of Pennsylvania). This construct was subsequently cloned into pcDNA3.1/V5-His-TOPO at HindIII/KpnI with a 5' Kozak sequence and a C-terminal poly-glycine linker. Human Sema3F without a signal sequence (residues 266–2467 of NCB Accession # XM_005265382.2) was then cloned into this plasmid at KpnI/XbaI. The plasmid coding for AP-Sema3F, used as a PCR template to make this construct, was provided by Dr. Roman J. Giger (University of Michigan).

Mammalian Cell Culture

COS-7 cells (ATCC) were maintained as recommended by ATCC, except media was supplemented with 1% (v/v) 100× Antibiotic/Antimycotic solution (100 U/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B; Hyclone) and subcultivation ratios ranged from 1:10 to 1:20. Transfections occurred via electroporation with a Bio-Rad Gene Pulser XCell using pre-set COS-7 parameters.

BRET^2 Assay

COS-7 cells were co-transfected via electroporation with a pGFP^2 and a pRLuc construct containing a fusion to full-length NRP2a. Each transfection was split across eight wells in a white, flat-bottomed 96-well plate. Each well represented one replicate, and each transfection represented one round for the specified condition. Cultures were allowed to grow for two days, and measurements on adherent cells were taken as previously described using a Tecan Infinite F200 multiwell plate reader.[37,38] Following measurements, levels of BRET^2 protein expression were confirmed consistent between mutants via western blots (1:1000 dilutions of EGFP mouse monoclonal antibody, Clontech; mouse monoclonal anti-tubulin, Abcam; anti-mouse IgG HRP-linked antibody, Cell Signaling; or 1:2500 dilution of MSX Renilla Luciferase, Millipore) (Supporting Information Figure S1).

For analysis, we first considered the total luminescence of individual wells for each round. Cultures with total luminescence values lower than that of the average ± one SD luminescence of mock-transfected cells were eliminated from subsequent analyses. The ratio of green luminescence to magenta luminescence was computed for each sample, then divided by the average ratio of green luminescence measurements to magenta luminescence measurements for the WT condition for that round of experiments. Results represent the average percent difference from WT and standard error of at least 24 independent replicates, with the standard error of WT samples added to the standard error of the mutants.

NRP2a Overexpression in Zebrafish

Capped NRP2a mRNA was made using the mMESSAGE mMACHINE T7 Transcription Kit (Ambion) as per manufacturer's instructions, with template NRP2a DNA generated from the pcDNA3.1/V5-His-TOPO constructs linearized with XhoI.

Embryos from adult fli1-GFP intercrosses were injected with either NRP2a RNA at 1 μg/μL or miR-2188 morpholino (miR-2188-MO, Gene Tools) at 0.5 mM while in the single-cell stage. At 48 hours post fertilization, chorions were popped and embryos were fixed with 4% paraformaldehyde for 2 hours at room temperature. Embryos were subsequently washed 3× with PBS and imaged at 20× magnification. Embryos were evaluated for ISV branching previously identified as a NRP2a overexpression phenotype.[39]

Semaphorin Binding

Cells were transfected via electroporation with WT or mutant NRP2a in pcDNA3.1/V5-His-TOPO. Two days after transfections, all media was removed from the plate and replaced with 50-fold concentrated media from hook-Sema3F transfected cultures, and incubated 1.5–2 hours at 37°C in 5% CO_2. Treated cells were rinsed three times in PBS, then fixed for 30 minutes at room temperature with 4% paraformaldehyde in PBS. Cells were rinsed once in PBS, blocked for one hour in PBS + 1% (w/v) BSA + 0.5% (v/v) TritonX-100 at room temperature, and incubated in mouse monoclonal anti-myc antibody (Cell Signaling) at 1:2000 in block solution over two nights. Secondary antibody incubation (anti-mouse IgG AlexaFluor 546 or 488) took place over the course of one hour at room temperature. For analysis, twenty fields of confluent cells per condition were then imaged at 20× magnification with consistent exposure and gain per round; subsequently, an observer counted the total number of fluorescent cells per condition.

RESULTS

Specific Cysteines in the MAM Domain Influence NRP2a Homooligomerization

Previous research suggests the NRP2a MAM domain influences homooligomerization, similar to MAM domains in meprins and protein tyrosine phosphatases.[8,30] In particular, four conserved cysteines in the MAM domains of meprin α and PTPμ have been shown to play an important role in stabilizing the homodimer through disulfide bond formation.[34,35,40] As these cysteines are also conserved in the NRP2a MAM domain (Figure 1B), we hypothesized disulfide bond formation via these four conserved cysteines may also influence NRP2a MAM oligomerization. Comparison of the *Danio rerio* NRP2a MAM domain with the PTPμ MAM domain suggested C636–C643 and C711–C794 are likely to form disulfide bonds (analogous to disulfide bonds C27–C36 and C96–C182 in the PTPμ MAM domain).[41] Therefore, we mutated one or both cysteines in each of the corresponding putative disulfide bonds in the NRP2a receptor and analyzed the effects using the BRET^2 assay.[37,38] Mutations were introduced into the full-length NRP2a receptor for both the C-terminal GFP^2-fused and *Renilla luciferase* (RLuc)-fused proteins. The BRET^2 assay relies upon distance between the GFP^2-fused and the RLuc-fused proteins; upon addition of the Deep Blue C substrate, the RLuc tag catalyzes a reaction that causes light to be emitted at 395 nm. This light then excites nearby GFP^2 tags, causing light to be emitted at 510 nm. Results are characterized by the ratio of intensity of light produced by GFP^2 (green fluorescence at 510 nm) to the luminescence signal generated by RLuc (magenta luminescence at 395 nm).[37,38]

As shown in Figure 2, the MAM mutants C643S, C711S, and C711S + C794S show significant differences from WT as an enhancement in energy transfer. Therefore, the BRET^2 results indicate mutants C643S, C711S, and C711S + C794S enhance oligomerization of full-length NRP2a. Interestingly, the double mutant C636S + C643S did not exhibit significant changes in energy transfer efficiency from the WT receptor, suggesting specific cysteines or combinations of cysteines in the MAM domain influence NRP2a homooligomerization. Collectively, the results imply specific cysteines in the NRP2a MAM domain influence receptor homomeric interactions and subsequent clustering and activation (Figure 1).

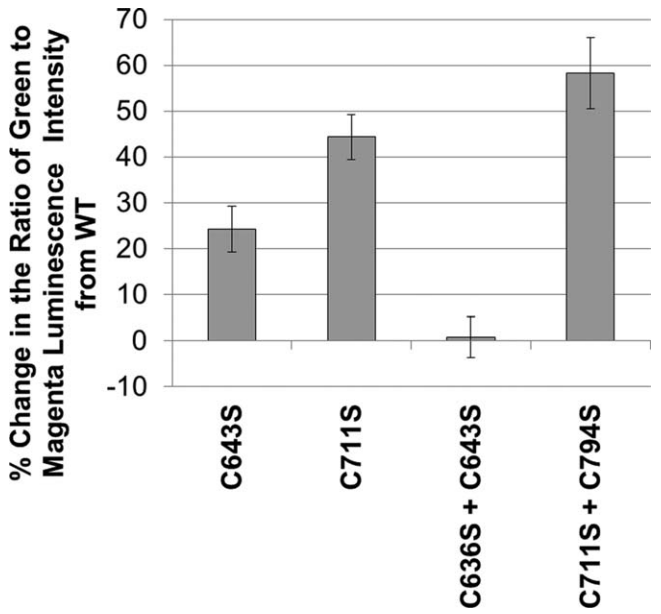

FIGURE 2 Cysteines in the NRP2a MAM domain influence receptor homooligomerization in a mammalian membrane, as determined by bioluminescent resonance energy transfer. Results were collected from at least three separate transfections of each condition and error bars represent standard error.

Mutations to the NRP2a MAM Domain Influence Overexpression Phenotypes in Zebrafish Vascular Patterning

Previous studies demonstrate that knockdown of a microRNA that suppresses NRP2a expression (miR-2188), hence inducing NRP2a overexpression, results in irregular ISV branches in zebrafish embryos. This phenotype is believed to manifest itself through the NRP2a-VEGF pathway.[39] One model suggests NRP2a influences VEGF signaling through NRP-VEGF-VEGFR clustering.[42] To determine if our cysteine mutations affect signaling in a NRP2a-VEGF pathway, we injected miR-2188-MO or NRP2a mRNA into zebrafish embryos and examined them for the NRP2a overexpression phenotype (Figure 3 and Table I).

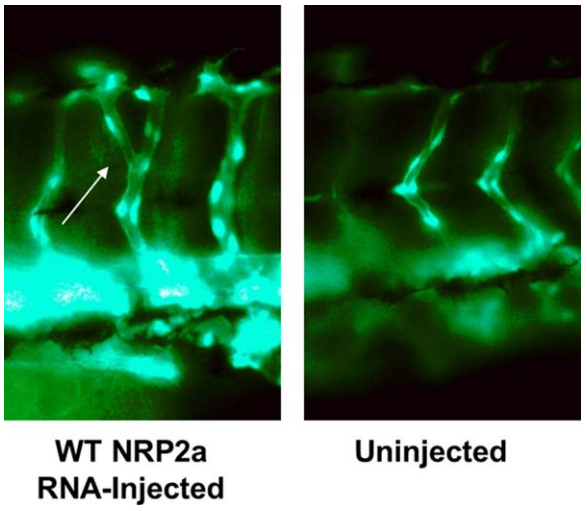

FIGURE 3 Wild-type (WT) NRP2a overexpression causes ISV branching in 48 hours post fertilization zebrafish embryos (arrow). This phenomenon is observed less frequently in uninjected embryos and embryos injected with dysfunctional mutant NRP2a.

Of the embryos examined, 16% of those injected with miR-2188-MO and 18% of those injected with WT NRP2a mRNA exhibited branches characteristic of the NRP2a overexpression phenotype. This was significantly more than the 2.5% of uninjected embryos exhibiting ISV branches ($P < 0.0005$, as determined via an unpaired two-tailed student *t*-test assuming equal variance). Embryos injected with cysteine-mutant NRP2a mRNA (C643S, C711S, and C711S + C794S) showed significant increases in NRP2a overexpression compared to the uninjected embryos, as well ($P < 0.05$ for C643S and C711S and $P < 0.0005$ for C711S + C794S). However, compared to the embryos injected with WT NRP2a mRNA, fewer embryos injected with C711S NRP2a mRNA exhibited the overexpression phenotype ($P < 0.05$). This suggests mutation C711S fails to elicit the same functionality as WT NRP2a, possibly due to alterations to receptor oligomeric state and/or tertiary structure as suggested by our BRET^2 results (Figure 2).

Mutations to the NRP2a MAM Domain Influence Sema3F Binding

Previous work suggests the NRP1 MAM domain promotes receptor *cis*- or *trans*-aggregation, and that these aggregates (of a higher-order than dimer) may be required for activation or interactions with a co-receptor.[30] While crystal structures suggest a 2:2:2 NRP1:PlexinA2:Sema3A stoichiometry,[28] it is possible that activation of the full-length receptors requires a higher order oligomer than dimer or promotes binding of the semaphorin ligand (Figure 1A).[30] Previous results with NRP2 have shown that deletion of the MAM domain reduces receptor affinity for the Sema3F ligand, as determined by reduction in absorbance measurements of COS-7 cells transfected with NRP2 and subsequently treated with an AP- Sema3F.[30] To determine a possible functional role of the MAM cysteines in semaphorin binding, we examined Sema3F binding to COS-7 cells transfected with either WT or mutant *Danio rerio* NRP2a. Sema3F acts as a negative regulator of growth cone formation guided by NRP2-PlexinA3, and thus binding of Sema3F is necessary for growth cone collapse.[29]

Expression of full-length FLAG-tagged WT NRP2a or mutant C711S + C794S resulted in significant binding of Sema3F compared to mock-transfected cells, as determined by

Table I Percentage of Fli1-GFP Embryos Exhibiting ISV Branching

Type of Injection	Number of Embryos Examined	Percentage of Embryos Exhibiting NRP2 Overexpression Phenotype	*P*-Value Compared to Uninjected	*P*-Value Compared to WT NRP2a RNA-Injected
Uninjected	204	2.5	1	6.8×10^{-6}
WT NRP2a RNA	62	18	6.8×10^{-6}	1
C643S NRP2a RNA	54	9.3	0.021	0.19
C711S NRP2a RNA	141	7.8	0.020	0.034
C711S + C794S NRP2a RNA	81	17	4.4×10^{-6}	0.94
miR-2188-MO	43	16	1.1×10^{-6}	0.29

P-values were determined using an unpaired equal variance two-tailed student *t*-test.

fluorescence microscopy (Figures 4A and 4B). Significantly fewer Sema3F-bound cells were observed in cultures transfected with NRP2a mutant C711S for the same exposure times, indicating a reduction in Sema3F-binding in cells transfected with this mutant. The reduction in Sema3F-binding is likely due to altered oligomeric states or conformational changes in the protein rather than expression levels, as western blots on NRP2a $BRET^2$ constructs exhibit similar expression levels. Our $BRET^2$ (Figure 2) results point to C711 as influential to NRP2a homomeric interactions. A model in which the oligomeric state of NRP2a regulates Sema3F-binding, and C711 regulates NRP2a oligomerization, is consistent with these results (Figure 1).

DISCUSSION

Previous studies have shown NRPs exhibit an intrinsic ability to dimerize and aggregate.[8,30–32] Here, we identify a conserved cysteine in the MAM domain of NRP2a (C711) that governs homooligomerization when mutated in the context of the full-length receptor in a mammalian cell membrane (Figure 2). This mutation also disrupts Sema3F-binding to the full-length receptor (Figure 4) and causes less of the NRP2a overexpression phenotype in zebrafish embryo vasculature compared to the WT receptor (Table I). We also identified mutations to conserved cysteines (C711S + C794S) that affect receptor homomeric interactions, as determined by $BRET^2$ (Figure 2), but do not disrupt Sema3F-binding (Figure 4) or a reduced NRP2a overexpression phenotype in zebrafish vasculature

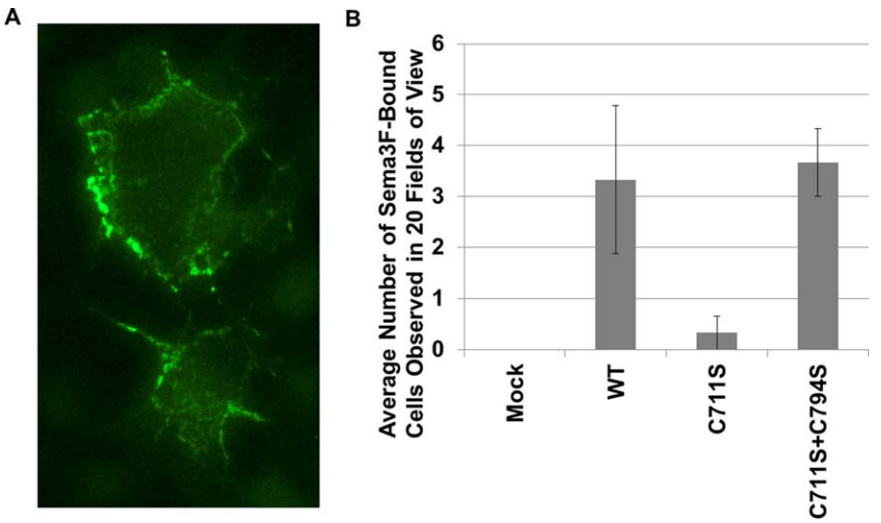

FIGURE 4 Mutant C711S reduces binding of Sema3F to the full-length NRP2a receptor. (A) Representative images of hook-Sema3F-bound cells. (B) Mutant C711S disrupts Sema3F-binding. Cells were transfected with water, WT, or mutant NRP2a constructs and treated with 50×-concentrated hook-Sema3F. Twenty random fields of view for each condition per round (three rounds total) were examined at an exposure time consistent within the round and the total number of Sema3F-bound cells were counted. Error bars indicate standard error.

(Table I). Our results collectively point to a model in which the MAM domain acts as a regulator of the equilibrium between oligomeric states (Figure 1) and possibly receptor extracellular domain tertiary structure, thereby controlling semaphorin binding and VEGFR clustering. Furthermore, conserved cysteines govern this equilibrium. Additional structural and functional studies will allow further elucidation of the functional significance of the cysteine mutations.

In particular, one of the principal observations from this work is that the C711S mutation, which mutates one of four conserved cysteines in the MAM domain, drives dimerization in the context of the full-length receptor (Figure 2), suggesting the disulfide bond involving C711 negatively regulates oligomerization. Consistent with previous studies on meprin α and PTPμ MAM domains in which disruption of disulfide bonds resulted in decreased function,[34,35,40] the NRP2a C711S mutant exhibits a reduction in Sema3F-binding capabilities as well as in ability to induce a NRP2a overexpression phenotype in zebrafish vasculature (Table I and Figure 4). In PTPμ, mutation of a conserved cysteine disrupted MAM domain interactions between receptors while maintaining the ability to induce cellular aggregation.[35] In meprin α, disrupting a conserved MAM domain cysteine resulted in decreased thermal stability of the enzyme, increased proteolytic degradation, and decreased activity towards protein substrates.[34] In NRP2a, we find disruption of the C711 disulfide bond triggers oligomerization of the full-length NRP2a receptor (Figure 2), which seemingly disrupts Sema3F binding (Figure 4) and reduces its ability to induce branching in zebrafish embryo vasculature (Table I). It is likely that the C711S mutation could disrupt the equilibrium between oligomeric states and thereby affect ligand binding (Figure 1). Alternatively, the mutation could introduce a conformational change in the monomeric NRP2a structure itself that not only leads to NRP2a oligomerization, but also disrupts ligand binding sites. Our studies do not distinguish between either of the possibilities, but do suggest that residue C711 is important for defining the oligomeric state of NRP2a and the receptor's ability to bind Sema3F and function in a VEGF-dependent system (Figure 1).

In summary, we have shown that residues in the NRP2a MAM domain regulate the equilibrium between NRP2a oligomeric conformations which lead to activated, clustered states (Figure 1). Cysteines, in particular C711, assist in regulating MAM dimerization, with intact disulfide bonds disrupting clustering of the full-length receptor (Figure 2). Regulation of NRP2a MAM domain cysteine chemistry ultimately affects Sema3F-binding, as observed with mutation of residue C711 (Figure 4), as well as its ability to act in a VEGF-dependent signaling cascade (Table I). Our results suggest that cysteine interactions in the NRP2a MAM domain regulate oligomeric state and may provide a target site for approaches to modulate NRP homomeric interactions.

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