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The proteolytic cleavage of SEMA3F may be mediated by non-furin proprotein convertases

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THE PROTEOLYTIC CLEAVAGE OF SEMA3F MAY BE MEDIATED BY NON-FURIN PROPROTEIN CONVERTASES

By

ERIK LI

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Approved by

First Reader _____

Gwynneth Offner, Ph.D.

Director, M.A. Medical Sciences Program

Associate Professor of Medicine

Second Reader _____

Diane Bielenberg, Ph.D.

Assistant Professor of Surgery, Harvard Medical School

Assistant Professor, Vascular Biology Program,

Boston Children's Hospital

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ABSTRACT

Class III Semaphorins (SEMA3) comprise a family of chemokines that have been implicated as negative regulators of axonal guidance, angiogenesis and tumor progression. It has been demonstrated previously that one SEMA3, SEMA3F, may have therapeutic potential in the treatment of cancer. When transfected with SEMA3F, the highly metastatic human melanoma cell line A375SM was found to exhibit a highly-encapsulated, avascular phenotype with limited metastasis. Members of SEMA3 are regulated on many levels, including proteolytic processing. SEMA3F, like other SEMA3, is expressed as a 100 kD proprotein that is seen to be processed *in vitro* and *in vivo* to 95 and 65 kD isoforms. This has been largely attributed to furin-like endoproteases on the basis of furin inhibition studies. However, currently available small chemical or peptide inhibitors against the family of subtilisin/kexin-type proprotein convertases (PCSK), to which furin belongs, do not have good selectivity between PCSKs. Cleavage of SEMA3 to 65 kD have been shown to have differing effects. SEMA3A loses its ability to repel sympathetic ganglia and SEMA3E reverses its phenotype from chemorepulsant to chemoattractant for developing vasculature following cleavage. In order to further develop therapeutic strategies based on

SEMA3F, it is therefore critical to better understand the proteolytic regulation of this molecule. In this study, it is shown that digest of purified SEMA3F with purified recombinant human furin does not result in proteolytic cleavage and suggested that the cleavage of SEMA3F to a 65 kD isoform may be mediated by other members of the PCSK family.

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LIST OF ABBREVIATIONS

3% milk ..... 3% Blotting-grade Blocker in Tris-buffered Saline

AMC 7-amino-4-methylcoumarin

BSA ..... Bovine Serum Albumin

ddH_2O ..... Double Distilled Water

DMEM ..... Dulbecco's Modified Eagle Medium

DMEM/F12 ..... Dulbecco's Modified Eagle Medium/Ham's F12 Medium

ECL ..... Enhanced Chemiluminescent Substrate

FBS ..... Fetal Bovine Serum

FPLC ..... Fast Protein Liquid Chromatography

GPS ..... Penicillin-Streptomycin-Glutamine

H6 ..... Furin Inhibitor II, hexa-D-arginine

HBSS ..... Hank's Balanced Salt Solution

HEK ..... Human Embryonic Kidney

MEM ..... Minimum Essential Medium

NRP ..... Neuropilin

PBS ..... Phosphate-buffered Saline

PCS ..... Putative Cleavage Site

PCSK ..... Subtilisin/Kexin-type Proprotein Convertase

PVDF ..... Polyvinylidene Fluoride

rhFurin ..... Recombinant Human Furin

RIPA ..... Radioimmunoprecipitation Assay Buffer

RVKR-CMK ..... Furin Inhibitor I, dec-RVKR-chloromethylketone

SDS-PAGE ..... Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SEMA3 ..... Class III Semaphorins

siNRP2 ..... siGENOME siRNA to NRP2

TBS ..... Tris-buffered Saline

TBST ..... Tris-buffered Saline with 0.1% Tween 20

VEGF ..... Vascular Endothelial Growth Factor

VEGFR ..... VEGF Receptor

INTRODUCTION

Class III Semaphorins (SEMA3) are a large class of secreted signaling molecules first described as chemorepulsive and inhibitory modulators of axonal guidance. An increasing number of semaphorins have been identified and they have been implicated not only in the nervous system but also in vascular and tumor biology.

Structurally, semaphorins are related by an N-terminal conserved ~500 amino acid *sema* domain. The SEMA3, which encompass the seven secreted members of the semaphorin family in vertebrate animals (SEMA3A-G), additionally share homology over C-terminally located immunoglobulin-like and basic domains ("Unified Nomenclature," 1999). SEMA3 mediate their effects by binding to a holoreceptor complex comprised of neuropilins (NRP) and plexins. NRP are able to recognize and bind the *sema* domain of SEMA3, but because their cytoplasmic domains are relatively short, it is believed that they have limited signal transduction ability. NRP therefore complex with plexins, which contain a cytoplasmic domain with GTPase-activating activity (Neufeld & Kessler, 2008). The signaling pathway of SEMA3F has been shown to involve Abelson 2 tyrosine kinase in the inactivation of the RhoA pathway, downstream activation of cofilin and depolymerization of f-actin (Figure 1, Shimizu et al., 2008). RhoA-mediated cytoskeletal reorganization and collapse has been implicated in the signaling pathways of many SEMA3 and is thought to contribute to their ability to inhibit angiogenesis, neurogenesis, cell migration, adhesion and survival (Bielenberg & Klagsbrun, 2007).

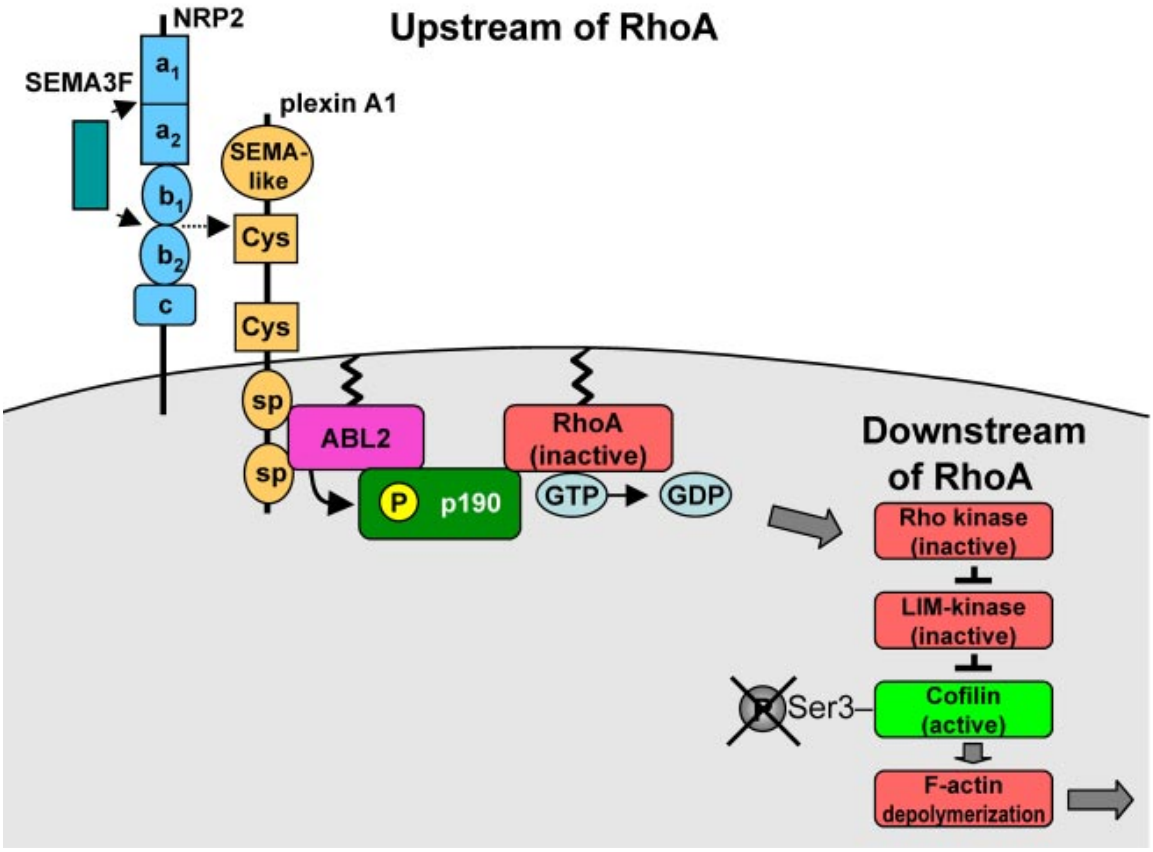

Figure 1: SEMA3F binding to neuropilin initiates an enzymatic cascade resulting in cytoskeletal reorganization. Binding of SEMA3F to NRP2 induces Plexin A1 to recruit Abelson 2 tyrosine kinase, which phosphorylates and activates p190 RhoGAP. This, in turn, leads to the deactivation cascade of RhoA, Rho kinase and LIM kinase. This activates cofilin and depolymerizes F-actin. Reproduced from Shimizu et al. **(2008)**.

NRP are also able to bind vascular endothelial growth factor (VEGF) and complex with VEGF receptors (VEGFR) to potentiate proangiogenic effects. NRP are structurally organized into a1/a2, b1/b2, c, transmembrane and short cytoplasmic domains. VEGF is thought to bind the NRP b1/b2 domain, whereas the *sema* domain of SEMA3 is thought to bind the a1/a2 domain. The C-terminal

end of SEMA3 may also compete with VEGF for binding the b1/b2 pocket. Functionally, SEMA3A has been shown to compete VEGF-induced endothelial cell motility (Miao et al., 1999). However, some structural studies have suggested that SEMA3 and VEGF may not directly compete for binding in the b1/b2 domain (Appleton et al., 2007).

The c domain of NRP is thought to have a role in dimerization, which is required for NRP functionality. Prematurely truncated transcripts of NRP are translated to soluble isoforms of NRP that contain only a1/a2 domains and b1/b2 domains (Bielenberg et al., 2006). These soluble NRP act to inhibit NRP function by competing with membrane-bound NRP for SEMA3 and VEGF.

The different isoforms of NRP, SEMA3 and plexin differ in tissue expression and binding specificity. During murine development NRP1 is predominantly found in the arterial vasculature and NRP2 in the venous and lymphatic vasculatures (Bielenberg & Klagsbrun, 2007). SEMA3A signals through NRP1 and SEMA3F/G signal through NRP2, while SEMA3B-D are able to signal through both. SEMA3E is an exception and binds directly through Plexin D1 (Figure 2). The size of the semaphorin and VEGF families as well as their differential affinities for NRP1 and NRP2 suggest that members of each family may have non-overlapping functionality, enabling fine-tuned control of vascular development and other SEMA3-mediated processes (Gaur et al., 2009).

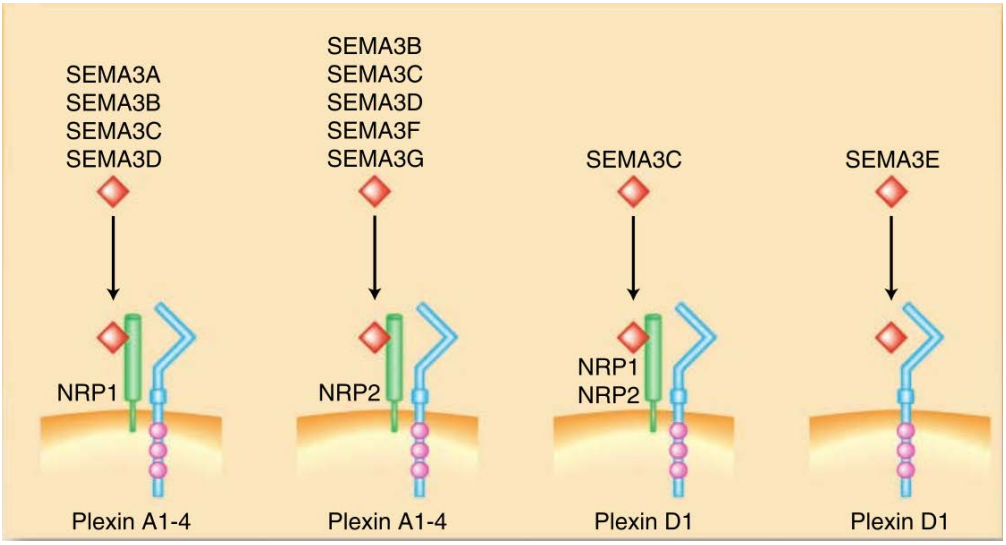

Figure 2: SEMA3, NRP and plexins show specificity in their interactions. Differences in ligand-binding affinity and tissue expression suggest that SEMA3, NRP and plexins each occupy specific biological niches. Reproduced from Gaur et al**. (2009)**.

In the tumor context, many researchers have posited the idea of an "angiogenic switch", which is a crucial step in tumor progression to provide sufficient nourishment for growth and a metastatic route (Varshavsky et al., 2008). The ability of NRP to bind both SEMA3 and VEGF suggests that it may play an important role in determining angiogenic balance. Many therapeutic approaches have therefore targeted NRP in order to prevent its proangiogenic effects or enhance its antiangiogenic effects. It has been shown previously that SEMA3F may have therapeutic potential in the treatment of cancer (Bielenberg et al., 2004). *SEMA3F* and *SEMA3B* genes are located in chromosomal region 3p21.3, which is commonly deleted in small cell lung cancer (Xiang et al., 2002). Progressive loss of SEMA3F expression also appears to be associated with tumor

progression and development of a metastatic phenotype. In our 2004 study, transfection of the highly metastatic human melanoma cell line A375SM with SEMA3F was shown to induce a highly encapsulated, avascular phenotype and almost completely prevented the formation of lung and lymph node metastasis when these cells were injected into nude mice (Bielenberg et al., 2004).

SEMA3 have also been shown to be regulated by proteolytic processing. They are expressed as 100 kD proproteins and subsequently processed to 95 and 65 kD isoforms. The functional consequence of these proteolytic processing events seems variable within the SEMA3 family. For SEMA3A, it has been shown that cleavage to the p65 form prevents dimerization by removing a downstream cysteine residue, resulting in the functional loss of repelling ability (Figure 3, Adams et al., 1997; Klostermann et al., 1998). SEMA3B is similarly inactivated by cleavage to the p65 form and wild-type SEMA3B demonstrates limited ability to retard the growth and metastasis of most cancers since it is particularly susceptible to cleavage in the tumor environment, which typically upregulate proteolytic pathways (Varshavsky et al., 2008). In contrast, p95 SEMA3E has a repulsive phenotype but has been shown to acquire a prometastatic phenotype upon cleavage to the p65 isoform (Christensen et al., 2005). Expanding our understanding of the role of proteolytic processing in the regulation of SEMA3F is therefore critical to the therapeutic application of SEMA3F.

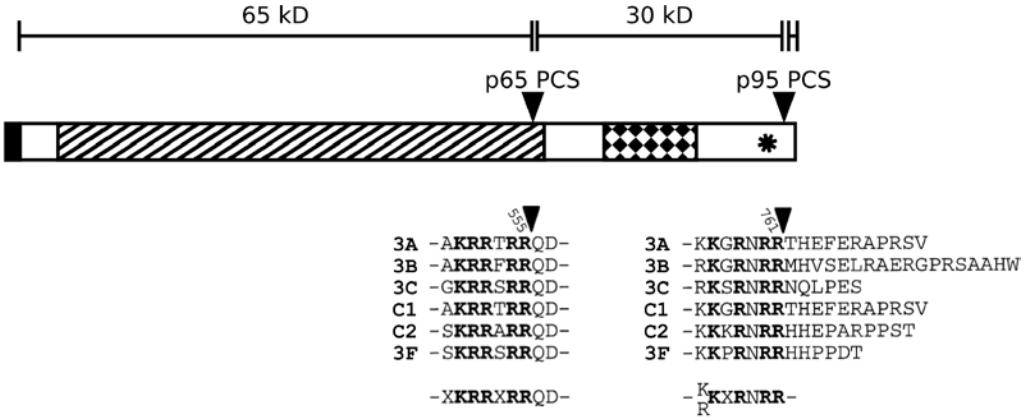

Figure 3: Two putative cleavage sites (PCS) are conserved across the SEMA3 family. These two cleavage sites process the SEMA3 family members to 95 and 65 kD isoforms. Aligned sequences from members of the SEMA3 family, including mouse orthologs Sema3A, Sema3B, Sema3C and Sema3F, chick SEMA3A ortholog collapsin-1 (C1) and chick SEMA3D ortholog collapsin-2 (C2) are shown above. From left to right, shaded regions in the schematic above correspond to the signal sequence, *sema* domain and Ig domain. Cleavage at the p95 site of Sema3A has been shown to be required for Sema3A to acquire an inhibitory phenotype. Asterisk represents the location of the conserved cysteine residue necessary for the formation of dimeric SEMA3, located at residue 723 in Sema3A. Cleavage of Sema3A at the p65 site therefore prevents dimerization of Sema3A, resulting in a functional loss of repulsive and collapsing phenotypes. Adapted from Guo et al. (2013) and Klostermann et al. (1998).

Furin or furin-like endoproteases have been implicated in this cleavage on the basis of furin inhibition and cleavage site mutation studies (Adams et al., 1997; Christensen et al., 2005; Guo et al., 2013; Parker et al., 2010; Varshavsky et al., 2008). Furin is a member of the subtilisin/kexin-type proprotein convertase

(PCSK) family of serine endoproteases, which cleave C-terminally to the R-X-(R/K/X)-R$^{\downarrow}$ motif (Remacle et al., 2008). At least two such motifs have been found to be conserved across the SEMA3 family, one located centrally and the other located C-terminally(Adams et al., 1997). Furin has been identified in almost all examined mammalian cell lines and plays a major role in the proteolytic activation of a wide range of enzymes and signaling molecules. Small chemical and peptide inhibitors of PCSKs suffer from poor selectivity between the PCSKs and limit the ability of currently published studies to identify which PCSK is specifically responsible for SEMA3 cleavage (Zhong et al., 1999).

The Vander Kooi laboratory has recently published two papers focusing on the interaction of SEMA3F and VEGF, two competitive ligands for the NRP2 receptor. In their studies, they used recombinant protein and small peptide synthesis of the C-terminal portion of SEMA3F to demonstrate that SEMA3F is unable to competitively inhibit VEGF binding unless it exposes a C-terminal arginine, as it would following furin cleavage (Parker et al., 2010). Mutational analysis of a small peptide corresponding to the last 40 amino acids of p95 SEMA3F demonstrated that point mutation of a conserved cysteine residue could disrupt dimer formation, resulting in a monomeric peptide with 40-fold decrease in inhibition as compared to the dimeric peptide (Figure 4, Guo et al., 2013).

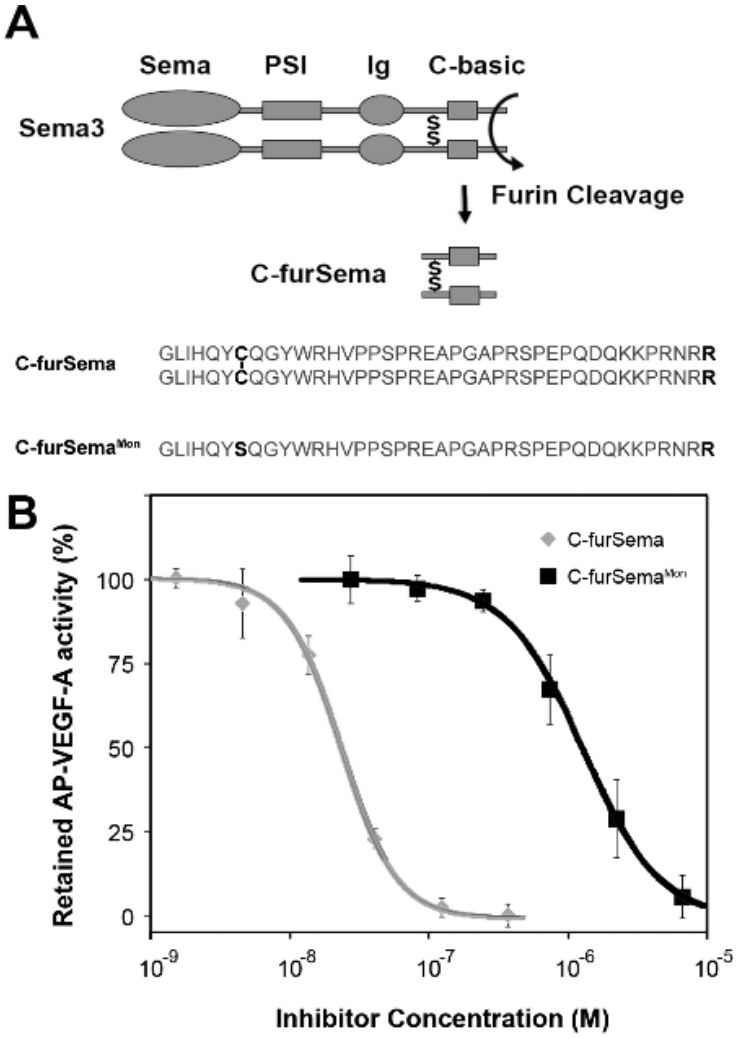

Figure 4: Mutation of a cysteine residue within a peptide construct corresponding to the SEMA3F C-terminal construct results in a monomeric peptide with reduced inhibition. *A,* A construct corresponding to the C-terminal residues of mouse Sema3F following a p95 cleavage event was constructed (C-furSema). Mutation of the internal cysteine residue of C-furSema to serine prevented the construct from forming dimers. *B,* The ability of monomeric C-furSema to compete with VEGF for the binding of mouse Nrp1 was significantly reduced as compared to that of dimeric C-furSema. Adapted from Guo et al. (2013).

SPECIFIC AIMS

The overarching aim of this study is to elucidate the role of specific PCSKs in the cleavage and regulation of SEMA3F, which the Bielenberg laboratory has previously investigated as a cancer therapeutic to prevent metastasis and retard growth. Proteolytic processing has been shown to be an important regulator of many members in the SEMA3 family, such as SEMA3A, SEMA3B and SEMA3E. Recent studies have suggested that C-terminal cleavage of SEMA3F to its 95 kD isoform by furin is important for SEMA3F to effectively compete with VEGF for NRP2 binding, but relatively little has been published on the 65 kD isoform regarding cleavage and resultant activity.

This study will pursue three approaches to investigate the p65 cleavage of SEMA3F. These encompass pure protein digests, transfection of PCSK-deficient and wild-type cell lines and siRNA knockdown of specific endoproteases. In pursuing the first, purified SEMA3F will be incubated with recombinant human furin (rhFurin), varying both digest duration and SEMA3F:rhFurin ratio. Secondly, this study will examine the presence of specific SEMA3F isoforms in the conditioned media of furin-deficient and furin-complemented CHO-K1 strains RPE.40 and 40.fur following transfection with SEMA3F. Finally, the competence of human melanoma A375SM cells previously transfected with SEMA3F for additional transfection with siRNA will be determined to assess their suitability for siRNA studies.

METHODS

Cell culture

Minimum Essential Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), 1:1 DMEM/Ham's F12 Medium (DMEM/F12), 0.05% Trypsin/EDTA and 100x Penicillin-Streptomycin-Glutamine (GPS) were purchased from Gibco Life Technologies. Dimethyl sulfoxide was purchased from Sigma-Aldrich. When complete medium is specified, medium was supplemented with 10% Fetal Bovine Serum (FBS, Denville Scientific) and 1x GPS. Human Embryonic Kidney (HEK) cells transformed with SV40 large T antigen (HEK293FT, Gibco Life Technologies) were cultured with complete DMEM supplemented with 4.5 mg/ml D-glucose. 500 μg/ml Geneticin (Gibco) was used to maintain SV40 transfection.

A375SM cells were previously obtained from the Fidler laboratory (MD Anderson Cancer Center, Houston, TX) and originally derived by orthotopically cycling the human melanoma cell line A375P in nude mice and isolating metastatic cancer cells (Li et al., 1989). Creation of a pSecTag2A vector encoding SEMA3F (Ascension #HSU38276) was previously described (Bielenberg et al., 2008). This vector replaced the SEMA3F signal sequence with the IgK leader sequence and C-terminally linked c-myc and His tags to SEMA3F. pSecTag2A also conferred transfected cells with resistance to zeocin. A375SM cells were subsequently transfected with this vector and stable transfectants were selected

and maintained using 500 μg/ml zeocin (Gibco Life Technologies). This paper will refer to this stably transfected A375SM cell line as SM/SEMA3F (Bielenberg et al., 2004).

RPE.40 cells are a furin-deficient CHO-K1-derived line of cells first identified for their resistance to Pseudomonas endotoxin due to an inability to proteolytically process and mature the pathological endotoxin. RPE.40 cells and RPE.40 cells stably transfected with mouse furin (40.fur cells) were kindly provided by Joseph Sucic (Moehring et al., 1993). Both cell types were cultured in complete DMEM/F12 media.

All cells were cultured at 37 °C in a humidified incubator. A375SM and SM/SEMA3F cells were cultured with 10% CO2. HEK293FT, RPE.40 and 40.fur cells were cultured with 5% CO2. 10 cm cell culture plates were seeded with 10^6 cells and maintained at 30–80% confluence.

SEMA3F Purification

C-terminally c-myc/His-tagged SEMA3F was purified as previously described (Bielenberg et al., 2008). Briefly, HEK293FT were transiently transfected with the pSecTag2A SEMA3F plasmid described above and the media was replaced with serum-free CD293 medium (Invitrogen). After 48 hours, conditioned media were collected, centrifuged 15 min at 16,900 g and filtered through a 0.22 μm polyvinylidene fluoride (PVDF) membrane. The C-terminal His tag allows SEMA3F to be purified by nickel affinity. A HiTrap high-performance chelating column (GE Healthcare Amersham Biosciences) was prepared with NiSO4 and

equilibrated with phosphate-buffered saline (PBS). The nickel column was connected to a fast protein liquid chromatography (FPLC) machine and SEMA3F was sequestered in the column. SEMA3F was eluted from the column using a high-salt buffer and desalted with a PD-10 gel filtration column (GE Healthcare). The expected yield from fifteen 15-cm tissue culture plates was about 5 mg. Purified SEMA3F was stored at -80 ℃.

Production of anti-SEMA3F antibody in rabbit

An antibody against the *sema* domain of SEMA3F was raised in rabbit and purified to a concentration of 0.781 mg/ml. Rabbit anti-SEMA3F antibody was produced previously by Akio Shimizu.

Lower limit of SEMA3F detection by Western blot

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The molecular weight of purified SEMA3F was confirmed by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blot. Protogel (30% acrylamide, 0.8% bis-acrylamide), 4x Resolving Buffer and 4x Stacking Buffer were purchased from National Diagnostics. 10x Running Buffer was purchased from Boston Bioproducts. Tetramethylethylenediamine was purchased from JT Baker and ammonium persulfate was purchased from Sigma-Aldrich.

10-well polyacrylamide gels were handcast at 1.5 mm thick using a Bio-Rad Mini-PROTEAN Tetra Cell system. The resolving layer (pH 8.8) was cast at 7.5 wt% polyacrylamide and the stacking layer (pH 6.8) at 3.9 wt%.

In order to determine the lower limit of detection for SEMA3F, differing quantities of SEMA3F were loaded into each well. 100, 200, 400 and 600 ng samples of SEMA3F were prepared in duplicate. Samples were prepared under reducing conditions using reducing sample buffer (Boston Bioproducts) and diluted to 36 µl loading volume using double-distilled water (ddH_2O).

SDS-PAGE gels were run at 90 V for 2 h until the Kaleidoscope Prestained Standards (Bio-Rad) were clearly separated. All subsequent SDS-PAGE gels were run using these parameters unless otherwise specified.

Transfer of SDS-PAGE to nitrocellulose membrane by Western blot

Gels were transferred to nitrocellulose membranes before probing with antibodies. 10x Transfer Buffer and 10x tris-buffered saline (TBS) was purchased from Boston Bioproducts.

0.45 µm nitrocellulose membrane (Bio-Rad) and two pieces of 3MM chromatography filter paper (Whatman) were cut to 6×9 cm. Nitrocellulose membrane, filter paper and two sponges were immersed in transfer buffer and polyacrylamide gels were removed from the SDS-PAGE apparatus. Nitrocellulose membrane was placed against the gel and then both were sandwiched between filter paper and sponges. Electrophoretic transfer was completed under constant amperage of 300 mA for 2 h on ice.

A 3% blotting-grade blocker (Bio-Rad) solution was prepared in TBS (3% milk) and the membrane was blocked for 1 h at room temperature with moderate agitation. The blot was cut in half with each side containing a full set of SEMA3F dilutions. 1:1000 dilutions of 9E10 mouse anti-c-myc antibody (Santa Cruz Biotechnology) and rabbit anti-SEMA3F antibody were prepared in a 0.01% Tween 20 (Sigma-Aldrich) in TBS solution (TBST). Each half of the blot was incubated with either the mouse anti-c-myc or the rabbit anti-SEMA3F for 2 h at room temperature with moderate agitation.

Blots were washed briefly with TBST and then washed an additional three times with TBST at room temperature with gentle agitation for 10 min each. 1:3000 dilutions of NA931V HRP-linked sheep anti-mouse IgG (GE Healthcare) and NA934V HRP-linked donkey anti-rabbit IgG (GE Healthcare) were prepared in TBST. Blots were incubated with the appropriate secondary antibody dilution at room temperature with gentle agitation. After 1 h, blots were washed briefly with TBST and then washed three times with TBST under gentle agitation for 10 min each.

Western Lightning Plus enhanced chemiluminescent substrate (ECL, Perkin Elmer) was prepared by combining 1 ml each of the luminol reagent and oxidizing reagent. ECL solution was incubated with the nitrocellulose membrane for 5 min. HyBlot CL autoradiography film (Denville Scientific) was exposed and developed to visualize probed proteins.

All subsequent Western blots were transferred and developed using these parameters unless otherwise specified.

SM/SEMA3F Expression of SEMA3F

The conditioned media of SM/SEMA3F cells were assayed for the presence of SEMA3F. Two SM/SEMA3F clones, C3 and D1, were plated at 10^6 cells in a 10 cm tissue culture plate. When cells reached 70% confluence, plates were washed with 10 ml serum-free MEM before adding 5 ml serum-free MEM to each plate. Cells were replaced in the incubator and conditioned media was collected after 24 h. Media was spun down at 1000 rpm for 5 min and supernatants were collected.

For each sample, 30 µl of conditioned media and 6 µl 6x reducing sample buffer were combined and boiled for 10 min before loading onto a SDS-PAGE gel. Serum-free MEM with reducing sample buffer was loaded as a negative control and 300 ng SEMA3F was diluted in serum-free MEM with reducing sample buffer as a positive control. This and all subsequently described SDS-PAGE gels were run with Kaleidoscope Precision Plus Protein molecular weight markers (Bio-Rad).

Gels were transferred by Western blot and probed with anti-SEMA3F antibody.

Furin inhibition of SM/SEMA3F cells

The effect of two furin inhibitors on the *in vitro* cleavage of SEMA3F by SM/SEMA3F clone C3 was assayed. Furin inhibitor I (RVKR-CMK, Calbiochem),

decanoyl-arginine-valine-lysine-arginine-chloromethylketone, is an irreversible competitive inhibitor of furin and other proprotein convertases. Furin inhibitor II (H6, Calbiochem), hexa-D-arginine, is a reversible competitive inhibitor of furin and other proprotein convertases. SM/SEMA3F cells were seeded into a 6-well plate at 10^5 cells/well and grown to 50% confluence. Wells were briefly washed with 1 ml serum-free MEM. 1 ml serum-free MEM was added to each well. Two wells were left untreated as controls and each of the other four were treated with one of 10 μM RVKR-CMK, 100 μM RVKR-CMK, 10 μM H6 or 100 μM H6.

Cells were incubated for an additional 20 h before collecting conditioned media. Conditioned media was spun down at 1000 rpm for 5 min to remove cells from suspension and the supernatant was collected. 30 μl of conditioned media from each well was collected and analyzed by SDS page and Western blot using a rabbit anti-SEMA3F antibody.

Thermal stability of SEMA3F in various buffers

The stability of SEMA3F at 37 ℃ in phosphate-buffered saline (PBS, Gibco Life Technologies), radioimmunoprecipitation assay buffer (RIPA, Boston Bioproducts), Hank's balanced salt solution (HBSS, Mediatech Cellgro) and 0.9% NaCl saline (Hospira) was assessed.

200 ng of SEMA3F samples were diluted in 20 μl PBS, RIPA, HBSS and saline, then incubated at 37 ℃ 10% CO2 for 24 h. A dilution of SEMA3F in PBS was stored at 4 ℃ for 24 h to serve as a positive control. 4 μl of 6x reducing

sample buffer was added to each sample at 24 h and the samples were boiled 10 min. Samples were loaded into a 15-well 1.5 mm SDS-PAGE gel and run at 175 V for 1 h. Separated proteins were transferred by Western blot to a nitrocellulose membrane and blocked for 30 min at room temperature. Blot was blocked 30 min at room temperature and incubated overnight at 4 ℃ with 1:1000 rabbit anti-c-myc diluted in 3% milk.

Furin activity assessed by fluorogenic assay

Recombinant human furin (rhFurin) was purchased from R&D Systems and its protease activity was assessed using the fluorogenic substrate pERTKR-AMC (R&D Systems). Assay buffer was prepared as a 25 mM Trizma (Sigma-Aldrich), 1 mM CaCl2 (Sigma-Aldrich), 0.5% (w/v) Brij35 (Biomeda) solution. rhFurin was diluted to 4 μg/ml and pERTKR-AMC was diluted to 100 μM in assay buffer.

Samples were loaded into a 96-well black, clear-bottomed plate (Corning). Experimental wells were prepared in triplicate by the addition of 50 μl rhFurin dilution to each well. Substrate blanks were prepared in triplicate by adding 50 μl assay buffer to each well. The reaction was started by the addition of 50 μl substrate to each well. Plate was read 8 times over 10 min using a Wallac Victor3V 1420 Multilabel Plate Counter (Perkin Elmer). Excitation and emission wavelengths were 355 nm and 460 nm, respectively. The optimal excitation wavelength of 7-amino-4-methylcoumarin (AMC) is 380 nm.

Effect of duration on furin digest of SEMA3F

Purified SEMA3F was digested with rhFurin over various durations in order to characterize the kinetics of SEMA3F cleavage by furin. 300 ng SEMA3F and 10 ng rhFurin were diluted in 30 µl PBS. 300 ng SEMA3F was added to each experimental sample and all samples were incubated 5 h at 37 ℃. Furin was withheld from one SEMA3F sample and three other samples were digested with 10 ng rhFurin at 0, 2 and 4 h, resulting in furin digest durations of 0, 1, 3 and 5 h. Two negative controls, ddH_2O and PBS, were also included.

At the 5 h time point, reducing sample buffer was added to each digest vessel and samples were boiled for 10 min. Samples were analyzed by SDS-PAGE and Western blot using mouse anti-c-myc.

An additional rhFurin digest examining the effect of digest time on SEMA3F cleavage was performed with longer digest times. This digest was set up in the same manner as the previous assay but considered 8, 16 and 24 h digest times. All samples were incubated at 37 ℃ for 24 h with one exception. Two positive controls were used: SEMA3F incubated at 37 ℃ and SEMA3F incubated at 4 ℃ for 24 h. BS and 10 ng furin diluted in PBS were used as negative controls.

Effect of furin quantity on furin digest of SEMA3F

The effect of furin quantity on the cleavage of SEMA3F was assayed using purified protein. 300 ng rhFurin was incubated at 37 ℃ for 22 h with 0, 10, 20, 30, 40 and 100 ng rhFurin. 300 ng SEMA3F incubated 22 h at 4 ℃ served as a

positive control. PBS and 10 ng rhFurin diluted in PBS served as negative controls.

A prolonged digest series was also performed investigating the digest of 300 ng SEMA3F with 10, 30 and 50 ng rhFurin over a 90 h incubation period.

Cleavage of SEMA3F assessed *in vitro* by transfection

Furin-deficient RPE.40 and furin-complemented 40.fur cells were transfected with SEMA3F to investigate the ability of furin to cleave SEMA3F *in vitro*. pSecTag2A SEMA3F was modified to include an N-terminal HA tag (pSecTag2A HA-SEMA3F) by a previous lab member, Akio Shimizu. Transfection with this plasmid was performed using the Fugene 6 reagent (Roche). Cells were seeded in a six-well plate at a density of 1–1.5 × 10^5 cells/well and were transfected at 50-75% confluence.

For each well, the transfection cocktail was prepared as follows. 1.5 µl Fugene was added directly to 50 µl of complete DMEM/F12 and allowed to incubate for five minutes. 0.5 µg pSecTag2A HA-SEMA3F plasmid was added directly to the Fugene/medium solution and allowed to incubate an additional 15 minutes.

Media in each well was replaced with 1 ml complete media and the transfection cocktail was added to the well. Cells were incubated 20 h at 37 ℃, 5% CO2. Wells were washed with 0.5 ml serum-free media and then 0.5 ml serum-free DMEM/F12. Cells were incubated for an additional 48 h before collecting conditioned media. Conditioned media was spun at 1000 rpm for 5 min to pellet

cells and the supernatant was collected. 30 µl of each conditioned media sample was analyzed by SDS-PAGE and Western blot using rabbit anti-SEMA3F antibody.

Assessment of RNAi transfection efficiency of SM/SEMA3F cells

RNAi knockdown of NRP2 in SM/SEMA3F cells

Stably transfected cells often have poor transfection efficiency for additional plasmids or RNAi. The ability of siRNA to silence NRP2 in SM/SEMA3F cells was assessed *in vitro*. SiGENOME siRNA to NRP2 (siNRP2) was purchased as a 20 µM stock from Dharmacon. SM/SEMA3F cells were seeded in a 6-well plate at a density of 10^5 cells/well. Cells were cultured to 70% confluence and media was replaced with 1.25 ml fresh complete MEM. Cells were divided into three treatment groups: control, 20 nM siNRP2 and 60 nM siNRP2 treatment groups.

For the 20 nM siNRP2 treatment group, 3 µl siLentFect Lipid reagent (Bio-Rad) and 122 µl Opti-MEM (Gibco Life Technologies) for each well were combined in a microcentrifuge tube and incubated 5 min at room temperature. Concurrently, in another microcentrifuge tube, 1.5 µl siNRP2 was combined with 123.5 µl Opti-MEM for each well and incubated for 5 min. Both solutions were combined and incubated for 15 min. Combined solution (250 µl) was added to each well, gently swirling to distribute transfection cocktail. SM/SEMA3F cells were returned to the tissue culture incubator for 4 h, after which media were replaced with complete MEM.

The 60 nM siNRP2 treatment group was prepared as above, except that the amount of 20 μM siNRP2 was increased to 4.5 μl/well and the Opti-MEM was decreased correspondingly. Untreated control groups received 250 μl Opti-MEM in addition to the 1.25 ml MEM per well.

Collection of cell lysates

Cell lysates were harvested 72 h post-transfection. Lysate buffer was prepared by dissolving one CompleteMini protease inhibitor tablet (Roche) in 10 ml RIPA. SM/SEMA3F cells were placed on ice and washed twice with PBS. 50 μl lysate buffer was added to each well and allowed to incubate 15 min. A 1.8 cm cell scraper (Corning) was used to completely suspend cells in the lysate buffer and plate was incubated for an additional 15 min on ice. Lysate solutions were transferred to microcentrifuge tubes and sonicated to ensure complete cell lysis. Lysates were spun down at 14,000 rpm for 10 minutes to pellet cell debris and supernatants were collected.

DC Protein Assay

Total protein concentration of cell lysates was measured using a DC Protein Assay Kit (Bio-Rad) in a flat-bottomed 96-well plate. 5 μl of sample was transferred into each well. Lysate samples were measured in duplicate wells. Standard dilutions of Bovine Serum Albumin (BSA) were prepared covering a range from 3 mg/ml BSA serially diluted 1:3 to a final concentration of 0.53 mg/ml BSA. Standard samples were measured in triplicate wells.

Because cell lysates were prepared in RIPA, which contains detergent, a 2% solution of Reagent S was prepared in Reagent A, resulting in Reagent A+S. 25 µl of Reagent A+S was added to each well, followed by 200 µl Reagent B. The plate was incubated for 15 minutes at room temperature and absorbance was read at 650 nm. A linear best fit curve was plotted against the standard dilutions and used to determine the total amount of protein in the experimental samples.

NRP2 knockdown visualization by Western blot

Lysates were diluted to the same total protein concentration using lysate buffer. 30 µl of each lysate was combined with 6 µl 6x reducing sample buffer and boiled for 10 min. Samples were analyzed by SDS-PAGE and Western blot. Membrane was blocked 30 min and probed with a 1:1000 dilution C-9 mouse anti-NRP2 (Santa Cruz Biotechnology) in 3% milk overnight at 4 ℃.

RESULTS

SEMA3F limit of detection by anti-c-myc and anti-SEMA3F antibodies

At primary antibody dilutions of 1:1000 and secondary antibody dilutions of 1:3000, mouse anti-c-myc and rabbit anti-SEMA3F had a lower limit of detection of 200 ng (Figure 5) and below 100 ng (Figure 6), respectively.

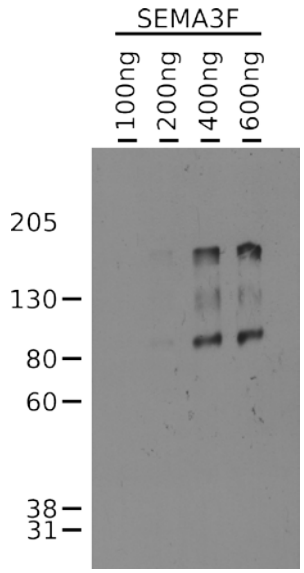

Figure 5: Visualization of SEMA3F with mouse anti-c-myc antibody. 100 ng SEMA3F is not visible and 200 ng SEMA3F appears much lighter than the 400 and 600 ng SEMA3F samples when labeled with anti-c-myc. For each of the 200, 400 and 600 ng lanes, bands were seen at roughly 95, 130 and 180 kD. Film was exposed 60 s before developing.

In addition to the primary expected band at 95 kD, anti-c-myc also labeled bands at molecular weights of ~180 kD and ~130 kD. It is possible that these

correspond to homodimeric p95:p95 species and heterodimeric p95:p35 species, respectively, suggesting that intermolecular disulfide bonds were not reduced and cleaved. Subsequent SDS-PAGE gels rarely show these bands, which is attributed to prolonged sample boiling time in these assays before gels were loaded.

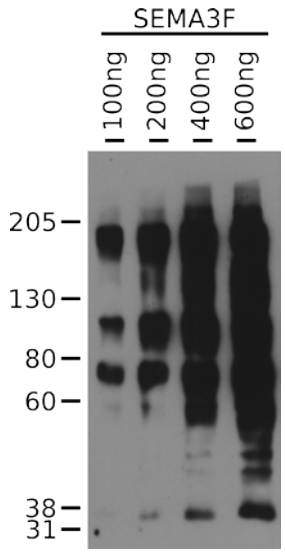

Figure 6: Visualization of SEMA3F with rabbit anti-SEMA3F antibody. Anti-SEMA3F-labeled bands can be seen in each of the 100, 200, 400 and 600 ng SEMA3F lanes. Three primary bands were observed in all four lanes with approximate molecular weights of 67, 95 and 180 kD. 400 and 600 ng SEMA3F lanes show additional bands at low molecular weights and one at ~130 kD. Film was exposed 30 s before developing.

The observed anti-SEMA3F-labeled bands at sizes 67, 95 and 130 and 180 kD are thought to respectively correspond to the cleaved p65 isoform, p95, p95:p35

heterodimer and p95:p95 homodimer. Lower molecular weight bands may represent additional cleavage products or, more likely, non-specific binding of anti-SEMA3F or secondary antibodies.

SEMA3F is partially cleaved in SM/SEMA3F conditioned medium

SEMA3F was detectable in the unconcentrated 24 h conditioned media collected from SM/SEMA3F clones C3 and D1 (Figure 7).

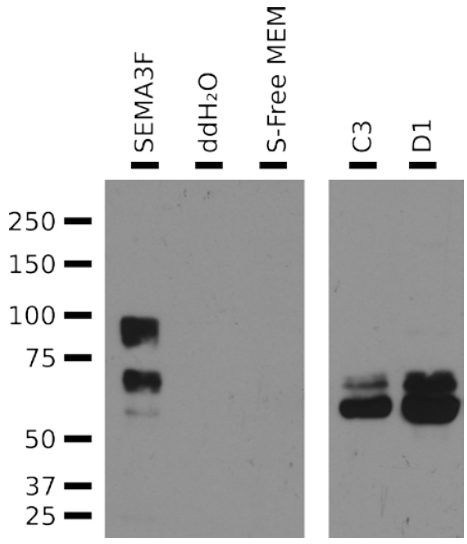

Figure 7: SEMA3F is detectable in the concentrated media of SM/SEMA3F cells. Film fragments shown here were both from the same blot. Intermediate lanes contained conditioned media from other cell types (data not shown) and were removed for clarity. One positive and two negative controls consisting of purified SEMA3F (+), ddH_2O (-) and serum-free MEM (-) were included. Proteins were labeled with anti-SEMA3F antibodies and film was exposed for 3 min.

The molecular weight of the predominant bands found in the conditioned media were approximately 60 and 70 kD, which are thought to represent alternative glycosylation of the N-terminally cleaved p65 fragment of SEMA3F. A very faint band at 95 kD is visible in the D1 lane and is thought to represent a very low level of p95 SEMA3F. These results indicate that SM/SEMA3F cells either proteolytically process SEMA3F intracellularly or excrete proteases that are capable of cleaving SEMA3F.

Furin inhibitors can prevent SEMA3F cleavage *in vitro*

On the basis of the previous assay, SM/SEMA3F clone C3 was cultured with two different furin inhibitors, RVKR-CMK and H6, in order to assess the ability of these inhibitors to restore the expected 95 kD SEMA3F band. Both RVKR-CMK and H6 are able to substantially inhibit the cleavage of SEMA3F, as demonstrated by shift in molecular weight from 65 kD to 95 kD between control and experimental lanes (Figure 8).

RVKR-CMK may inhibit SEMA3F cleavage slightly better than did H6. Interestingly, for both RVKR-CMK and H6, a significant difference in inhibition was not observed between the 10 μM and 100 μM levels.

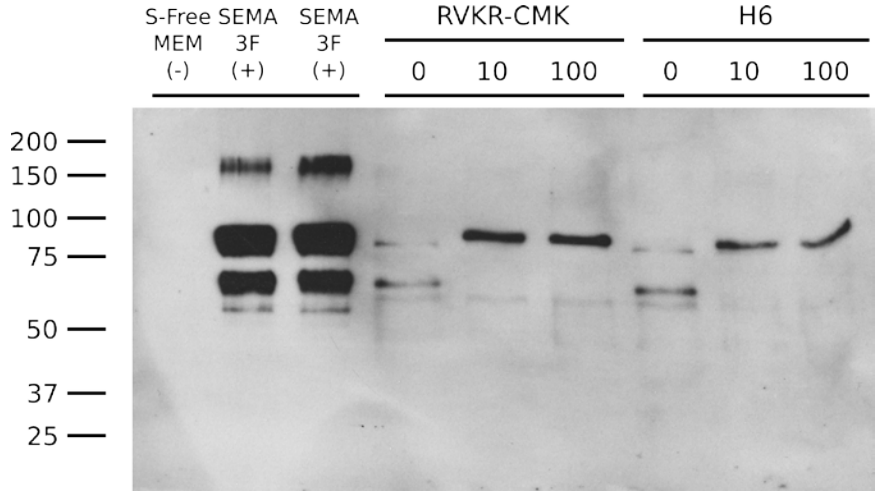

Figure 8: Furin inhibitors reduce cleavage of SEMA3F *in vitro*. Unconcentrated 20 h conditioned media were collected from SM/SEMA3F cells treated with RVKR-CMK and H6 at two different concentrations. A mixture of p65 and p95 SEMA3F cleavage isoforms can be seen in the conditioned media of untreated control cells. Conditioned media from RVKR-CMK and H6-treated SM/SEMA3F cells primarily contain the p95 isoform. Serum-free MEM was used as a negative control and two different batches of purified SEMA3F were used as a positive control. 180 kD band in control lanes are attributed to SEMA3F p95:p95 homodimers. Blot was labeled using anti-SEMA3F antibody and film was exposed for 3 min.

SEMA3F is thermally stable in several buffers

SEMA3F was incubated in RIPA, HBSS, 0.9% saline and PBS for 24 h at 37 ℃. SEMA3F suspended in PBS and stored at 4 ℃ was used as a positive control. SEMA3F does not appear to degrade significantly when incubated at 37 ℃ in RIPA, HBSS, saline or PBS for 24 h, although there were small variations in the quantity of p65 and smaller bands. Notably, incubation with RIPA induced a

band at roughly 180 kD. This is tentatively suggested to be caused by increased aggregation of SEMA3F molecules due to the high detergent levels in RIPA.

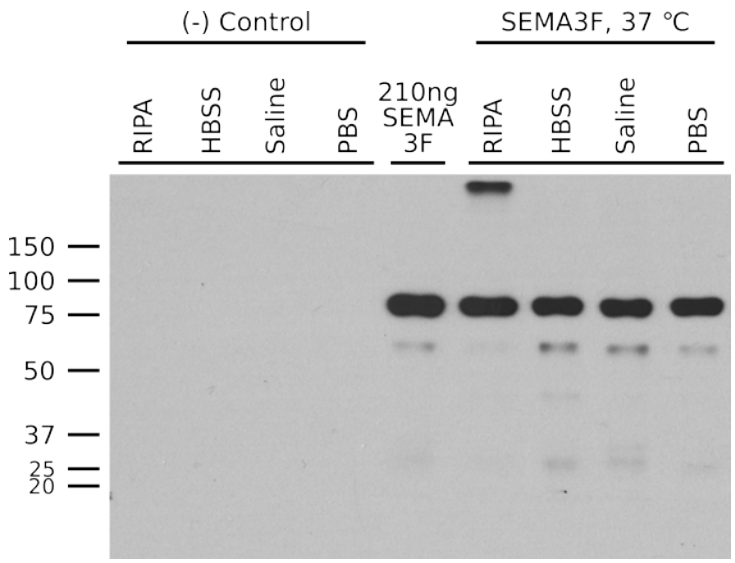

Figure 9: SEMA3F is thermally stable for up to 24 h in 37 ℃ RIPA, HBSS, saline and PBS. Lanes corresponding to incubation in HBSS, saline and PBS look identical to the control lane. Incubation of SEMA3F in RIPA did not display significant changes in p95 levels, but did induce a 180 kD band. Each buffer was loaded separately as a negative control. Blot was labeled with anti-c-myc antibody and film was exposed for 10 s.

rhFurin actively cleaves pERTKR-AMC

Cleavage of the fluorogenic substrate pERTKR-AMC was used in order to assess the proteolytic activity of rhFurin. Substrate alone maintained a relatively constant level of fluorescence, whereas the addition of rhFurin induced an increasing level of fluorescence over time. This suggests that rhFurin is able to actively cleave pERTKR and release the fluorogenic molecule AMC.

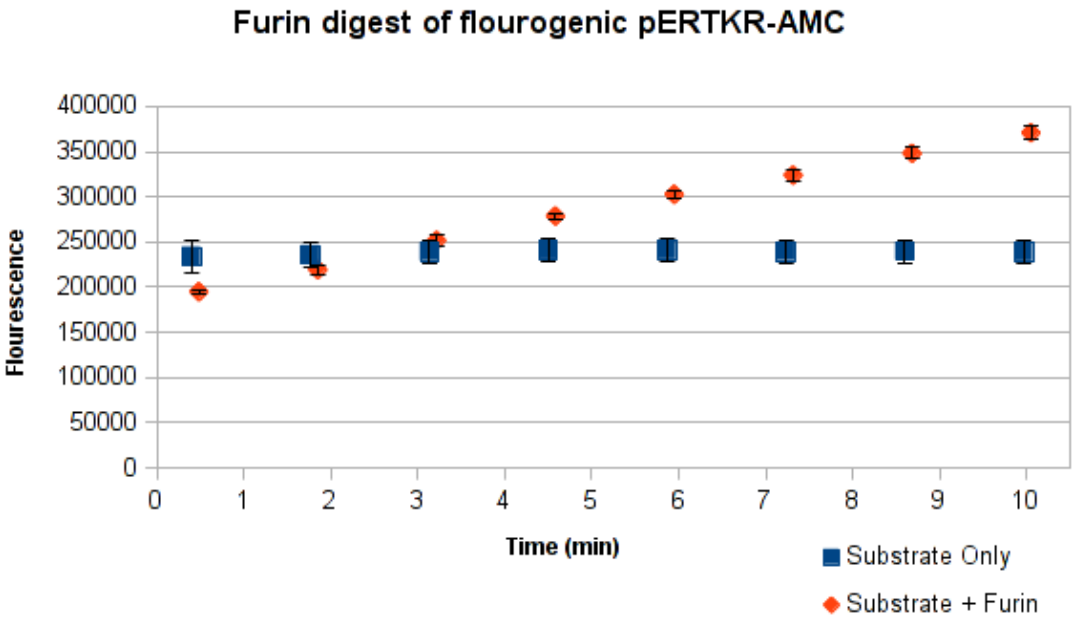

Figure 10: Furin digest of fluorogenic pERTKR-AMC. Over a time period of 10 minutes, the fluorescence's of an rhFurin/pERTKR-AMC solution was observed to increase linearly, suggesting that rhFurin was processing pERTKR-AMC and releasing the fluorogenic compound AMC. The fluorescence of pERTKR-AMC without rhFurin remained constant. Standard deviation for each time point (n=3) are shown as error bars.

SEMA3F does not exhibit significant cleavage by low rhFurin levels

SEMA3F did not appear to be significantly processed when incubated with 10 ng rhFurin up to 5 h (Figure 11). Control and digest lanes appear largely identical for the intensity of 95 and 65 kD bands. The 1 h digest may be an exception, with a slightly darker 65 kD band than the other lanes. However, higher p65 levels at 1 h digest than at 3 and 5 h digests is inconsistent with rhFurin-mediated cleavage.

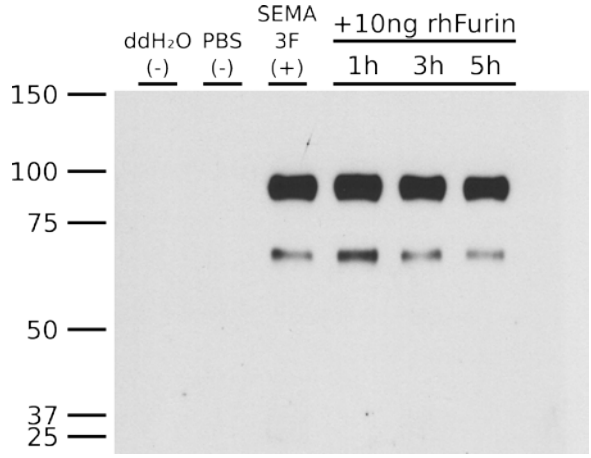

Figure 11: Cleavage of SEMA3F is not evident up to five hours. At the 95 kD size, there was no change between the SEMA3F positive control, which was incubated at 37 ℃ 5 h without furin, and the rhFurin-incubated samples. The 1 h incubation may have higher levels of the 65 kD isoform, but this elevation was not seen at 3 and 5 h. Blot was labeled with anti-c-myc antibody and film was exposed for 3 min.

In order to determine if cleavage might be observed with longer digest times, SEMA3F was incubated with furin for up to 24 h (Figure 12). Again, no cleavage was observed.

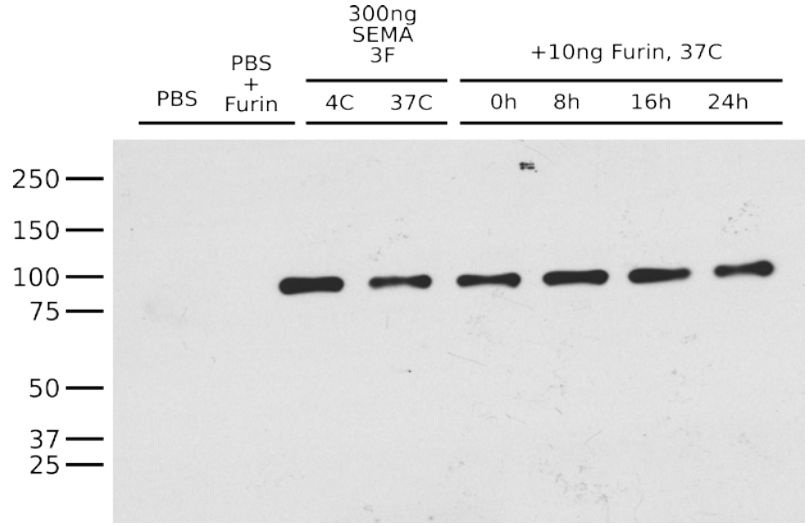

Figure 12: Cleavage of SEMA3F is not evident after incubating with 10 ng rhFurin up to 24 h. No change in band intensity was observed between control samples and experimental samples incubated with rhFurin. Blot was labeled with anti-c-myc antibodies and film was exposed 30 s.

Cleavage of purified SEMA3F is still not evident at high rhFurin levels

In order to identify if SEMA3F cleavage was not observed due to insufficient rhFurin levels, up to 100 ng rhFurin was incubated with 300 ng SEMA3F at 37 ℃ for 22 h (Figure 13). Although the banding pattern in some lanes appears slightly different than that in the 4 ℃-incubated sample or the 0 ng rhFurin sample, there is no discernable pattern between banding pattern and rhFurin levels. Even at a 3:1 ratio of SEMA3F to rhFurin, no cleavage was observed.

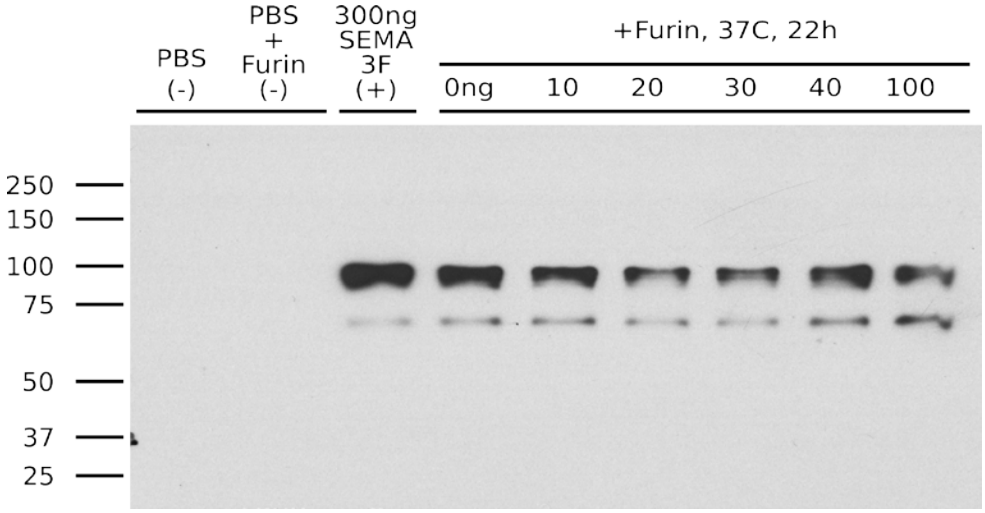

Figure 13: SEMA3F is not cleaved in the presence of furin up to a ratio of 3:1 SEMA3F:rhFurin. Bands corresponding to 95 kD and 65 kD SEMA3F isoforms were visible in all SEMA3F lanes. No relationship between rhFurin level and isoform levels was observed. Blot was labeled with anti-SEMA3F antibody and film was exposed for 15 s.

In order to test if cleavage might have been observed with even longer digest times, SEMA3F was incubated with up to 50 ng rhFurin for 90 h (Figure 14). Even at these furin levels and digest durations, SEMA3F cleavage was not observed. It appears that the 10 ng rhFurin lane has a relatively higher level of the p65 SEMA3F isoform as compared to positive controls. However, the level of p65 SEMA3F in this sample is also higher than that in samples with still higher rhFurin levels. Therefore, no discernable relationship between rhFurin levels and SEMA3F cleavage was observed.

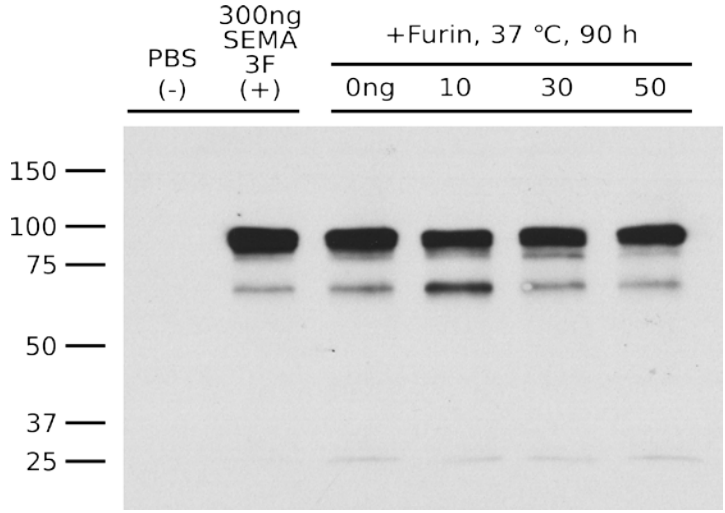

Figure 14: SEMA3F is not cleaved by rhFurin over a 90 h period. Two bands can be observed between the 75 and 100 kD molecular weight markers and are thought to represent alternative glycosylation of SEMA3F. No discernable difference between the higher molecular weight glycosylation was observed between SEMA3F controls and rhFurin-incubated samples. Lower molecular weight glycosylation appeared to be reduced in samples with higher levels of rhFurin, but no corresponding increase in a lower molecular weight isoform was observed, as would be expected if cleavage had occurred. The 10 ng rhFurin digest appears to have higher levels of the 65 kD SEMA3F isoform, but no trend between rhFurin and p65 levels were observed. Faint 25 kD bands are thought to represent non-specific binding of the anti-HA antibody. Blot was labeled with anti-HA antibody and film was exposed for 30 min.

All digests included in this paper were conducted in a reaction solution whose primary component was PBS. In addition, digests were performed in HBSS and serum-free MEM (data not shown) in order to account for the possibility that

rhFurin cofactors might not have been present. Digest results appear very similar to those included here.

SEMA3F Transfection of RPE.40 and 40.fur cells is inconclusive

Furin-deficient RPE.40 cells and furin-transfected 40.fur cells were transfected with pSecTag2A HA-SEMA3F in order to assess the ability of furin to cleave SEMA3F *in vitro* (Figure 15 and Figure 16).

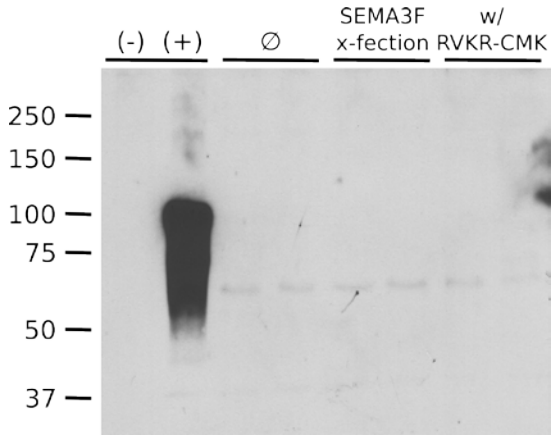

Figure 15: RPE.40 cells did not appear to secrete SEMA3F 48 h post-transfection. Conditioned media was collected from biological replicates of untransfected (∅) cells, SEMA3F-transfected cells and SEMA3F-transfected cells cultured with RVKR-CMK. Faint bands were observed at 60 kD, but did not differ in level between groups. Blot was labeled with anti-SEMA3F and film was exposed for 30 min.

Conditioned media were collected from three groups: untransfected cells, SEMA3F-transfected cells and SEMA3F-transfected cells cultured in the presence

of 10 μM RVKR-CMK. Cells were cultured 48 h post-transfection before collecting conditioned media.

Both RPE.40 and 40.fur cells appear to excrete low-levels of a 60 kD protein. However, no difference was observed in the levels of this protein between control groups and the two experimental groups. This suggests that either RPE.40 and 40.fur cells transfected with SEMA3F do not excrete the expressed protein or that the transfection was unsuccessful.

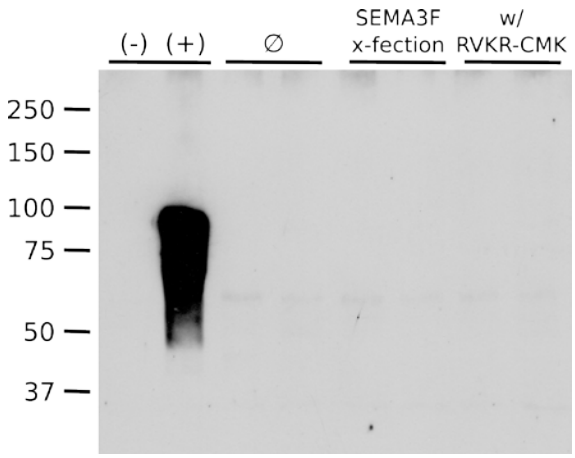

Figure 16: 40.fur cells did not appear to secrete SEMA3F 48 h post-transfection. Conditioned media was collected from biological replicates of untransfected (∅) cells, SEMA3F-transfected cells and SEMA3F-transfected cells cultured with RVKR-CMK. Faint bands were observed at 60 kD, but did not differ in level between groups. Blot was labeled with anti-SEMA3F and film was exposed for 30 min.

siNRP2 shows tentative knockdown of NRP2 in SM/SEMA3F

In order to assess the ability of RNAi to successfully downregulate gene expression in SM/SEMA3F cells, siRNA was used to knockdown NRP2 levels (Figure 17).

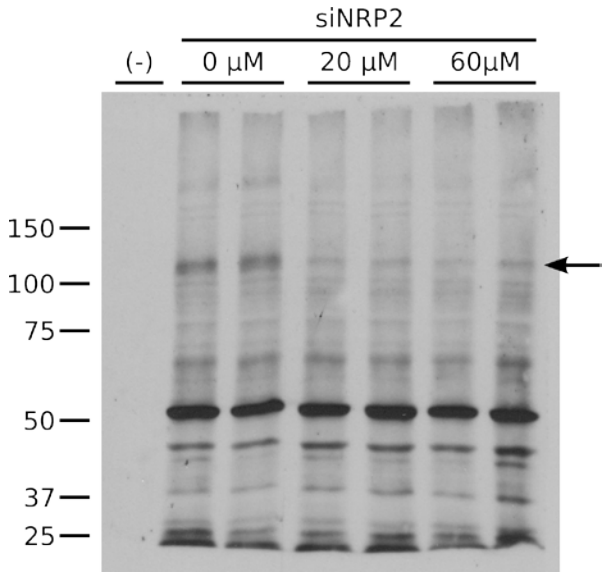

Figure 17: Knockdown of NRP2 in SM/SEMA3F cells by siNRP2 was tentatively successful. A row of bands at ~120 kD (indicated by arrow), potentially corresponding to NRP2, was found to decrease in intensity when SM/SEMA3F cells were treated with siNRP2. However, this blot shows a high level of non-specific antibody binding interactions. Blot was labeled with an anti-NRP2 antibody and film was exposed for 10 min

The molecular weight of NRP2 is approximately 120 kD, which corresponds to a row of bands that reduce in intensity following treatment of SM/SEMA3F cells with siNRP2. However, this blot displayed a high level of non-specific anti-NRP2 antibody binding, so it is difficult to confirm that this row of bands truly

corresponds to NRP2. Promisingly, the non-specific banding patterns appear identical between lanes, suggesting that cell lysates were loaded at equal concentrations.

The most intensely labeled bands were located at a molecular weight of approximately 55 kD. It is unknown to what protein this band may correspond.

DISCUSSION

This series of experiments has shown that rhFurin is not capable of directly cleaving SEMA3F within the synthetic digest environment. Using the anti-SEMA3F antibody previously raised in rabbits, as low as 100 ng SEMA3F could be detected by Western blot. *In vitro*, the change in observed molecular weight from predominantly 65 kD to predominantly 95 kD SEMA3F in SM/SEMA3F cells following the administration of RVKR-CMK or H6 is striking. However, these two inhibitors have been shown to interact with PCSK other than furin (PCSK3). RVKR-CMK is able to inhibit PCSK1-7 and H6 has been shown to inhibit at least PCSK1 and PCSK6 (Cameron et al., 2000; Tian & Jianhua, 2010). Therefore, the observed molecular weight shift in these cells primarily demonstrates that SEMA3F cleavage is PCSK mediated. Interestingly, H6 has been shown to upregulate PCSK2 activity, suggesting that PCSK2 is most likely not involved in the processing of SEMA3F.

Under synthetic conditions, even with SEMA3F:rhFurin ratios approaching 3:1 and digest durations of up to 90 h, no evidence was found to support furin-mediated cleavage of SEMA3F in the context of purified protein interactions. We have confirmed that the rhFurin used in these experiments was able to cleave the fluorogenic substrate pERTKR-AMC and therefore do not believe that activity of the furin used is in question. Furin does rely on a calcium cofactor that is absent from PBS, which was used as a diluent for SEMA3F and rhFurin in these digests. However, because rhFurin is supplied with high levels of

calcium, it is not thought that this was related to the observed absence of cleavage. Additional digests were also conducted in a variety of buffers, including serum-free MEM, which is supplemented with calcium salts. Data from these digests are not shown, but the results were similar, again suggesting that any potential cleavage was not calcium-limited. Another possibility is that furin requires a biological cofactor in order to cleave SEMA3F. As SEMA3F has already been demonstrated to be cleaved in the conditioned media of SM/SEMA3F cells, one option to investigate this possibility might be to collect SM/SEMA3F conditioned media and supplement it with purified SEMA3F and varying levels of rhFurin. It is hypothesized that secreted proteases in the conditioned media could cause SEMA3F cleavage, so an additional assay comparing different incubation durations of SEMA3F in SM/SEMA3F conditioned media should also be performed.

A weakness in this study is the absence of a good positive control for the rhFurin digest of SEMA3F. Furin is part of the constitutive secretory pathway and has been implicated in the proteolytic maturation and/or inactivation of many biological enzymes, hormones and peptides, such as the transforming growth factor β (TGF-β) family. TGF-β1 has important roles in early embryonic development and arteriogenesis. When co-transfected with furin in the furin-deficient human LoVo cell line, the ~48 kD TGF-β1 was seen to be cleaved into ~33 and ~14.5 kD fragments (Dubois et al., 2001; Thomas, 2002). Another

possibility is VEGF-C, which when cleaved by furin, PCSK5 or PCSK7 exhibits a molecular weight shift from 59 to 29 kD (Siegfried et al., 2003).

Therapeutic application of SEMA3F is expected to benefit from increased biological half-life, one component of which is thermal stability at physiological temperatures. Although this study only specifically assessed the thermal stability of SEMA3F for periods up to 24 h, SEMA3F levels also remained stable over a 90 h incubation period with rhFurin at 37 ℃. In another set of experiments conducted by Matthew Migliozzi, Meetu Seth and Diane Bielenberg, two week administration of purified SEMA3F was achieved using a slow release osmotic pump co-implanted into mice with B16F10 mouse melanoma cells over a period of 27 days (unpublished data, 2013). When the osmotic pumps were implanted four days prior to tumor cells injections, but not when they were implanted nine days after, the resulting tumors exhibited decreased tumor volumes. Taken together, these data suggest that SEMA3F may be able to retain biological activity over durations of up to two weeks when protected from cleaveage. If so, delayed release delivery modalities for SEMA3F may be able to reduce the burden of receiving daily cancer treatment. It is important to note that none of these experiments are able to directly address the change in SEMA3F activity over prolonged exposures to 37 ℃ temperatures. In order to do so, collapse or migration assays utilizing SEMA3F subjected to varying 37 ℃ incubation times would need to be performed.

In an attempt to confirm the results of our purified protein digests *in vitro*, we examined the transfection of furin-deficient and furin-complemented CHO-K1 strains, respectively RPE.40 and 40.fur, with SEMA3F plasmid. At this point in time, the data suggest that the transfection of these cells lines was either not successful or that SEMA3F was not secreted into the assayed conditioned media, but was instead sequestered by the CHO-K1 cells. As CHO-K1 cells are widely used for protein synthesis and protein expression studies because they are easily transfected and have highly active secretory pathways, it seems unlikely that they are sequestering the SEMA3F.

In the transfection of CHO-K1 strains RPE.40 and 40.fur, 0.5 μg plasmid DNA per well in a 6-well plate was utilized. It has been found previously that this level is sufficient for high-efficiency transfection of HEK293 cells (Bielenberg et al., 2008). However, in transfecting RPE.40 cells with mouse furin to produce 40.fur, Moehring et al. (1993) used 20 μg of plasmid DNA per 5×10^5 cells in a calcium phosphate-based transfection method (Chen & Okayama, 1987). As each of the wells in this study was seeded with $1–1.5 \times 10^5$ cells, this represents at least a four-fold increase over the levels of plasmid DNA used in these transfections. Similarly, Kadlecova et al. (2012) transfected CHO-K1 cells using the Fugene reagent, as was done here, but utilizing 0.8 μg of plasmid DNA per well of a 12-well plate. Accounting for the difference in surface area between wells of 6-well and 12-well plates, this again represents a four-fold difference in plasmid amount used for transfection of CHO-K1 cells.

Future assays will increase the amount of plasmid used to transfect these cells in order to increase the transfection efficiency. If the transfection was successful in some cells, an alternative approach may be to enrich the conditioned media for glycoproteins in order to increase the concentration of SEMA3F to levels detectable by Western blot. One method to accomplish this might involve the use of sepharose beads incorporating concanavalin A.

The finding that rhFurin was unable to cleave SEMA3F in pure protein studies was surprising given the large body of literature that implicates furin or furin-like proteases in the cleavage of the semaphorin family. However, these studies primarily rely on the usage of non-specific furin inhibitors, especially RVKR-CMK, that are unable to selectively inhibit specific PCSK (Adams et al., 1997; Cameron et al., 2000; Christensen et al., 2005; Parker et al., 2010; Tian & Jianhua, 2010; Varshavsky et al., 2008; Zhong et al., 1999).

In experiments involving a fusion protein of human growth hormone and the C-terminal portion of SEMA3F (residues 605-785), Parker et al. (2010) reported that the furin-deficient CHO mutant FD11 was unable to cleave this fusion protein, whereas cleavage was observed in a furin-overexpressing CHO cell line. As they were primarily interested in the p100 to p95 cleavage, their fusion protein incorporated only residues C-terminal to the predicted p65 cleavage site KRRSRR. If furin does cleave the p95 cleavage site and not the p65 cleavage site, this may suggest a mechanism for the specific activation and inhibition of SEMA3F by proteolytic processing.

Discovery of a mechanism by which SEMA3 are selectively cleaved either to p95 or p65 forms would have profound implications. As previously mentioned, cleavage of SEMA3 have isoform-specific effects. Cleavage at the p65 site inactivates the repelling signals SEMA3A-B and converts the repelling signal SEMA3E to a prometastatic phenotype (Adams et al., 1997; Christensen et al., 2005; Klostermann et al., 1998; Varshavsky et al., 2008). As tumors typically upregulate PCSK activity, it follows that cleavage of the SEMA3 should result in tumor-favorable conditions, suggesting a trend wherein the p95 isoform is either more tumor-inhibiting or less tumor-enhancing than the corresponding p65 isoform. However, this trend may not be generalizable. Esselens et al. (2010) have suggested that SEMA3C which has been processed at the p95 site by the metalloproteinase ADAMTS1 is in fact prometastatic.

The activity of SEMA3F fragments has not been well characterized. Using a recombinant construct corresponding to the C-terminal end of the p30 fragment liberated from the cleavage of p95 to p65 SEMA3F, Guo et al. (2013) have suggested that the p30 fragment may be able to inhibit the interaction of VEGF with NRP1. If so, it is possible that this could present an additional therapeutic effect of administered full-length SEMA3F even after initial cleavage. It is also interesting that the p30 fragment of SEMA3F may interact with NRP1 when SEMA3F typically interacts with NRP2. However, SEMA3G displays similar activity, binding only NRP2 when full-length but both NRP1 and NRP2 post-processing (Kutschera et al., 2011).

Given the diverse nature of the bioactive SEMA3 cleavage fragments, more experiments will need to be conducted in order to specifically determine the role of p65 SEMA3F. If the protease responsible for SEMA3F cleavage is identified, one option to generate p65 SEMA3F may be to simply digest purified SEMA3F and use a size-exclusion filter to isolate the p65 isoform. One concern with this method is that the yield might be very low. Another option is to create an expression vector corresponding to p65 SEMA3F based on cleavage at the p65 putative cleavage site. In order to purify such a protein, it might be prudent to shift the His-tag from its current C-terminal position in the SEMA3F construct used for these studies to the N-terminal position in order not to interfere with any interactions occurring at the "cleaved" C-terminal end. To our knowledge, there have been no reports at this time of SEMA3F inducing a protumorigenic or prometastatic effect. As such, we do hypothesize that the p65 SEMA3F isoform at the very least does not encourage tumor growth or metastasis and may simply be inactive.

In addition to studying the cleavage of SEMA3F in RPE.40 and 40.fur cells, another approach would be to use siRNA as a method to downregulate specific proprotein convertases. This study suggests that SM/SEMA3F cells may be amenable to the use of siRNA. When SM/SEMA3F cells were exposed to siNRP2, there was a corresponding decrease in the levels of a ~120 kD protein that may correspond to NRP2. Follow-up assays are currently being conducted to

confirm this finding. In addition, the specificity of the NRP2 knockdown will be assessed by stripping and reprobing the blot with anti-NRP1 antibodies.

Data from the RVKR-CMK and H6 inhibition of SM/SEMA3F cells suggest that all PCSK except PCSK2 may be potential SEMA3 protease candidates for silencing by siRNA. Siegfried et al. (2003) have reported that VEGF-C, a competitive ligand for SEMA3F receptor NRP2, is cleaved and activated by furin, PCSK5 and PCSK7. Upon cleavage of proVEGF-C, mature VEGF-C is able to bind VEGFR2 and VEGFR3, activating both lymphangiogenesis and angiogenesis. Additional binding of NRP2 by mature VEGF-C is able to enhance the activation of VEGFR2 and VEGFR3 (Neufeld & Kessler, 2008; Xu et al., 2010). Interestingly, proVEGF-C is also able to bind VEGFR2 and VEGFR3, but it results in no autophosporylation of VEGFR2 and only weak phosphorylation of VEGFR3, demonstrating that proVEGF-C acts as an inhibitor of VEGF-C function (Joukov et al., 1997). The VEGF-C/NRP2/VEGFR2-3 axis is therefore a highly interesting therapeutic target for the prevention of blood and lymph vessel ingrowth which can facilitate tumor growth and metastasis. As SEMA3F also binds to NRP2 and is a negative regulator of lymphangiogenesis, an attractive hypothesis is therefore that one of these VEGF-C-cleaving proteases may also cleave and inactivate SEMA3F, facilitating the switch from an antiangiogenic state to a proangiogenic state.

As previously mentioned, wild-type SEMA3B is a particularly poor inhibitor of tumor progression because it is predominantly cleaved in the tumor

microenvironment. Therefore, studies investigating SEMA3B typically use a recombinant variant that lacks the p65 cleavage site (Varshavsky et al., 2008).

Similarly, not all tumors respond to SEMA3F, presumably because they either process and inactivate SEMA3F or lack the functional receptor, such as MCF7 breast tumor cells (Kigel et al., 2008). Identifying the proteases responsible for SEMA3F cleavage would help to better target SEMA3F therapy to receptive tumor targets and also help clarify why some tumors are refractory to SEMA3F treatment, such as NCI-H1299 or GLC45 lung cancer lines (Bielenberg et al., 2006). Identification of proteases for SEMA3F might also enable co-administration of SEMA3F with protease inhibitors in the future.

As another approach, this lab has engineered a recombinant SEMA3F construct, SEMA3FR, with a similar mutation in the p65 cleavage site (RRSRR → RASRA). Under the assumption that cleavage of SEMA3F to the p65 isoform inactivates SEMA3F, it is expected that SEMA3FR would exhibit greater therapeutic effect in the treatment of cancer due to its increased half-life.

In summary, these experiments suggest that despite numerous literature reports of furin-like PCSK cleavage of the SEMA3 family, furin itself may not cleave SEMA3F. Understanding the protease-specificity of the putative cleavage sites in SEMA3 family members and the activity of the resulting cleavage fragments of SEMA3F is expected to enhance the ability of SEMA3F to be used therapeutically. Future approaches to the study of SEMA3F cleavage will incorporate purified protein digests conducted within conditioned media,

expression of SEMA3F in PCSK-positive and PCSK-negative cell lines and siRNA approaches to silencing potential PCSK candidates for the cleavage of SEMA3F.

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