

1 **RGMs: Structural Insights, Molecular Regulation and Downstream Signaling**

2

3 Christian Siebold<sup>1,6</sup>, Toshihide Yamashita<sup>2,6</sup>, Philippe P. Monnier<sup>3,6</sup>, Bernhard K.

4 Mueller<sup>4,6</sup>, and R. Jeroen Pasterkamp<sup>5\*</sup>

5

6 <sup>1</sup>Division of Structural Biology, Wellcome Trust Centre for Human Genetics, University of  
7 Oxford, Oxford, UK

8 <sup>2</sup>Department of Molecular Neuroscience, Graduate School of Medicine, Osaka University, 2-  
9 2, Yamadaoka, Suita, Osaka, 565-0871, Japan

10 <sup>3</sup>Krembil Research Institute, 60 Leonard Street, M5T 2S8, Toronto, Ontario, Canada

11 <sup>4</sup>Neuroscience Discovery Research, Abbvie, Knollstrasse 50, 67061 Ludwigshafen, Germany

12 <sup>5</sup>Department of Translational Neuroscience, Brain Center Rudolf Magnus, University Medical  
13 Center, Universiteitsweg 100, 3584 CG, Utrecht, The Netherlands

14 <sup>6</sup>These authors have contributed equally to this work.

15

16 \*Correspondence: r.j.pasterkamp@umcutrecht.nl (R.J. Pasterkamp)

1   **Abstract**

2

3   Although originally discovered as neuronal growth cone collapsing factors, repulsive  
4   guidance molecules (RGMs) are now known as key players in many fundamental  
5   processes such as cell migration, differentiation, iron homeostasis and apoptosis during  
6   the development and homeostasis of many tissues and organs, including the nervous,  
7   skeletal and immune systems. Further, four RGMs (RGMa, RGMb/DRAGON,  
8   RGMc/hemojuvelin, RGMd) have been linked to the pathogenesis of various disorders  
9   ranging from multiple sclerosis to cancer and juvenile hemochromatosis. While the  
10   molecular details of these (patho)biological effects and signaling modes have long  
11   remained undiscovered, recent studies unveil several exciting and novel aspects of  
12   RGM processing, ligand-receptor interactions and downstream signaling. This review  
13   highlights recent advances in the mechanisms-of-action and function of RGM proteins.

14

15   Keywords: Axon guidance; BMP; Immune system; Iron metabolism; Neogenin;  
16   Proteolytic cleavage

17

## **RGMs: A Small Gene Family with Widespread Effects**

Guidance molecules, initially observed to direct growing axons during embryogenesis [1], also play crucial roles in the morphogenesis and homeostasis of non-neuronal tissues by controlling a plethora of cellular processes, ranging from cell division and migration to differentiation and death. The discovery of repulsive guidance molecule a (RGMa) in 2002 revealed a new family of guidance molecules (see Text Box 1). Since then, four RGMs have been found in vertebrate species; RGMa, RGMb (or DRAGON), RGMc (or hemojuvelin) and RGMd (only present in fish). Invertebrate species such as *C. elegans* possess one *RGM* gene.

RGMs are membrane-associated glycosylphosphatidylinositol (GPI)-linked proteins that harbor an N-terminal signal peptide, an RGD motif (RGMa and RGMc) and a partial von Willebrand type-D (vWFD) structural domain (Figure 1A). Each RGM displays tissue-specific expression, is subjected to distinct biosynthetic and processing steps, and has unique but also shared biological functions [2]. RGMs bind the type-1 transmembrane protein Neogenin (Figure 1A) and many of the reported biological effects of RGMs rely on Neogenin receptor functions, e.g. axon guidance or neuronal survival [3,4]. RGMs also serve as co-receptors for bone morphogenetic proteins (BMPs) (Figure 1A) to regulate iron metabolism and skeletal development [5–10] and axon regeneration [11]. In addition to these physiological roles, and as discussed below, RGMs have been implicated in various diseases and are considered to be promising targets in the treatment of multiple sclerosis (MS), spinal cord injury, stroke, anemia and inflammation [5,11–15].

Although the molecular mechanisms underlying the biological effects and signaling modes of RGMs have long remained undiscovered, recent work has unveiled

several exciting and novel aspects of RGM processing, ligand-receptor interactions and downstream signaling. These insights include high-resolution structural data of binary or tertiary protein complexes, the unique processing of RGMs into protein fragments with distinct functions, and the identification of a novel molecular mechanism to control ligand-induced ectodomain shedding of Neogenin. This review discusses recent highlights in RGM research starting from novel structural data to previously unexplored signaling mechanisms and cellular functions.

## **Structural Insight into Ligand-Receptor Interactions**

For many years, RGMs posed a molecular puzzle because of a general lack of structural homologies to any known protein fold. Recent studies have shed light on their 3-dimensional structure and identified two ordered and disulphide-stabilized domains that are connected by a flexible linker [16,17](Figure 1A-C). The RGM N-terminal domain (N-RGM) is composed of a three-helix bundle that harbors the “RGD” motif. RGD motifs are traditionally known to be important in integrin-mediated adhesion but no binding of RGMs to or signaling through integrins has been reported. The C-terminal domain (C-RGM) forms a tight  $\beta$ -sandwich structure and harbors a “GDPH” cleavage site, which mediates autoproteolysis (Figure 1A).

C-RGM is the major high-affinity interaction site for Neogenin [16], with an additional Neogenin binding site positioned in N-RGM, as suggested by antibody blocking experiments [11]. In the C-RGM-Neogenin (NEO1) complex structure, two RGM molecules act as a molecular staple bringing together the juxtamembrane fibronectin-type III (FNIII) domains of two Neogenin receptors (Figure 1B)[16]. This Neogenin region is necessary and sufficient for high affinity RGM-Neogenin

1 interaction as single point mutants of interface residues (either in RGM or Neogenin)  
2 abolish binding [16,18]. The RGM-Neogenin complex architecture is proposed to  
3 induce dimerization of Neogenin and to position its C-termini in close proximity to  
4 each other. These data hint at intracellular dimerization as a mechanism of signal  
5 transduction through the plasma membrane.

6

7 RGMs are crucial activators of BMP signaling and bind to BMP ligands with high  
8 affinity [17–19]. Crystal structures of all human RGMs in complex with the BMP  
9 ligand BMP2 have revealed a common mode of binding and have identified N-RGM  
10 as the high affinity interaction site for BMP ligands [17] (Figure 1C). This analysis also  
11 informed a potential molecular mechanism for **Juvenile Hemochromatosis** (JHH, see  
12 Glossary)-linked mutations in RGMc that occur at the binding interface with the BMP  
13 ligand, as these mutations disrupt the RGMc-BMP interaction. RGMc controls levels  
14 of **hepcidin** through BMP signaling and reduced hepcidin expression leads to the iron  
15 overload in the liver, heart and pancreas observed in JHH [8–10].

16 A comparison between the structures of the RGM-BMP and BMP-BMP type I receptor  
17 ectodomain complexes showed that RGM and the BMP type I receptor ectodomain of  
18 BMP-R1A share the same binding site on the BMP ligand. This was an unexpected  
19 discovery, because simultaneous binding of the BMP type I and type II receptors to the  
20 BMP ligand is an essential requirement for canonical, SMAD-dependent downstream  
21 signaling. So, how can RGM activate canonical BMP signaling despite it competing  
22 with BMP receptor binding? Since the RGM-BMP interaction is pH-dependent, unlike  
23 the BMP-BMP receptor complex [17], an endocytosis-linked mechanism for RGM-  
24 activated BMP signaling has been proposed. In this model, the RGM-BMP complex  
25 (potentially together with the BMP type II receptor) might be targeted into endosomes,

1 which are enriched with BMP type I receptors [20]. The lower pH of the endosomal  
2 environment might then lead to dissociation of the RGM-BMP complex and  
3 replacement by the BMP type I receptor leading to potentiation of SMAD signaling  
4 provided by the endosomal environment compared to the cell surface [20,21]. However,  
5 further work will be required to test this model, for example demonstrating the  
6 involvement of RGMs in BMP ligand endocytosis and showing that the endosome  
7 functions as a platform for BMP signaling. It will be interesting to see whether all RGM  
8 family members act via the same mechanism or whether the biological context,  
9 including available interaction partners, plays a role in directing how RGMs affect the  
10 BMP pathway. However, localization of the signaling machinery in close vicinity to  
11 the nucleus is a favorable way of activating target gene transcription. Such a mechanism  
12 has been suggested for other extracellular signaling systems including the epidermal  
13 growth factor and transforming growth factor signaling pathways [22,21,23].

14       Much evidence suggests that the Neogenin and BMP signaling pathways are  
15 functionally linked, most likely through the actions of RGMs [9,24–27]. The most  
16 compelling evidence is the iron overload observed in the livers of *Neogenin* knockout  
17 mice [28]. Structural experiments showing a direct physical interaction with RGM  
18 acting as a link between these two signaling pathways led to the crystal structure of a  
19 ternary complex composed of the BMP ligand BMP2, the full-length extracellular  
20 domain of RGMb, and the two membrane proximal FNIII domains of Neogenin [17].  
21 These results together with X-ray solution scattering and super-resolution microscopy  
22 [17] suggest a model by which RGMs mediate clustering of BMP and Neogenin dimers  
23 on the cell membrane. These data show that RGM is a structural bridge between  
24 Neogenin and BMP signaling, and inform that a clustering mechanism may be  
25 important in the activation of these signaling pathways.

## Cell Biology, Molecular Regulation and Downstream Signaling of RGMs

RGMs signal through both *trans* (intercellular) and *cis* (same cell) interactions. *Trans* signaling is relevant for cell functions such as axon growth and guidance [29,30], early stages of neurulation [31], CD4+ T-cell adhesion and activation [32], and leukocyte migration [33]. This can be either contact-dependent (adhesive) or mediated by gradients established by cleaved extracellular RGM isoforms. The location of the RGM-binding site on the Neogenin-FNIII domains, close to the plasma membrane, and the RGM-Neogenin complex architecture [16] indicate a binding mode for which the release of the RGM ectodomain expressed by neighboring cells might be a prerequisite (Figure 1D). In contrast, RGM ectodomain shedding may not be required in situations where both Neogenin and RGM are expressed on the same cell surface (*cis* signaling), such as in hepatocytes [25] and chondrocytes [9] (Figure 1E).

Work in the past decade has led to the identification of several (co)receptors for RGMs as well as components of downstream signaling pathways that mediate their biological effects. Further, RGMs have been implicated in a plethora of cell biological effects (e.g. in axon growth and iron homeostasis), in different organs (e.g. in the brain, skeleton and immune system), and in relation to diverse human disorders (e.g. in spinal cord injury and cancer). Furthermore, recent data indicate that both RGMs and Neogenin are proteolytically processed. This processing serves to release RGMs from the cell surface in certain situations as well as to diversify the effects of the RGM signaling pathway and to control signaling duration following ligand binding. Here, we highlight some of the most recent insights into RGM processing and signaling in the

context of specific biological functions, and refer to other reviews for a detailed discussion on other signaling mechanisms and biological effects [2,8,10,34,35].

#### *Autocatalytic and Proteolytic Cleavage of RGMc in the Control of Body Iron Levels*

All RGMs contain an autocatalytic Gly-Asp-Pro-His (GDPH) cleavage site (Figure 1A), which is known to be unstable under mildly acidic conditions due to a specific conformation and/or general acid catalysis [24]. Incubation of purified recombinant RGMc at pH 5.5 increases autocatalytic cleavage, supporting the idea that RGMc undergoes partial autocatalytic cleavage within the GDPH sequence [24]. The GDPH site is found in the loop connecting two  $\beta$  sheets (Figure 1A, 3A) and is highly conserved across species. Autocatalytic cleavage does not lead to a secreted fragment of RGM, since the resulting two polypeptides are joined covalently together by disulfide bonds to form a stable structural unit. Autocatalytic cleavage seems to be important for the correct folding of the protein [16]. A clue about the functional importance of RGM autocatalytic cleavage comes from genetic studies in patients suffering from JHH. Several JHH-associated mutations are located in the vicinity of the autocatalytic cleavage site for RGMc [16,36,37]. These mutants are often retained in the ER and have low signaling activities, suggesting that autocatalytic processing is necessary for RGMc plasma membrane expression and for its iron regulatory function. Further, experiments using non-cleavable RGMa mutants reveal that RGMa autocatalytic processing is also required for its growth inhibitory effects on axons [38]. Thus, autocatalytic processing appears to be a general and important feature of RGMs.

In addition to autocatalytic cleavage, proteolytic processing of RGMc by furin and serine protease matriptase-2 (TMPRSS6) plays a role in the regulation of body iron levels. Membrane-bound RGMc acts as a co-receptor for BMPs to regulate



1 hepcidin expression resulting in increased iron absorption. RGMc cleavage by furin  
2 at a specific C-terminal cleavage site, not present in RGMa or RGMb, releases a 42  
3 kDa soluble protein, which acts as a decoy receptor that competes with membrane-  
4 bound RGMc for binding to BMP ligands, thereby suppressing hepcidin expression  
5 [39]. In addition, the serine protease matriptase-2 (TMPRSS6) binds and cleaves cell  
6 surface RGMc. However, the RGMc fragment shed by matriptase-2 has reduced  
7 ability to bind BMPs and fails to repress BMP-induced hepcidin expression *in vitro*  
8 [40,41]. Unlike furin, it is thought that matriptase-2 impacts iron homeostasis mainly  
9 by reducing levels of membrane-bound RGMc. However, other work suggests that  
10 the interaction between matriptase-2 and RGMc may be more complex, e.g.  
11 independent of protease activity [42]. Further work is needed to assess the  
12 requirement of RGMc cleavage by matriptase-2 *in vivo* and the precise link between  
13 matriptase-2, RGMc, BMP signaling and hepcidin [10]. Nevertheless, these studies  
14 reveal important roles for proteolytic processing of RGMc in the regulation of  
15 hepcidin expression and iron levels.

#### 17 *Ectodomain Shedding of Neogenin*

18 Over the past years many groups have confirmed the ability of RGMs to induce axon  
19 repulsion, neurite growth inhibition and growth cone collapse using different types of  
20 neurons [13,29,43–46]. The role of RGMs in axon guidance and neurite growth  
21 inhibition during development has been shown in frog and chick embryos, while *in vivo*  
22 evidence for the neurite growth inhibitory effects of RGMs in mammalian species is  
23 provided by the ability of RGM blockage to promote CNS axon regeneration. Other  
24 important effects of RGM on cell death, migration and differentiation have also been  
25 confirmed (see Text Box 1 and 2)[11,13,47–49]. The neurite growth inhibitory effects

1 of RGMs have been studied extensively and rely on signaling by Rho-GTPases  
2 downstream of RGMa. Binding of RGMa to Neogenin activates RhoA through the GEF  
3 LARG in an Unc5B-dependent manner [50]. In parallel, RGM-Neogenin interactions  
4 trigger a reduction in Ras activity through focal adhesion kinase (FAK) and p120GAP  
5 [51](Figure 2). However, these signaling events provide a simplified view of RGM  
6 signaling and, as discussed, precise regulation of the sensitivity of neurons to RGM of  
7 processing of RGMs into distinct polypeptides appears to be a prerequisite for the  
8 formation of appropriate neuronal networks.

9       The finding that cell surface shedding of Neogenin by **ADAM17** desensitizes  
10 axons to RGMa suggested a role for proteolysis in Neogenin signaling [52]. The  
11 extracellular part of Neogenin binds and is cleaved by ADAM17. This cleavage event  
12 induces ectodomain shedding and thereby reduces Neogenin cell surface expression.  
13 However, how cleavage of Neogenin by ADAM17 is initially prevented to allow  
14 cleavage only after ligand binding remained unknown. Recent findings show that the  
15 transmembrane leucine-rich repeat protein Lrig2 negatively regulates ADAM17-  
16 mediated guidance receptor proteolysis in neurons [48]. Lrig2 binds Neogenin and  
17 prevents premature Neogenin shedding by ADAM17 (Figure 2). RGMa reduces Lrig2-  
18 Neogenin interactions, providing ADAM17 access to Neogenin and allowing this  
19 protease to induce ectodomain shedding. This study identified a unique ligand-gated  
20 mechanism that controls receptor shedding by ADAMs and shows that the functions of  
21 Lrig2 are required for effects of RGM on neurite growth, cortical neuron migration and  
22 regenerative failure.

23

24 *Proteolysis of RGMs Into Different Functional Protein Fragments*

1   Proprotein Convertases (PCs) form a family of nine proteinases. Two of them, furin  
2   and subtilisin kexin isozyme-1 (SKI-1), process RGMa into C-terminal membrane-  
3   bound (C-RGM) and N-terminal soluble (N-RGM) fragments (Figure 3A)[38,46].  
4   Interestingly, these cleavage events are dependent on RGM autoproteolysis. D149A  
5   and H151A mutations in the autocatalytic cleavage sequence do not alter RGMa  
6   processing toward the cell surface, but abolish processing by both SKI-1 and furin [38].  
7   The functional significance of C-RGMa and N-RGMa fragments *in vivo* has been  
8   studied using the chick retinotectal system. Retinal ganglion cell (RGC) neurons reside  
9   in the eye and send their axons in a topographic manner through the optic nerve to the  
10   tectum in the brain (Figure 3B). RGMa is expressed in an anterior low to posterior high  
11   gradient in the embryonic tectum, whereas Neogenin is expressed in the retina in a  
12   temporal high to nasal low gradient (Figure 3B). The RGMa gradient in the tectum  
13   restricts temporal RGC axons to the anterior part of the tectum, whereas nasal axons  
14   can target the posterior part. When retinal axons reach the tectum, they will first extend  
15   within the most superficial layer of the optic tectum, the stratum opticum (SO). Once  
16   axons reach the appropriate anterior-posterior coordinates in the tectum, they turn into  
17   deeper layers to establish terminal arbors within layers a-f of the stratum griseum et  
18   fibrosum superficiale (SGFS)(Figure 3B)[27]. Ectopic expression of C-RGMa  
19   throughout the tectum leads to axon overshooting mainly in the superficial layer, while  
20   ectopic N-RGMa induces overshooting of RGC axons into deeper layers of the tectum  
21   (Figure 3B). In agreement with a role for C-RGMa and N-RGMa in retinotectal  
22   pathfinding, *in vivo* inhibition of C-RGMa or N-RGMa with recombinant antibodies in  
23   chick leads to defects in the targeting of RGC axons [46].

24         It is unknown why ectopic tectal expression of C-RGMa and N-RGMa  
25   differently affects retinal axon targeting *in vivo*. A possible explanation is that the

1 peptides activate distinct downstream signaling pathways that exert quantitatively or  
2 quantitatively distinct effects on embryonic axons. Indeed, while both C-RGMa and N-  
3 RGMa require Neogenin to regulate axon development, N-RGMa influences axon  
4 growth and guidance *in vitro*, while C-RGMa only affects axon growth. Furthermore,  
5 these effects are induced through the activation of distinct downstream pathways. C-  
6 RGMa leads to stimulation of the signaling cascade involving activation of LARG,  
7 RhoA and ROCK, whereas signaling downstream of N-RGMa is thought to rely on  $\gamma$ -  
8 secretase cleavage of the intracellular domain of Neogenin [53] (Figure 2). Many cell  
9 surface receptors undergo cleavage by  $\gamma$ -secretase triggering the subsequent release of  
10 their intracellular domain (ICD). Often this intracellular cleavage event is preceded by  
11 proteolytic release of the receptor ectodomain followed by shuttling of the ICD into the  
12 nucleus. NeICD harbors NLS and NES sequences, binds various nuclear proteins and  
13 acts as a transactivator of gene transcription [54].  $\gamma$ -secretase cleavage is required for  
14 RGMa-mediated axon repulsion *in vitro* and ectopic NeICD expression induces RGC  
15 axon targeting effects *in vivo* [53]. One of the binding partners of NeICD is LIM-only  
16 protein 4 (LMO-4), a transcriptional coactivator [55]. LMO-4 is required for the axon  
17 repulsive activity of RGMa and for targeting of RGC axons in the tectum by N-RGMa.  
18 Recent evidence indicates that Frazzled (Fra), a close homologue of Neogenin, is also  
19 processed by  $\gamma$ -secretase to release its intracellular domain (Fra-ICD) [56]. Fra-ICD  
20 shuttles between the cytoplasm and the nucleus where it works as a transcriptional  
21 factor that regulates Commissureless expression to control axon midline crossing.  
22 NeICD may serve a similar function and act as a transcription factor. It is interesting to  
23 note that LMO-4 functions as a novel co-factor of NGN2 (Neurogenin) in the  
24 developing cortex [57]. LMO-4 binds NGN2 to form a multi-protein transcription  
25 complex. This complex is recruited to the E-box containing enhancers of NGN2-target

1 genes, which regulate various aspects of cortical development and activate NGN2-  
2 mediated transcription [57]. It will be interesting to determine whether LMO-4 and  
3 NeICD (and perhaps NGN-2) form a transcription complex that regulates genes  
4 involved in axon growth. While *in vivo* work suggests that the axon repulsive effects of  
5 N-RGMA are independent of LARG, *in vitro* studies have shown that LMO-4  
6 knockdown inhibits RhoA activation by RGMA [55]. Therefore, further studies are  
7 needed to probe the role of RhoA downstream of N-RGMA and to address other open  
8 questions such as is  $\gamma$ -secretase required *in vivo* for the effects of N-RGMA.

9       Together these studies reveal that RGMA processing by PCs generates distinct  
10 RGMA fragments that signal through different signaling cascades (LARG – RhoA –  
11 ROCK versus NeICD – LMO-4) to exert specific biological effects. This suggests that  
12 proteolytic processing of RGMs, together with the ability of RGMs to signal in *trans*  
13 and *cis* and their link to different signaling systems (Neogenin, BMP), functions to  
14 diversify the effects of these proteins. This helps to explain how a small family of  
15 proteins can regulate a disproportionately large number of biological events in different  
16 tissues and organ systems. Finally, it is interesting to note that pre-incubation of  
17 Neogenin with C-RGMA abolishes Neogenin-N-RGMA binding, while vice versa N-  
18 RGMA reduces interactions between C-RGMA and Neogenin [38]. It is therefore  
19 possible that the local concentration of C-RGMA or N-RGMA determines which of the  
20 two peptides will prevalently interact with Neogenin to influence axons.

21

## 22 *Lipid Raft Localization of Neogenin*

23 RGMs play a crucial role in BMP signaling. For an extensive description of the  
24 signaling pathways involved in these effects and the proposed role of Neogenin we refer  
25 to other reviews [8,10,58]. However, it should be noted that whereas early studies failed

1 to implicate BMPs in RGM-mediated effects on developing neurons, more recent work  
2 suggests that RGMs may function through BMPs to affect neurons [27,45,59]. A  
3 compelling example of the functional interplay between RGMs, Neogenin and BMPs  
4 is the role of these proteins in endochondrial bone development during skeleton  
5 formation. During this process, Neogenin controls chondrocyte maturation by  
6 promoting BMP-induced receptor association with lipid rafts, thus enhancing effective  
7 BMP receptor concentration or BMP binding affinity and increasing SMAD  
8 phosphorylation and downstream gene transcription [9]. How does Neogenin localize  
9 BMP receptors to lipid rafts? RGMs were found to form a protein bridge between  
10 Neogenin and BMP receptors thereby inducing the formation of a multimeric receptor  
11 complex. Since RGMs contain GPI domains that localize these proteins to lipid rafts  
12 [29], the authors proposed that RGMs may be responsible for moving the Neogenin-  
13 RGM-BMP receptor complex into lipid rafts. The presence of Neogenin in lipid rafts  
14 is not only required during endochondrial bone development but also for its neurite  
15 growth inhibitory and neuron death-inducing effects in the nervous system [15,27].  
16 Interference with RGM-Neogenin binding using specific protein fragments or anti-  
17 RGMa antibodies causes Neogenin to move out of lipid rafts and prevents pro-apoptotic  
18 and neurite growth inhibitory effects. Application of these tools in models for brain or  
19 axonal injury (e.g. middle cerebral artery occlusion or optic nerve crush) promotes  
20 regeneration and functional recovery [15,27]. This suggests that interfering with the  
21 lipid raft localization of Neogenin may represent a powerful means of neutralizing the  
22 detrimental effects of RGM-Neogenin following injury or disease.

23  
24 *Actin Regulation During Epithelial Cell Adhesion*

1 Epithelial morphogenesis is fundamental to organogenesis in the embryo. Epithelial  
2 sheets undergo choreographed movements to generate complex structures such as the  
3 neural tube. Early depletion of Neogenin or RGMa in the neuroepithelium leads to loss  
4 of adhesion and apicobasal polarity, and as a result a failure in neural tube closure  
5 [31,47,60,61]. E-cadherin-mediated cell-cell adhesion found at **adherens junctions**  
6 (AJs) plays a key role in maintaining the fidelity of the epithelium. Junctional stability  
7 requires reciprocal interactions between the cadherins and the circumferential actin ring  
8 running parallel to the AJ. The actin ring undergoes continuous turnover and failure to  
9 rebuild the ring causes loss of adhesion. Interestingly, recent work identifies Neogenin  
10 as a key component of the actin nucleation machinery governing AJ stability [62].  
11 Neogenin promotes the formation of stable actin rings at AJs by spatially coupling  
12 Arp2/3-mediated actin nucleation to the AJ via recruitment of the wave regulatory  
13 complex (**WRC**) (Figure 2). A direct interaction between the Neogenin **WIRS** domain  
14 and the WRC is crucial for the restricted localization of the WRC and Arp2/3 to the  
15 junction. Neogenin is not sufficient to activate Arp2/3, which requires activation of Rac  
16 and Sra1 binding. Neogenin also affects E-cadherin recycling, but whether this effect  
17 depends on its ability to control actin dynamics is unknown. Knockdown of RGMa  
18 induces defects in AJ stability and WRC localization similar to those observed  
19 following Neogenin depletion [62]. This suggests that RGMa and Neogenin act  
20 together at the AJ. However, the precise mode-of-action of RGMa at the AJ, e.g.  
21 whether it functions in *cis* or *trans* with Neogenin, remains unknown. As growth cone  
22 steering is highly dependent on actin regulation, it will be interesting to determine  
23 whether the induction of actin nucleation by Neogenin plays a role at neuronal growth  
24 cones as well.

25

# 1 *Immune Cell Signaling and Multiple Sclerosis*

2 A new role of RGMA in the immune system has become recently apparent [32,33].  
3 Bone marrow-derived dendritic cells express RGMA and Neogenin is expressed by  
4 CD4<sup>+</sup> T-lymphocytes. Binding of RGMA to Neogenin-positive CD4<sup>+</sup> T cells induces  
5 activation of the small GTPase Rap1, thereby increasing adhesion to intracellular  
6 adhesion molecule-1 (ICAM-1). Thus, in contrast to Neogenin-induced growth cone  
7 collapse, which results in rapid loss of adhesion [45], binding between RGMA and  
8 Neogenin on immune cells can trigger enhanced adhesion. This increase in ICAM-1-  
9 adhesion may facilitate invasion of immune cells into the MS brain, making RGMA a  
10 novel therapeutic target for disease. An antibody blocking RGMA was able to improve  
11 disease scores in commonly used MS mouse models. Treatment of mice with the anti-  
12 RGMA antibody reduced invasion of inflammatory cells into the CNS. The antibody  
13 also affected T cell proliferation and cytokine production in a mouse model and in  
14 isolated PBMCs (peripheral blood mononuclear cells) from MS patients [32],  
15 indicating a T cell immune suppressive effect. These experiments suggest that blocking  
16 RGMA may reduce inflammatory disease. However, another study reported that RGMA  
17 inhibits migration of RGMA-expressing leukocytes (T- and B-lymphocytes, monocytes,  
18 granulocytes) via chemo- and contact-repulsion and RGMA suppressed inflammation  
19 in a zymosan-induced peritonitis model [33]. These seemingly contrasting results might  
20 be explained by the different types of models used and by differences in underlying  
21 signaling complexes. Whereas the focus of both studies was the adaptive immune  
22 system, a third study focused on the innate immune system (microglia cells and  
23 macrophages) and its role in MS-associated neurodegeneration [11]. Highly  
24 inflammatory microglial cells and macrophages have been postulated to play an  
25 important role in progressive MS and are the target of several new therapeutic drug



1 approaches. Systemic treatment of **EAE** rats with RGMa-specific antibodies resulted  
2 in significant and highly reproducible functional improvement, reduction of the size of  
3 the microglial lesion, enhanced axon regeneration into the inflammatory lesion and  
4 signs of remyelination [11]. One of the first symptoms of MS in 20-30% of patients is  
5 an optic neuritis, an inflammatory attack of the optic nerve. Such an attack can have a  
6 strong impact on RGCs and their axons forming the retinal nerve fiber layer (RNFL).  
7 In a tEAE optic neuritis model, systemic application of RGMa-specific antibodies  
8 dramatically reduced degeneration of the RNFL, suggesting that RGMa is not only  
9 involved in inhibition of axon regeneration but also in cell death regulation [11]. How  
10 does RGMa contribute to neurodegeneration in MS? Recent work shows that IL-17-  
11 expressing CD4<sup>+</sup> T cells (Th17 cells) strongly express RGMa and that Th17 cells induce  
12 neuronal cell death probably via RGMa-Neogenin-induced dephosphorylation of Akt  
13 [14]. Together, these studies implicate RGMa in immune regulation and disease. Future  
14 work is needed to unravel the precise molecular details of these effects.

15

## 16 **Concluding Remarks**

17 It is an exciting time to study RGMs. During the first RGM symposium (Awaji, Japan,  
18 April 2-3, 2016), leading RGM experts from all over the world presented new and very  
19 promising data that showed that since their original discovery as tectum-derived axon  
20 repellents, RGM proteins have emerged as pleiotropic regulators of a multitude of cell  
21 biological processes in many different tissues. Often these novel cellular functions have  
22 only been probed in one specific tissue and an important future goal is therefore to  
23 assess whether some of the newly discovered functions of RGMs also contribute to the  
24 development or homeostasis of other tissues (see Outstanding Questions). For example,  
25 given the important role of regulation of the actin cytoskeleton in growth cone collapse

1 is tempting to speculate that Neogenin may also control WRC-Arp2/3 signaling in  
2 growth cones as has been shown recently at AJs. Further, while the ability of RGMs to  
3 control BMP signaling in regulating iron homeostasis and endochondral bone formation  
4 have been firmly established, the contribution of BMPs to other RGM-mediated effects  
5 remains largely unexplored. It has become clear that RGMs have diverse binding  
6 partners (e.g. BMPs and Neogenin), while these binding partners can also interact with  
7 proteins unrelated to RGMs such as Netrin-1 or BMP receptors. An important challenge  
8 is to understand how these interactions are regulated. Do different binding partners  
9 compete for binding on RGMs or Neogenin? For example, since RGMa and Netrin-1  
10 both bind the Neogenin FNIII region do these proteins compete for binding to  
11 Neogenin? Do ligands such as RGMs and Netrins activate similar or distinct signaling  
12 cascades downstream of Neogenin? How can RGMs signal axon repulsion and cell  
13 adhesion through the same receptor? Thus far, many studies have employed vertebrate  
14 models to dissect the functions and signaling pathways of RGMs. However, many  
15 invertebrate species have a RGM gene and therefore represent excellent models to  
16 address outstanding questions about RGM biology.

17 RGMs are being implicated in an ever increasing number of diseases, ranging  
18 from cancer and MS to JHH. An interesting observation in the CNS is the consistent  
19 upregulation of RGMs following a wide variety of insults (e.g. immune-mediated,  
20 neurodegeneration or trauma). The first results of blocking RGMs in experimental  
21 models are very promising and a first clinical trial of anti-RGMa blocking antibodies  
22 in MS patients is currently being conducted. In addition to blocking RGM function it  
23 will be important to better understand how RGMs normally function as this will  
24 undoubtedly contribute to our ability to define the pathogenic mechanisms underlying

- 1 specific disorders and to eventually design novel and more effective therapeutic
- 2 strategies.
- 3
- 4

1    **Acknowledgements**

2    We thank Hidekiyo Harada for help with preparing Figure 3. Work on RGMs in the  
3    laboratories of the authors was supported in part by a VICI grant from the Netherlands  
4    Organization for Scientific Research (ALW) and by a grant from the UU strategic  
5    theme Dynamics of Youth to R.J.P. C.S. is supported by a Cancer Research UK Senior  
6    Research Fellowship (C20724/A14414) and a European Research Council (ERC)  
7    Consolidator award (647278).

## 1   **References**

- 2   1     Kolodkin, A.L. and Pasterkamp, R.J. (2013) SnapShot: Axon guidance II. *Cell*
- 3       153, 722.e1
- 4   2     Severyn, C.J. *et al.* (2009) Molecular biology, genetics and biochemistry of the
- 5       repulsive guidance molecule family. *Biochem. J.* 422, 393–403
- 6   3     Rajagopalan, S. *et al.* (2004) Neogenin mediates the action of repulsive
- 7       guidance molecule. *Nat. Cell Biol.* 6, 756–62
- 8   4     Matsunaga, E. *et al.* (2004) RGM and its receptor neogenin regulate neuronal
- 9       survival. *Nat. Cell Biol.* 6, 749–55
- 10  5     Babitt, J.L. *et al.* (2006) Bone morphogenetic protein signaling by hemojuvelin
- 11       regulates hepcidin expression. *Nat. Genet.* 38, 531–9
- 12  6     Babitt, J.L. *et al.* (2005) Repulsive guidance molecule (RGMa), a DRAGON
- 13       homologue, is a bone morphogenetic protein co-receptor. *J. Biol. Chem.* 280,
- 14       29820–7
- 15  7     Samad, T.A. *et al.* (2005) DRAGON, a bone morphogenetic protein co-
- 16       receptor. *J. Biol. Chem.* 280, 14122–9
- 17  8     Corradini, E. *et al.* The RGM/DRAGON family of BMP co-receptors. *Cytokine*
- 18       *Growth Factor Rev.* 20, 389–98
- 19  9     Zhou, Z. *et al.* (2010) Neogenin regulation of BMP-induced canonical Smad
- 20       signaling and endochondral bone formation. *Dev. Cell* 19, 90–102
- 21  10    Tian, C. and Liu, J. (2013) Repulsive guidance molecules (RGMs) and
- 22       neogenin in bone morphogenetic protein (BMP) signaling. *Mol. Reprod. Dev.*
- 23       80, 700–17
- 24  11    Demicheva, E. *et al.* (2015) Targeting repulsive guidance molecule A to
- 25       promote regeneration and neuroprotection in multiple sclerosis. *Cell Rep.* 10,

1 1887–98

2 12 Feng, J. *et al.* (2012) RNA interference against repulsive guidance molecule A  
3 improves axon sprout and neural function recovery of rats after  
4 MCAO/reperfusion. *Exp. Neurol.* 238, 235–242

5 13 Hata, K. *et al.* (2006) RGMa inhibition promotes axonal growth and recovery  
6 after spinal cord injury. *J. Cell Biol.* 173, 47–58

7 14 Tanabe, S. and Yamashita, T. (2014) Repulsive guidance molecule-a is  
8 involved in Th17-cell-induced neurodegeneration in autoimmune  
9 encephalomyelitis. *Cell Rep.* 9, 1459–70

10 15 Shabanzadeh, A.P. *et al.* (2015) Uncoupling Neogenin association with lipid  
11 rafts promotes neuronal survival and functional recovery after stroke. *Cell*  
12 *Death Dis.* 6, e1744

13 16 Bell, C.H. *et al.* (2013) Structure of the repulsive guidance molecule (RGM)-  
14 neogenin signaling hub. *Science* 341, 77–80

15 17 Healey, E.G. *et al.* (2015) Repulsive guidance molecule is a structural bridge  
16 between neogenin and bone morphogenetic protein. *Nat. Struct. Mol. Biol.* 22,  
17 458–65

18 18 Yang, F. *et al.* (2008) Neogenin interacts with hemojuvelin through its two  
19 membrane-proximal fibronectin type III domains. *Biochemistry* 47, 4237–45

20 19 Wu, Q. *et al.* (2012) Repulsive guidance molecule (RGM) family proteins  
21 exhibit differential binding kinetics for bone morphogenetic proteins (BMPs).  
22 *PLoS One* 7, e46307

23 20 Hartung, A. *et al.* (2006) Different Routes of Bone Morphogenic Protein  
24 (BMP) Receptor Endocytosis Influence BMP Signaling. *Mol. Cell. Biol.* 26,  
25 7791–7805

1    21    Di Guglielmo, G.M. *et al.* (2003) Distinct endocytic pathways regulate TGF-  
2        beta receptor signalling and turnover. *Nat. Cell Biol.* 5, 410–21

3    22    Vieira, A. V *et al.* (1996) Control of EGF receptor signaling by clathrin-  
4        mediated endocytosis. *Science* 274, 2086–9

5    23    Le Roy, C. and Wrana, J.L. (2005) Clathrin- and non-clathrin-mediated  
6        endocytic regulation of cell signalling. *Nat. Rev. Mol. Cell Biol.* 6, 112–26

7    24    Zhang, A.-S. *et al.* (2005) Interaction of hemojuvelin with neogenin results in  
8        iron accumulation in human embryonic kidney 293 cells. *J. Biol. Chem.* 280,  
9        33885–94

10   25    Zhang, A.-S. *et al.* (2009) Hemojuvelin-neogenin interaction is required for  
11       bone morphogenic protein-4-induced hepcidin expression. *J. Biol. Chem.* 284,  
12       22580–9

13   26    Tian, C. *et al.* (2013) The neogenin/DCC homolog UNC-40 promotes BMP  
14       signaling via the RGM protein DRAG-1 in *C. elegans*. *Development* 140,  
15       4070–80

16   27    Tassew, N.G. *et al.* (2014) Modifying lipid rafts promotes regeneration and  
17       functional recovery. *Cell Rep.* 8, 1146–59

18   28    Lee, D.-H. *et al.* (2010) Neogenin inhibits HJV secretion and regulates BMP-  
19       induced hepcidin expression and iron homeostasis. *Blood* 115, 3136–45

20   29    Monnier, P.P. *et al.* (2002) RGM is a repulsive guidance molecule for retinal  
21       axons. *Nature* 419, 392–5

22   30    Wilson, N.H. and Key, B. (2006) Neogenin interacts with RGMa and netrin-1  
23       to guide axons within the embryonic vertebrate forebrain. *Dev. Biol.* 296, 485–  
24       98

25   31    Kee, N. *et al.* (2008) Neogenin and RGMa control neural tube closure and

1        neuroepithelial morphology by regulating cell polarity. *J. Neurosci.* 28, 12643–  
2        53

3    32    Muramatsu, R. *et al.* (2011) RGMa modulates T cell responses and is involved  
4        in autoimmune encephalomyelitis. *Nat. Med.* 17, 488–94

5    33    Mirakaj, V. *et al.* (2011) Repulsive guidance molecule-A (RGM-A) inhibits  
6        leukocyte migration and mitigates inflammation. *Proc. Natl. Acad. Sci. U. S. A.*  
7        108, 6555–60

8    34    Yamashita, T. *et al.* (2007) Neogenin and repulsive guidance molecule  
9        signaling in the central nervous system. *Curr. Opin. Neurobiol.* 17, 29–34

10   35    De Vries, M. and Cooper, H.M. (2008) Emerging roles for neogenin and its  
11        ligands in CNS development. *J. Neurochem.* 106, 1483–92

12   36    Lanzara, C. *et al.* (2004) Spectrum of hemojuvelin gene mutations in 1q-linked  
13        juvenile hemochromatosis. *Blood* 103, 4317–21

14   37    Lee, P.L. *et al.* (2004) Hemojuvelin (HJV) mutations in persons of European,  
15        African-American and Asian ancestry with adult onset haemochromatosis. *Br.*  
16        *J. Haematol.* 127, 224–9

17   38    Tassew, N.G. *et al.* (2012) SKI-1 and Furin generate multiple RGMa fragments  
18        that regulate axonal growth. *Dev. Cell* 22, 391–402

19   39    Silvestri, L. *et al.* (2007) Defective targeting of hemojuvelin to plasma  
20        membrane is a common pathogenetic mechanism in juvenile hemochromatosis.  
21        *Blood* 109, 4503–10

22   40    Silvestri, L. *et al.* (2008) Furin-mediated release of soluble hemojuvelin: a new  
23        link between hypoxia and iron homeostasis. *Blood* 111, 924–31

24   41    Maxson, J.E. *et al.* (2010) Matriptase-2- and proprotein convertase-cleaved  
25        forms of hemojuvelin have different roles in the down-regulation of hepcidin



1 expression. *J. Biol. Chem.* 285, 39021–8

2 42 Guillem, F. *et al.* (2012) Inactive matriptase-2 mutants found in IRIDA patients  
3 still repress hepcidin in a transfection assay despite having lost their serine  
4 protease activity. *Hum. Mutat.* 33, 1388–96

5 43 Brinks, H. *et al.* (2004) The repulsive guidance molecule RGMa is involved in  
6 the formation of afferent connections in the dentate gyrus. *J. Neurosci.* 24,  
7 3862–9

8 44 Matsunaga, E. *et al.* (2006) Repulsive guidance molecule plays multiple roles  
9 in neuronal differentiation and axon guidance. *J. Neurosci.* 26, 6082–8

10 45 Conrad, S. *et al.* (2007) Neogenin-RGMa signaling at the growth cone is bone  
11 morphogenetic protein-independent and involves RhoA, ROCK, and PKC. *J.*  
12 *Biol. Chem.* 282, 16423–33

13 46 Tassew, N.G. *et al.* (2009) Sustained in vivo inhibition of protein domains  
14 using single-chain Fv recombinant antibodies and its application to dissect  
15 RGMa activity on axonal outgrowth. *J. Neurosci.* 29, 1126–31

16 47 Niederkofler, V. *et al.* (2004) Repulsive guidance molecule (RGM) gene  
17 function is required for neural tube closure but not retinal topography in the  
18 mouse visual system. *J. Neurosci.* 24, 808–18

19 48 van Erp, S. *et al.* (2015) Lrig2 Negatively Regulates Ectodomain Shedding of  
20 Axon Guidance Receptors by ADAM Proteases. *Dev. Cell* 35, 537–52

21 49 Kam, J.W.K. *et al.* (2016) RGMB and neogenin control cell differentiation in  
22 the developing olfactory epithelium. *Development* 143, 1534–46

23 50 Hata, K. *et al.* (2009) Unc5B associates with LARG to mediate the action of  
24 repulsive guidance molecule. *J. Cell Biol.* 184, 737–50

25 51 Endo, M. and Yamashita, T. (2009) Inactivation of Ras by p120GAP via focal

1        adhesion kinase dephosphorylation mediates RGMa-induced growth cone  
2        collapse. *J. Neurosci.* 29, 6649–62

3    52    Okamura, Y. *et al.* (2011) TACE cleaves neogenin to desensitize cortical  
4        neurons to the repulsive guidance molecule. *Neurosci. Res.* 71, 63–70

5    53    Banerjee, P. *et al.* (2016) γ-secretase and LARG mediate distinct RGMa  
6        activities to control appropriate layer targeting within the optic tectum. *Cell*  
7        *Death Differ.* 23, 442–53

8    54    Goldschneider, D. *et al.* (2008) The neogenin intracellular domain regulates  
9        gene transcription via nuclear translocation. *Mol. Cell. Biol.* 28, 4068–79

10   55    Schaffar, G. *et al.* (2008) LIM-only protein 4 interacts directly with the  
11        repulsive guidance molecule A receptor Neogenin. *J. Neurochem.* 107, 418–31

12   56    Neuhaus-Follini, A. and Bashaw, G.J. (2015) The Intracellular Domain of the  
13        Frazzled/DCC Receptor Is a Transcription Factor Required for Commissural  
14        Axon Guidance. *Neuron* 87, 751–763

15   57    Asprer, J.S.T. *et al.* (2011) LMO4 functions as a co-activator of neurogenin 2  
16        in the developing cortex. *Development* 138, 2823–2832

17   58    Mueller, T.D. (2015) RGM co-receptors add complexity to BMP signaling.  
18        *Nat. Struct. Mol. Biol.* 22, 439–40

19   59    Ma, C.H.E. *et al.* (2011) The BMP coreceptor RGMb promotes while the  
20        endogenous BMP antagonist noggin reduces neurite outgrowth and peripheral  
21        nerve regeneration by modulating BMP signaling. *J. Neurosci.* 31, 18391–400

22   60    Mawdsley, D.J. *et al.* (2004) The Netrin receptor Neogenin is required for  
23        neural tube formation and somitogenesis in zebrafish. *Dev. Biol.* 269, 302–15

24   61    Kee, N. *et al.* (2013) Netrin-1 is required for efficient neural tube closure. *Dev.*  
25        *Neurobiol.* 73, 176–87

1 62 Lee, N.K. *et al.* (2016) Neogenin recruitment of the WAVE regulatory  
2 complex maintains adherens junction stability and tension. *Nat. Commun.* 7,  
3 11082

4 63 Stahl, B. *et al.* (1990) Biochemical characterization of a putative axonal  
5 guidance molecule of the chick visual system. *Neuron* 5, 735–43

6 64 Müller, B.K. *et al.* (1996) Chromophore-assisted laser inactivation of a  
7 repulsive axonal guidance molecule. *Curr. Biol.* 6, 1497–502

8 65 Xia, Y. *et al.* (2011) Dragon (Repulsive Guidance Molecule b) Inhibits IL-6  
9 Expression in Macrophages. *J. Immunol.* 186, 1369–1376

10 66 Li, J. *et al.* (2016) Repulsive guidance molecule B inhibits metastasis and is  
11 associated with decreased mortality in non-small cell lung cancer. *Oncotarget*  
12 DOI: 10.18632/oncotarget.7463

13 67 Hesson, L.B. *et al.* (2016) Integrated Genetic, Epigenetic, and Transcriptional  
14 Profiling Identifies Molecular Pathways in the Development of Laterally  
15 Spreading Tumors. *Mol. Cancer Res.* DOI: 10.1158/1541-7786.MCR-16-0175

16 68 Li, V.S.W. *et al.* (2009) Frequent Inactivation of Axon Guidance Molecule  
17 RGMA in Human Colon Cancer Through Genetic and Epigenetic Mechanisms.  
18 *Gastroenterology* 137, 176–187

19 69 Papanikolaou, G. *et al.* (2004) Mutations in HFE2 cause iron overload in  
20 chromosome 1q–linked juvenile hemochromatosis. *Nat. Genet.* 36, 77–82

21 70 Huang, F.W. (2005) A mouse model of juvenile hemochromatosis. *J. Clin.*  
22 *Invest.* 115, 2187–2191

23 71 Tawfik, A. *et al.* (2014) Deletion of Hemojuvelin, an Iron-Regulatory Protein,  
24 in Mice Results in Abnormal Angiogenesis and Vasculogenesis in Retina  
25 Along With Reactive Gliosis. *Investig. Ophthalmology Vis. Sci.* 55, 3616

1

## 2 **Legends**

3

4 **Figure 1.** Molecular determinants of RGMs and their interactions with Neogenin  
5 (NEO1) and BMP ligands. **(A)** Upper part. Schematic representation and domain  
6 organization of RGMs, Neogenin and BMPs. Lower part. Both the N-terminal domain  
7 (N-RGM, lower left panel, PDB ID 4UI1) and C-terminal domain (C-RGM, lower right  
8 panel, PDB ID 4BQ6) form distinct domains stabilized by several intramolecular  
9 disulphide bonds. Structures are shown in cartoon in rainbow coloring (blue: N-  
10 terminus, red: C-terminus). Note that RGD motifs are potential integrin binding sites  
11 but that no binding of RGMs to integrins has been reported so far. **(B)** The RGM-  
12 Neogenin complex (PDB ID 4BQ6). Two C-RGM molecules (blue) act as a molecular  
13 staple bringing together two Neogenin receptors (red). **(C)** The N-RGM-BMP complex  
14 (PDB ID 4UI1). The disulphide-linked BMP2 dimer binds two molecules of N-RGM  
15 in its wing region. The yellow asterisk indicates the position of the “RGD” motif. **(D)**  
16 Model for RGM-mediated signaling *in trans*. The RGM ectodomain can be shed by  
17 proteolytic or phospholipase activity (open triangle). RGM-binding to pre-clustered  
18 Neogenin results in stabilization and dimerization of the Neogenin ectodomain,  
19 subsequently activating downstream signaling (grey lightning bolt). The grey box  
20 highlights the RGM-Neogenin signaling hub observed in the crystal structure. **(E)**  
21 Model for RGM-mediated signaling *in cis*. RGMs can act as a physical protein bridge  
22 bringing together Neogenin and the BMP ligand resulting in clustering. FN, fibronectin;  
23 GDPH, autoproteolysis cleavage motif; GPI: glycosylphosphatidylinositol anchor;  
24 ICD, intracellular domain; IG, immunoglobulin; L, flexible linker; RGD, Arg-Gly-Asp;  
25 SP, signal peptide; TM, transmembrane; vWFD, von Willebrand Factor type D.

1

2 **Figure 2 (Key Figure).** Signaling mechanisms downstream of RGMs. RGMs act as  
3 co-receptors for BMPs and have been proposed to act as a structural bridge between  
4 BMPs and Neogenin. A recently proposed model suggests that RGM may induce  
5 endocytosis of the BMP receptor complex thereby activating canonical SMAD  
6 signaling. Interactions between RGMs and BMP signaling have been implicated in iron  
7 homeostasis and endochondrial bone development.

8 Binding of RGM to Neogenin inhibits interactions between Lrig2 and Neogenin  
9 allowing ectodomain shedding by ADAM17 leading to signal termination. In general,  
10 RGM-Neogenin binding leads to the activation of RhoA through Unc5 and LARG, and  
11 inactivation of Ras through focal adhesion kinase (FAK) and p120 RasGAP to induce  
12 growth cone collapse. However, signaling is dependent on the proteolytic processing  
13 of RGMs since C-RGM triggers RhoA-dependent signaling, while the effects of N-  
14 RGM rely on shedding of the Neogenin intracellular domain by  $\gamma$ -secretase and LMO-  
15 4. The Neogenin intracellular domain has been proposed to move into the nucleus,  
16 possible together with LMO-4, and regulate gene transcription.

17 In epithelial cells, Neogenin binds and localizes the WAVE regulatory complex (WRC)  
18 leading to actin nucleation by Arp2/3, which also requires activation by Rac1 and  
19 adherence junction stability. The extent to which these signaling pathways are specific  
20 to select cell types or cellular functions remains to be determined.

21

22 **Figure 3.** Proteolytic processing of RGMa generates N- and C-RGMa fragments that  
23 regulate distinct aspects of retinotectal pathfinding *in vivo*. (A) Autocatalytic  
24 processing (arrowhead) and proteolysis by SKI-1 and furin generates C- and N-RGMa  
25 fragments. (B) Ectopic expression of C- and N-RGMa peptides in the chick optic

1 tectum results in distinct axon targeting defects. Normally two gradients of Neogenin  
2 (blue) in the eye and RGMa (in the tectum) allow correct anterior-posterior targeting of  
3 retinal axons. In control experiments, all axons from retinal ganglion cells in the eye  
4 terminate before the terminal front (TF) in the tectum and arborize in layers a-f of the  
5 SGFS (stratum griseum et fibrosum superficiale). Ectopic expression of C-RGMa  
6 results in axonal overshooting beyond the TF with aberrant retinal axons remaining  
7 restricted to the superficial SO (stratum opticum) layer. In contrast, ectopic expression  
8 of N-RGMa induces overshooting beyond the TF and into deeper layers (beyond SGFS  
9 layer g). N, nasal; T, temporal.

10

## **Box 1. A Brief History of the Repulsive Guidance Molecule**

During nervous system development, axons travel long distances to reach their synaptic partner cells. Axons are guided to their targets by many different attractive and repulsive guidance molecules present in their environment [1]. A neuronal system in which this process of axon guidance has been studied extensively is the retinotectal system, i.e. axonal projections from retinal neurons in the eye to the tectum in the brain. Gradients of topographic guidance cues had been postulated decades ago to drive the process of retinotectal map formation. However, identification of these cues remained elusive for nearly 50 years. In 1990, Bonhoeffer and colleagues discovered a tectum-derived lipid-anchored repulsive guidance molecule with a molecular weight of 33/35 kDa [63], later named repulsive guidance molecule (RGM)[64]. RGM was the first graded topographic guidance molecule and its amino acid sequence was published in 2002 [29]. RGM is part of a small gene family that contains four members: RGMa (or RGM), RGMb (DRAGON), RGMc (Hemojuvelin) and RGMd (only present in fish). Work from different groups has shown that RGMa and RGMb are expressed in a largely non-overlapping pattern in the central nervous system (CNS) and other tissues, whereas RGMc is mostly absent from the CNS and expressed in liver and skeletal muscle. In addition to axon guidance, RGMs are now known to subserve a multitude of physiological functions ranging from immune system function to the regulation of iron homeostasis. Furthermore, in adult humans suffering from traumatic brain injury, cerebral stroke, Multiple Sclerosis (MS), Parkinson's disease and Alzheimer's disease, RGM proteins (mostly RGMa but also in some indications RGMb) are re-expressed and accumulate at sites of damage or injury. This suggests that targeting RGMs may be promising therapeutic strategy for different brain diseases. Consequently, several

1 studies have successfully explored the effect of neutralizing RGMs in experimental  
2 disease models. This showed, for example, that neutralization of RGMa, a known  
3 inhibitor of axon regeneration, following spinal cord injury results in enhanced  
4 functional recovery [13]. The first clinical trials targeting RGM with a highly selective  
5 RGMa-specific antibody (ABT-555) will tell if such regeneration promotion of  
6 damaged axons is also observed in humans suffering from MS or from spinal cord injury.

7

## 8 **Box 2. Mutations and Knockouts of RGMs.**

9

10 With the exception of RGMc not much is known about the effects of *in vivo* loss-of-  
11 function of RGMs. *RGMb* maps to chromosome 5q15 and *RGMb* gene knockout in  
12 mice results in death at 2-3 weeks after birth [65]. The olfactory epithelium of *RGMb*  
13 knockout mice displays an increase in dividing progenitor cells in addition to  
14 supernumerary sustentacular support cells [49]. A role for RGMb in proliferation is also  
15 suggested in different cancers. Reduced RGMb expression, probably by *RGMb*  
16 promoter hypermethylation, is associated with poor prognosis in non-small lung cancer  
17 patients and overexpression of RGMb in a highly metastatic mouse model has a  
18 suppressive effect on cancer progression [66]. This suggests that RGMb may act as a  
19 tumor suppressor, possibly by inhibiting SMAD activation. This idea is also supported  
20 by the observation that the *RGMb* gene is inactivated by frameshift mutations in a  
21 subtype of colorectal cancer [67].

22 A potential tumor suppressor role for *RGMa* is suggested in human colon cancer where  
23 (epi)genetic inactivation of *RGMa* results in strongly decreased levels of RGMa in  
24 colorectal cancer (CRC) tissue, but also in CRC cell lines and adenomas [68]. The  
25 *RGMa* gene maps to chromosome 15q26.1 and *RGMa* gene knockout in mice can result



1 in early embryonic lethality, due to failure of neural tube closure (only 50% of the  
2 expected homozygous mice are born). Surviving *RGMa* knockout mice show no defects  
3 in retinotectal map formation [47]. This is not an unexpected finding since *RGMb* may  
4 compensate for *RGMa* loss-of-function. Unfortunately, double *RGMa/RGMb* knockout  
5 mice are subject to early embryonic or postnatal lethality, while conditional  
6 *RGMa/RGMb* double knockout mice are not yet available.

7 *RGMc* maps to chromosome 1q21.1 and was identified as the second gene mutations  
8 in which result in JHH [69]. *HAMP*, encoding for hepcidin, was the first gene linked  
9 to JHH. Many mutations have been identified in *RGMc* resulting in premature stop  
10 codons or miss-sense substitutions of highly conserved residues. Two studies  
11 analyzing *RGMc* knockout mice show massively increased serum iron levels and very  
12 low hepcidin expression [47,70]. *RGMc* knockout mice also display an interesting  
13 retinal phenotype, with abnormal vasculogenesis and angiogenesis, and reactive  
14 gliosis of microglial and Müller-type glial cells [71].

1   **Glossary**

2

3   *ADAM17* - a disintegrin and metalloproteinase domain-containing protein 17 (also  
4   known as tumor necrosis factor- $\alpha$  converting enzyme (TACE)) is a protease that  
5   induces ectodomain shedding of Neogenin and other cell surface proteins.

6

7   *Adherens Junction* - a protein complex located at the junction between epithelial cells.  
8   These proteins mediate cell-cell adhesion and are linked to the cell's actin  
9   cytoskeleton.

10

11   *EAE* – experimental autoimmune encephalomyelitis. Animal model for studying brain  
12   inflammation. Administration of different antigens (e.g. myelin or MOG) induces  
13   demyelinating disease of the CNS.

14

15   *GEF* - guanine nucleotide exchange factor. Proteins that stimulate monomeric  
16   GTPases by triggering the exchange of GDP for GTP.

17

18   *Hepcidin* - a small peptide secreted predominantly by hepatocytes that is essential for  
19   iron metabolism. It functions to degrade the iron exporter ferroportin.

20

21   *Juvenile Hemochromatosis (JHH)* - a rare genetic disorder characterized by the  
22   accumulation of iron in various organs of the body. Mutations in *RGMc* can cause  
23   JHH and cause a reduction of hepcidin.

24

1    *WRC* - wave regulatory complex. Group of proteins that regulates actin dynamics by  
2    stimulating the actin-nucleating activity of the Arp2/3 complex at the plasma  
3    membrane.

4

5    *WIRS* - WRC interacting receptor sequence. Conserved peptide motif that mediates  
6    binding to WRC.

7