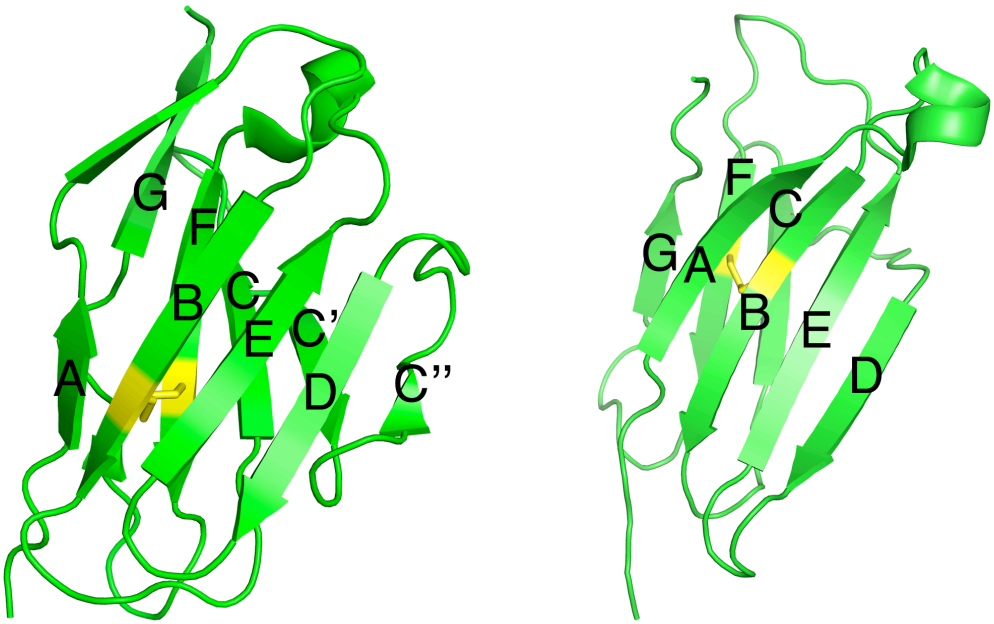
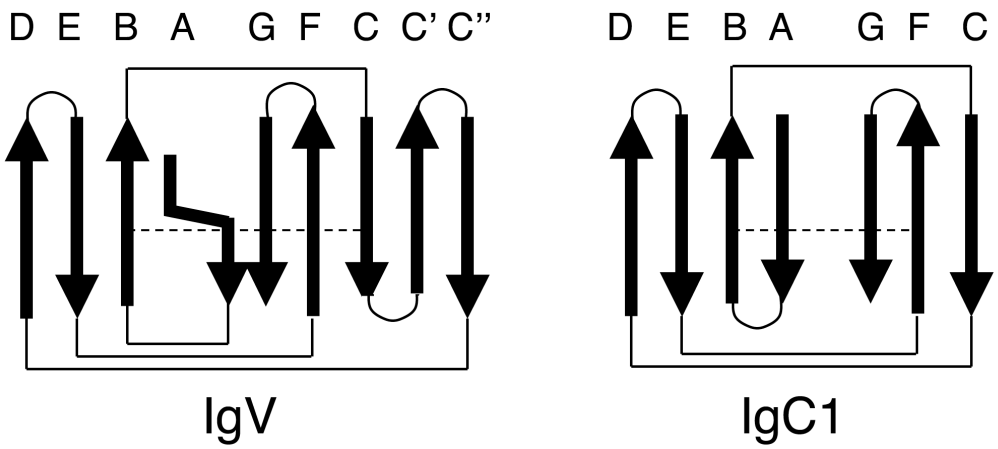
Chapter 1:
Introduction

Immunoglobulin (Ig) superfamily molecules

The concept of the immunoglobulin superfamily originated from the observation that domains within a variety of proteins share sequence similarity with immunoglobulin constant and variable domains, and such domains may have evolved from a common ancestral protein of ~100 amino acids in length (*1*). Initially, Ig or Ig-like domains were identified based on sequence similarity, but as more 3-D structures became available, this criterion was replaced by similarity based on structural features, which greatly broadened the definition of the superfamily. A typical Ig-like domain (Figure 1) has a sandwich-like fold formed by two sheets of antiparallel beta strands, and often a conserved disulfide bond between two cysteines, and an "invariant" tryptophan residue (*1*).

Most Ig superfamily molecules are located on cell surface, with exceptions including the secreted forms of antibodies. The most common functions of Ig superfamily receptors are adhesion/recognition and initiation of signaling cascade in the cytoplasm (*1*). One group of Ig superfamily receptors consists of tandem Ig-like domains followed by fibronectin type III (FNIII) domains, which is a common structure motif originally found in fibronectin (*2*). Both FNIII domains and Ig-like domains belong to the Greek key superfold (*3*), but the sequence similarity between these types of domains is usually quite low. The folding topology of a FNIII domain strongly resembles the IgC2 domain, but lacks the disulfide bond linking the two opposed beta sheets (*3*). Adhesion complexes formed by Ig superfamily molecules are not just static. Instead they are capable of sensing the signal from the extracellular space and modulating cellular activities (*4*).





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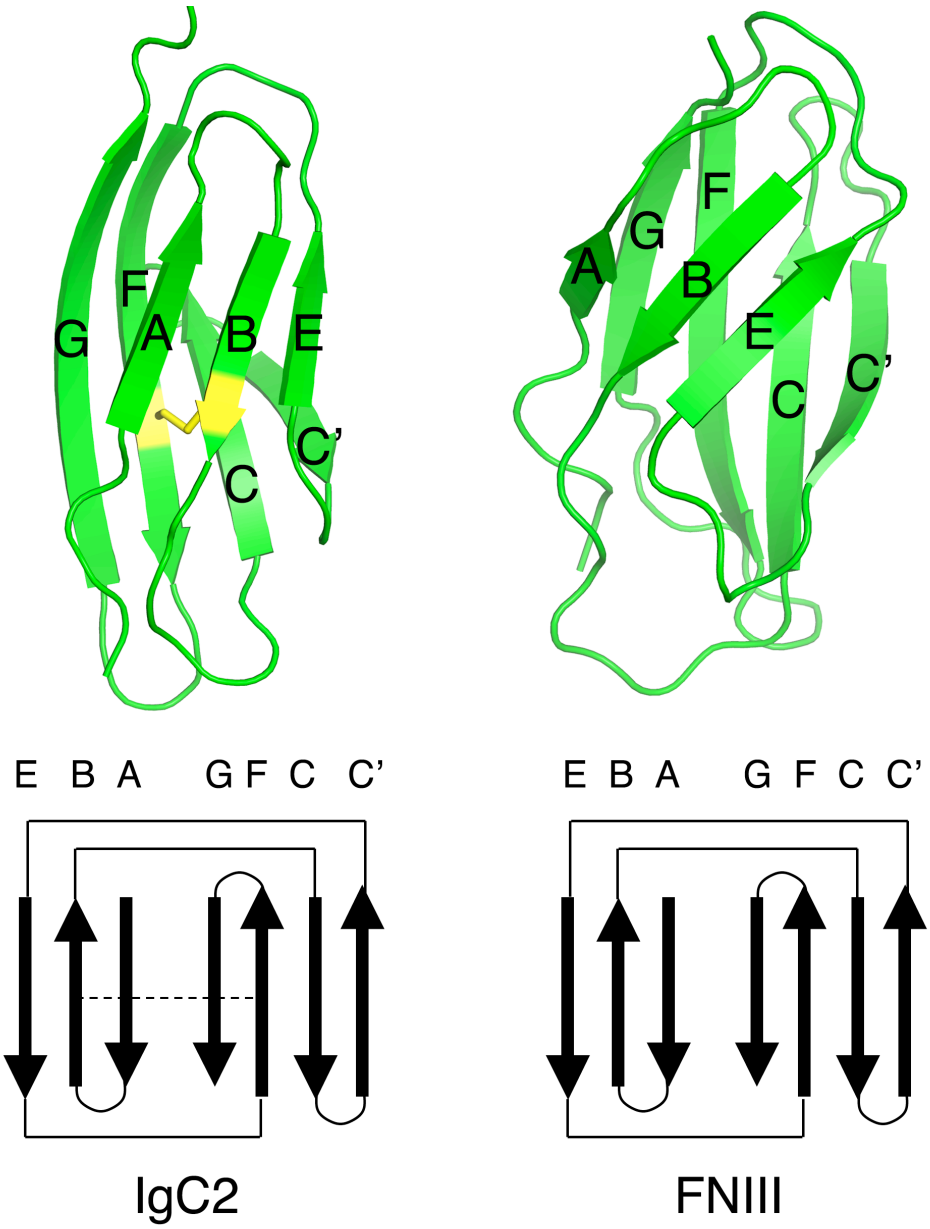

Figure 1. Ribbon and topology diagrams of typical Ig folds and FNIII fold. Disulfide bonds are shown as yellow sticks in ribbon diagram and dashed lines in topology diagram. The IgV and IgC1 domains are found in the immunoglobulin variable and constant regions, respectively. IgC2 domain shares sequence similarity to IgV but topologically looks like IgC1. FNIII domain has the same domain arrangement as the IgC2 but without the disulfide bond. PDB IDs used are 1YQV, 2YXF, 1HNF, and 1QR4.

Many Ig superfamily proteins function as adhesion molecules in the nervous system and they have been implicated in various roles during the development of the nervous system (*5*). Based on the composition of their extracellular domains, neural cell adhesion molecules of the Ig superfamily (IgCAMs) can be divided in three groups: containing Ig folds only, containing Ig folds followed by FNIII domain(s), and Ig folds linked to protein modules other than an FNIII domain. Figure 2 shows a schematic view of several neural cell adhesion molecules of the Ig superfamily, including the most extensively characterized proteins NCAM, L1, and DCC/neogenin. IgCAMs are known to interact with themselves (homophilic binding) and with other proteins (heterophilic binding), which can be other IgCAMs (*6*).

The thesis work described here is the characterization of the Ig superfamily receptors neogenin and L1 using biochemical and biophysical approaches. Although neogenin and L1 are both neural adhesion molecules of the Ig superfamily, neogenin interacts with a broader range of ligands and function in multiple aspects of development and metabolism other than CNS development, for example, iron homeostasis (*7*). Here we present studies aimed at elucidating the role of neogenin in the mammalian iron-regulatory network through its interactions with hemojuvelin. Chapter 2 presents the mapping of the hemojuvelin-binding epitope on neogenin and Chapter 3 presents the crystal structure of the hemojuvelin-binding fragment of neogenin and comparison with existing tandem FNIII domain structures. These results are also relevant to interactions between neogenin and repulsive guidance molecules (RGMs), which regulate neuronal survival and are related to hemojuvelin. The second part of the thesis describes a

biophysical approach to studying L1-mediated homophilic adhesion using L1 reconstituted into liposomes.

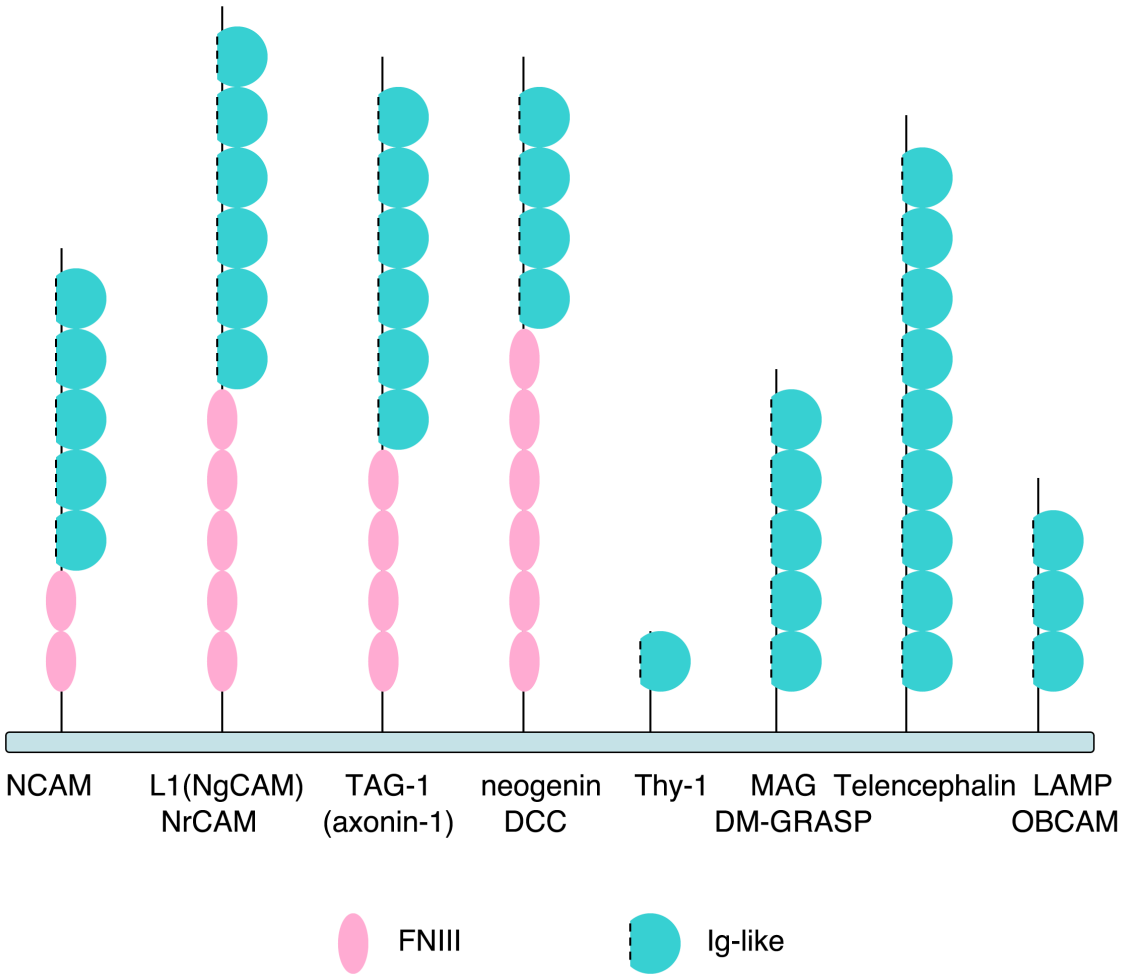

Figure 2. Neural adhesion molecules of the Ig superfamily. Proteins composed of Ig-like domains connected to FNIII domains (left) and proteins composed of Ig-like domains alone (right) are included. Ig superfamily proteins with Ig-like domains linked to motifs other than FNIII fold are not shown. Synonymous names are in parentheses. These molecules are associated with the membrane either by a single transmembrane segment or a glycosylphosphatidylinisotol (GPI)-anchor. For a more detailed list of Ig superfamily neural adhesion molecules, see reference (*5*).

Iron homeostasis

Iron is essential to almost all organisms on earth. The conversion between ferric (Fe^{3+}) and ferrous (Fe^{2+}) states enables it to donate and receive electrons and thus participate in many redox reactions. Well-known iron-containing proteins include the nitrogen fixation enzyme nitrogenase, ferrodoxin in photosynthesis, and the oxygen transporter hemoglobin. In order to maintain a normal level of iron availability and compensate for daily loss, dietary iron is absorbed through the mammalian intestinal epithelium, chelated by transferrin with extremely high affinity, and delivered to the rest of the body through the transferrin/transferrin receptor (Tf/TfR) system via a receptor-mediated endocytosis (*8*). Iron-loaded Tf undergoes a pH-dependent conformation change in the acidic environment of intracellular early endosomes and releases the iron for cellular usage or storage in ferritin, within which iron is kept in a non-toxic form and can be released for later application.

Ionic iron has the potential to initiate lipid peroxidation, a free radical chain reaction involving molecular oxygen that can lead to cell death. Therefore, iron usually exists in a tightly coordinated form such as within a heme or iron-sulfur cluster rather than the free ionic form. Mammals have evolved a complicated iron regulatory pathway in order to handle iron in a safe manner (*9*). Strict regulation of iron not only prevents it from damaging cellular structures, but also limits its availability to bacteria, thus preventing infection-induced inflammation. Dysfunction of regulation can lead to iron deficiency or iron overload, also known as hemochromatosis, which can result in severe damage to the liver, heart, and pancreas, and in the worst cases, organ failure (*10*).

Over the past two decades, many molecules in the iron-regulatory network have been identified, including HFE (*11*), transferrin receptor 2 (TfR2)(*12*), ferroportin (*13-16*), hepcidin (*17-19*), and hemojuvelin (*20*). Among these molecules, hepcidin, an anti-microbial peptide hormone secreted predominantly by liver, is the principal iron regulator (*21*). Hepcidin controls iron flux through binding and inducing internalization and degradation of ferroportin, the only known membrane iron exporter highly expressed in duodenal cells, hepatocytes, macrophages, and placental cells (*22*). Elevation in hepcidin expression prevents dietary iron uptake as well as iron efflux to the plasma and can lead to anemia. On the other hand, insufficient hepcidin production due to mutation in the hepcidin gene or its upstream regulators (HFE, TfR2, and hemojuvelin) results in most causes of hereditary hemochromatosis (*21*).

The hemochromatosis protein HFE is type I transmembrane protein and related to class I major histocompatibility complex (MHC) molecules, but lacking their peptide binding ability (*23*). HFE competes with iron-loaded Tf, the whole body iron status flag, for binding to TfR (*24, 25*). HFE also interacts with TfR2, a type II transmembrane protein with an N-terminal cytoplasmic domain and a large ectodomain homologous to TfR. TfR2 does bind iron-loaded Tf (*26*), and has been suggested to serve more as a sensor for body iron status than in Tf uptake (*27*). Despite the considerable sequence similarity between TfR and TfR2, HFE binds at different locations on these two molecules: the ectodomain in the case of TfR and the transmembrane region in the care of TfR2 (*28, 29*). Since HFE does not have any identifiable internalization sequence in its cytoplasmic domain, the TfR2/HFE interaction was proposed to transduce signals through the TfR2 intracellular domain when serum iron saturation is changed (*30*).

Compared with HFE and TfR2, the role of hemojuvelin in regulating hepcidin level is better understood thanks to growing information in recent years.

Iron regulatory protein hemojuvelin

In 2004, the *HJV* (originally called *HFE2)* gene, encoding the iron-regulatory protein hemojuvelin, was positionally cloned using samples from patients with juvenile hemochromatosis, an early-onset hereditary iron overload disorder (*20*). In HJV knocked-out mice, hepcidin mRNA is almost undetectable (*31*), consistent with the low urine hepcidin concentration in patients with HJV mutations.

Expressed in fetal and adult liver, heart and skeletal muscle, human *HJV* encodes a protein of 426 amino acids, including a secretion signal peptide, a conserved RGD triamino acid motif, a partial von Willebrandt factor domain, and a glycosylphosphatidylinisotol (GPI) anchor for attaching the mature protein to the cell membrane. The closest homologues of hemojuvelin are repulsive guidance molecules (RGMs), which have multiple functions in neural development. Hemojuvelin can undergo a proteolytic cleavage at a conserved Asp-Pro bond and forms two fragments that usually associate together (*32*). This feature was also observed in mouse and chick RGM family members (*32*). Hemojuvelin can exist in both soluble and membrane-associated forms. Soluble hemojuvelin is found in serum, serving as a competitor with its membrane-bound counterpart in a dose-dependent manner in regulating hepcidin expression (*32*).

The Ig superfamily receptor neogenin

Soon after the discovery of hemojuvelin, neogenin, a cell surface receptor belonging to the Ig superfamily, was found to guide axon growth and regulate neuronal survival through interacting with repulsive guidance molecule A (RGMa) (*33, 34*), the closest homolog of hemojuvelin. The high sequence similarity between hemojuvelin and RGMa suggested the possibility that neogenin might also function as the receptor for hemojuvelin, which was confirmed by co-immunoprecipitation experiment (*35*). The disease-causing mutant hemojuvelin G320V does not bind neogenin, indicating that the hemojuvelin-neogenin interaction is critical in iron homeostasis (*35*). Unlike the tissue-specific expression pattern for hemojuvelin, expression of human neogenin appears ubiquitous (*36*), with the highest mRNA level detected in skeletal muscle, one of the few places where hemojuvelin is highly expressed. Since hemojuvelin does not seem to play a major role in muscle morphogenesis (*20*), it has been proposed that the function of the hemojuvelin/neogenin interaction in skeletal muscle is to provide soluble hemojuvelin in serum (*37*).

Neogenin consists of a large ectodomain with four Ig-like domains and six FNIII domains, a transmembrane region and an intracellular domain (*38*). Sharing nearly 50% sequence identity, neogenin is closely related to the DCC (Deleted in Colorectal Cancer) protein. Both neogenin and DCC function as receptors for netrins, a group of proteins playing fundamental roles in the development of nervous system (*39, 40*). Neogenin is also involved in a broad range of developmental and metabolic processes. In addition to interacting with RGMs and netrin in axon guidance in the brain, neogenin is also critical in establishing organ architectures (*41*), in stimulating myogenic differentiation (*42*) and promoting mammary gland morphogenesis (*40*).

We initiated biochemical studies of the hemojuvelin/neogenin interaction to elucidate the mechanism of the interaction on the molecular level. The stoichiometry of the binding was found to be 1:1 and the hemojuvelin-binding epitope was mapped to the membrane-proximal FNIII 5-6 domains on neogenin as described in Chapter 2. In collaboration with Dr. An-Sheng Zhang in the Enns group at Oregon Health and Sciences University, we proved that this fragment is as effective as the intact neogenin ectodomain in competing with cell membrane neogenin both *in vitro* (Appendix A) and *in vivo* (Appendix B). The crystal structure of this hemojuvelin-binding fragment was solved and presented in Chapter 3. However, attempts to crystallize hemojuvelin alone and hemojuvelin/neogenin complexes have not yet been successful.

Hemojuvelin-assisted bone morphogenetic protein pathway

Belonging to the transforming growth factor β (TGF-β) superfamily, bone morphogenetic proteins (BMPs) are a group of secreted molecules that play important roles in the cell growth, differentiation, and apoptosis throughout the animal kingdom (*43*). Originally identified as inducer of bone formation *in vivo* when injected in mice, BMPs have been intensively studied after the cloning of the human BMP-2 gene in late 1980s (*44*). Like other molecules in the TGF-β superfamily, BMPs are synthesized as large precursors, which later become glycosylated and processed to form single disulfide bond-linked dimer with each polypeptide chain containing the C-terminal 114 residues of the propeptide (*45*).

Significant advances concerning the functions of BMPs and the receptor mediated signal transduction pathway have been achieved in recent years (*46*). Classic BMP

pathway is initiated by the binding of the ligand on the plasma membrane, inducing the heterodimerization of type I and type II transmembrane serine/threoinine kinase receptors, which in turn activates the Smad proteins by phosyphorylation (*47, 48*). The receptor-activated Smad will then recruit another protein called Smad4 and translocate into nucleus to regulate the transcription of target genes (*47, 48*). There are several different type I and type II receptors and their combination determines the molecules subsequently involved in the signal transduction (*47*).

An important advance in the iron field occurred in 2006, when hemojuvelin was identified as the co-receptor for BMP (*49*). Evidences showed that hemojuvelin-aided BMP signaling triggers hepcidin expression through the classic Smad1/5/8 activation upon binding of BMP to its type I and type II receptors (*49*). This pathway was found to be independent of other iron-regulatory proteins such as HFE, TfR2, and Interleukin-6 (IL-6) (*50*). BMP-responsive elements, STAT, and bZIP/HNF4/COUP motifs, were located in the promoter of hepcidin by two groups independently (*51, 52*). BMP-6 was then identified as the central endogenous regulator of hepcidin expression among all BMP family proteins *in vivo* and the phenotype of *BMP-6* null mice resembles hereditary hemochromatosis (*53*).

The major players in hemojuvelin-related hepcidin expression are depicted in Figure 3. How does neogenin fit into the picture of hemojuvelin-mediated BMP signaling? Shedding of hemojuvelin from cell membrane was observed to be responsive to the concentration of iron-loaded transferrin and hemojuvelin shedding is mediated by neogenin and independent of BMP or its antagonist (*37*). The group that initially discovered hemojuvelin as a co-receptor for BMPs claimed that hemojuvelin signaled

hepcidin expression only through a subset of BMP ligands (BMP-2, BMP-4, and BMP-6) and BMP receptors, and that this process was independent of neogenin (*54*). However, another study showed that neogenin-hemojuvelin interaction was critical in BMP-4-induced hepcidin expression (*55*), contradicting the previous conclusion. We also discovered that neogenin and BMP-2 do not bind to hemojuvelin at overlapping site (see Chapter 2), supporting the possibility that neogenin is part of the multi-protein complex that initiates the intracellular signaling that ultimately leads to hepcidin expression (*56*). Most recently, a third group succeeded in making neogenin$^{-/-}$ mice that exhibit iron overload, impaired BMP signaling and low levels of hepcidin (*57*). These researchers argued that neogenin regulates hemojuvelin/BMP-induced hepcidin expression through stabilizing GPI-anchored hemojuvelin and inhibiting hemojuvelin secretion.

Another way that neogenin may be involved in signaling is through the cleavage and translocation of its intracellular domain, which was suggested in a recent report (*58*). However, this study focused on the role of neogenin in axon guidance via interaction with the neuronal RGMa protein and thus does not directly address the questions in iron regulation, which mostly takes place in the liver. The exact role of neogenin in this pathway remains to be elucidated.

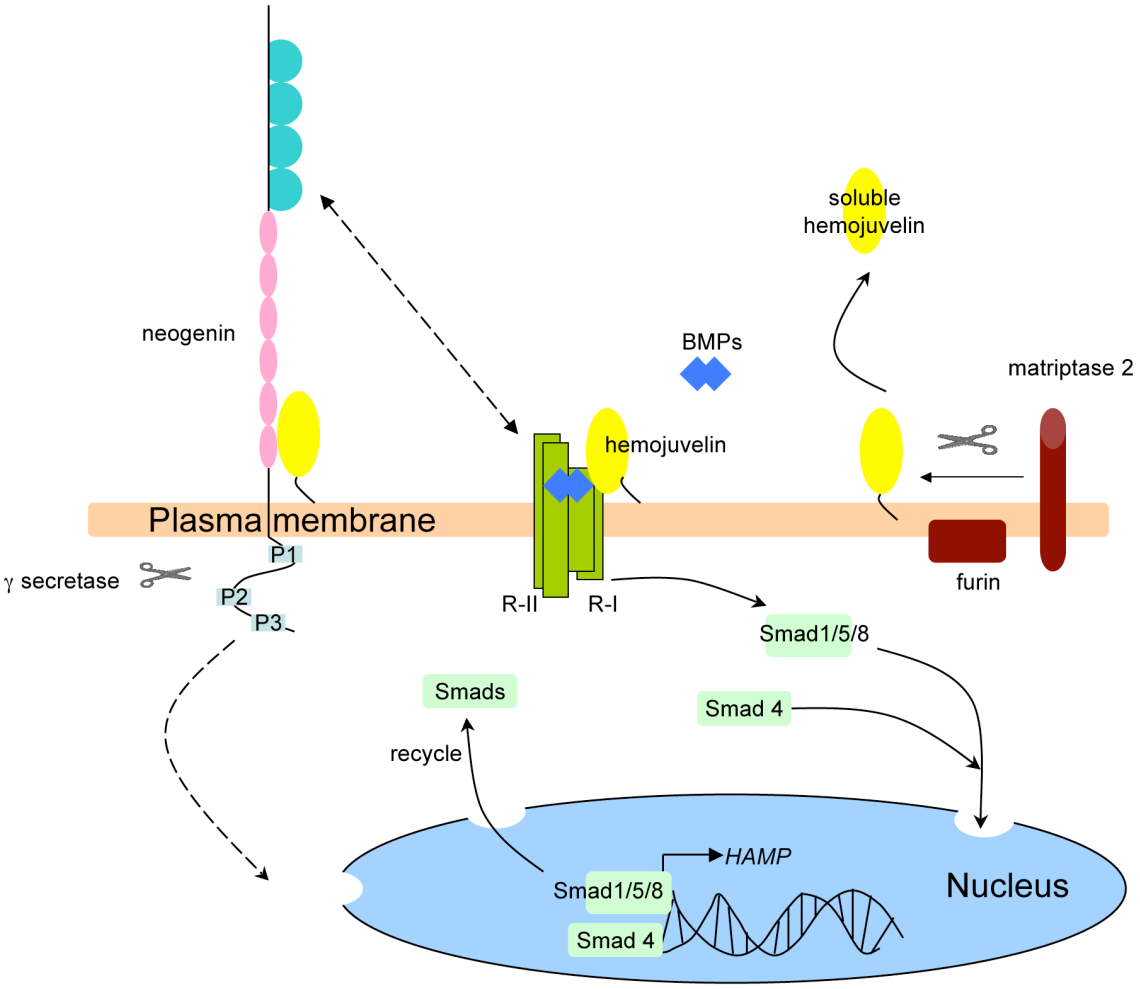

Figure 3. Molecular network of hemojuvelin-induced hepcidin expression. P1, P2 and P3 are the conserved regions on neogenin intracellular domain. R-I and R-II are type I and type II transmembrane serine/threoinine kinase receptors for BMP. γ-secretase is responsible for releasing the intracellular domain of neogenin to the cytosol. Two dashed lines indicate potential interaction or pathway suggested by previous studies (*56, 58*).

Neural cell adhesion molecule L1

The L1 gene is located on the X-chromosome (*59*) and mutations in the L1 gene are associated with a broad spectrum of neurological diseases (*60*) including mental retardation (*61*), MASA syndrome (*62*), X-linked hydrocephalus (*63*), impairment of sensorimotor gating (*64*). The positions of these pathological mutations were mapped onto a structural model of the L1 ectodomain (*65*), based on the crystal structures of domains in telokin (*66*) and neuroglian (*67*), where the latter is the *Drosophila* homologue of human L1. Over half of the mutations are clustered at N-termini of individual domains as well as the C-D strand region on the Ig-like domain, potentially causing the phenotype by destabilizing the protein (*65*).

Primarily expressed in the developing and adult nervous system, L1 consists of six Ig-like domains, five FNIII domains, a single transmembrane domain and a cytoplasmic tail of just over 100 amino acids (*68*) (Figure 2). The multi-domain structure of L1 enables it to interact with distinct partners such as integrins, fibroblast growth factor receptor and other cell adhesion molecules (*69*), resulting in dynamic regulation of cell adhesion in response to different ligands. The cytoplasmic domain of L1 contains a conserved region capable of binding to the cytoskeletal protein ankyrin (*68*), and a conserved tyrosine residue within this region was found to control binding by its phosphorylation (*70*). Downstream signaling induced by L1 after ankyrin binding is complicated, including recruitment of the microtubule-associated protein doublecortin (DCX) and sequential activation of a series of kinases (*69, 71, 72*).

L1-mediated homophilic adhesion

How L1 achieves homophilic adhesion has been under intensive study for many years. The first four Ig domains are critical in homophilic adhesion and neurite outgrowth but the potency of molecules containing only these domains is lower than the intact molecule (*73*). Based on existing crystal structures of the L1 homologues hemolin (*74*) and axonin-1 (*75*), the first four Ig domains of L1 are believed to form a horseshoe-shaped structure, with the first and second Ig domains folding back to interact with the third and fourth Ig domains. The FNIII domain region of the molecule appears to adopt a relatively extended conformation (*76*).

Severals models have been proposed to predict how the horseshoe pairs arrange with respect to each other in homophilic adhesion. Previous models, including a domain swapping model (*74*) and a zipper model (*75*), were based on crystal structures of proteins closely related to L1. Regularly spaced adhesion spots were observed in the more recent electron tomography studies and it was proposed that the separation distance is controlled by interactions either between negatively charged carbohydrates and positive surfaces of the neighboring protein or between uncharged carbohydrate pairs (*77*).

Giant unilamellar vesicle as model membranes

We sought to address some thermodynamic and kinetic issues related to L1-mediated homophilic adhesion. For example, what is the average adhesion energy for one pair of L1 molecules or the energy for a given L1 density? Is there cooperativity in L1-mediated adhesion? Does the adhesion zone actively recruit L1 from other regions of a

membrane? In order to address these questions, we used L1 incoporated into giant unilamellar vesicles as the experimental platform.

As an essential component of all biomembranes, the lipid bilayer has the unique feature of two-dimensional fluidity, which is critical in lipid/protein diffusion, distribution, and local enrichment (*78, 79*). Due to the complex nature of biomembranes in cells, researchers have used lipid model systems to understand basic membrane activities (*80*). These cell-free assays make it possible to track down essential components of the membrane trafficking processes and distinguish the order of events, while at the same time preserving the two-dimensional fluidity of cellular membranes. One of the most broadly used model membranes is spherical liposome, also known as vesicle. Many methods have been established to prepare liposomes using natural or synthetic lipids while varying the chemical composition of the lipid bilayer. Giant unilamellar vesicles (GUVs) are particularly of interest due to their cell-size dimensions (*81*). Although the observations from these *in vitro* experiments involving GUVs does not always translate into what happens in cells because cellular membranes are more rigid due the cytoskeleton, they often provide great insight essential to understanding events taking places on these membranes at a molecular level (*82, 83*). Chapter 4 summarizes our work on L1-mediated homophilic adhesion using both theoretical and experimental approaches involving GUVs.

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