Presenilins (PSs) are required for Notch signaling in the development of vertebrates and invertebrates. Mutations in human PS1 and PS2 homologs are a cause of familial Alzheimer’s disease (AD). The function of the recently identified ancient family of IMPAS proteins (IMP/SPP/PSH) homologous to PSs is not yet known. We show here that, unlike PSs, IMPs (orthologous C. elegans Ce-imp-2 and human hIMP1/SPD) do not promote Notch (C. elegans lin-12,glp-1) proteolysis or signaling. The knock-down of Ce-imp-2 leads to embryonic death and an abnormal molting phenotype in Caenorhabditis elegans. The molting defect induced by Ce-imp-2 deficiency was mimicked by depleting cholesterol or disrupting Ce-Irp-1 and suppressed, in part, by expression of the Ce-Irp-1 derivate. C. elegans lrp-1 is a homolog of mammalian megalin, lipoprotein receptor-related protein (LRP) receptors essential for cholesterol and lipoprotein endocytosis and signaling. These data suggest that IMPs are functionally distinct from related PSs and implicate IMPs as critical regulators of development that may potentially interact with the lipid-lipoprotein receptor-mediated pathway.

Materials and Methods

C. elegans Strains and Clones. The following strains were used: N2 Bristol as a wild-type strain; sel-12(ar131), sel-12(ar131)unc-1, sel-12(ar171)unc-1 (e538) (provided by I. Greenwald, Columbia University, New York), glp-1(e2142ts) (provided by C. Goutte, Amherst College, Amherst, MA), lin-12(n3173)/unc-32(e189)III;him-5(e1467)V, unc-32(e189)lin-12(n676n930)III, lrp-1(ku156)I;glp-1(q266)I (provided by the Caenorhabditis Genetics Center, which is funded by the National Institutes of Health National Center for Research Resources).

cDNA clones yk671a5, yk336d12, yk1191b02, and yk260f8 were obtained from Y. Kohara (Genome Biology Laboratory, National Institute of Genetics, Mishima, Japan). We used standard methods of C. elegans handling and culture (18) with OP50 Escherichia coli strain as a food source. Worms were observed by using a dissecting microscope with a maximum magnification of 40–60× or a fluorescent dissecting stereo-microscope with a Nomarski optic and magnification of up to ×100. Filipin staining was performed as described (19).

Double-Stranded RNA (dsRNA) Synthesis and Injections. To generate dsRNA for injection, a T7 sequence was added at both ends of psiRNA, double-stranded RNA, RNAi, RNA interference; LRP, lipoprotein receptor-related protein.

Abbreviations: AD, Alzheimer’s disease; ISC, incomplete shedding of cuticle; PS, presenilin; dsRNA, double-stranded RNA; RNAi, RNA interference; LRP, lipoprotein receptor-related protein.

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the cDNA template by PCR reaction with corresponding primers. PCR product was then purified on Qiagen (Valencia, CA) columns and used for RNA synthesis and purification by using Ambion (Austin, TX) MEGAscript T7 Transcription Kit. RNA was then checked on standard agarose gel, was quantified (≈ 1–5 μg/μl), and was used for pseudocoecklepmic injections [in head and tail regions beyond the positions of the two gonad arms (20, 21)] with an average of 0.5–1 × 10⁶ RNA molecules per animal. Four to six hours after injection, worms were transferred individually to fresh plates, and the phenotype of F1 progeny was analyzed within 2–3 days.

**RNA Interference (RNAi) by dsRNA Feeding.** RNAi by dsRNA feeding was performed as described (22–24). cDNAs for *C. elegans Ce-imp-1, Ce-imp-2, and Ce-imp-3* were cloned directly from yk clones or as PCR subfragments into an L4440 vector containing a double T7 promoter sequence (Fig. 5, which is published as supporting information on the PNAS web site).

**Germ-Line Injections and Extrachromosomal Array.** For *Ce-lrp-1* rescue experiments, germ-line injections of plasmid containing *Ce-lrp-1 L-ICD* were performed as described (25). *Ce-lrp-1 L-ICD* fragment was cloned into BamHI-PstI L4440 vector. *Ce-lrp-1 L-ICD* contains a 996-bp *Ce-lrp-1* promoter genomic region upstream of the ATG and a 1,026-bp 3′ genomic region downstream of stop codon TAA. Encoding sequence contains a 996-bp *Ce-lrp-1* promoter genomic region upstream of the ATG and a 1,026-bp 3′ genomic region downstream of stop codon TAA. Encoding sequence contains ICD fragment of *Ce-lrp-1* CDS with introduced ATG (5′-ATGGGACTTTTGGTATG and ends on the last coding triplet of *Ce-lrp-1* CDS fused to myc-epitope sequence (Fig. 6, which is published as supporting information on the PNAS web site). DNA was injected in concentrations of 10–50 ng/μl into gonadal distal cells of young adult animals. The coinjection marker *sur-5-GFP* for easy detection of transgenic progeny was used in concentration at 10 ng/μl. Four to six hours after injection, the worms were transferred individually to fresh plates. To establish individual lines with extrachromosomal maintenance of the transgene, marker-positive F1 animals were picked and placed separately.

**Results**

**Imp-genes in C. elegans.** Multiple IMP/SPP-related genes were identified in a variety of species from yeast to human. At least five IMP genes in human and three diverged homologous genes similar to presenilins in *C. elegans* were identified. Comparison of the most conserved domains suggests links to a family of proteins in Archaea and to bacterial peptidases (12, 15), indicating that *C. elegans Ce-imp-1, Ce-imp-2, and Ce-imp-3* have divergent N termini but have substantial amino acid conservation in three hydrophobic regions predicted to be transmembrane (Tm) domains (Fig. 1; Fig. 7, which is published as supporting information on the PNAS web site). The amino acid signature 5DxxxV-LGxGD-PxL (where 5-F,Y,W; ‘x’ is any amino acid, and ‘-’ connects the three most conserved domains of the signature) is invariant in *C. elegans Ce-imp-1 and Ce-imp-2* and members of IMPAS or PS families from distant taxons. Ce-imp-3 is more diverged family member, lacking this consensus signature but still harboring conserved aspartate and proline residues. The predicted Tm protein structure and membrane topology also resemble those described for human/C. elegans PS/SPP, homologous to PSs, may also facilitate cleavage of the Notch1 receptor. Constructs with human IMP1 were generated that efficiently express hIMP1 in transiently and stably transfected mammalian cells. hIMP1 in cell lysates is

![Fig. 1. Similarity of Ce-IMP-2 to other members of IMPAS and presenilin families of proteins. (A) Predicted transmembrane structure of Ce-IMP-2, C. elegans, and hIMP1, *Homo sapiens*. The arrowheads denote the positions of conserved aspartate and PAL-motif residues. The letters show invariant amino acid residues in eukaryotic PSs and IMPASs; the underlined letters are identical also in related proteins from archaea and bacterial polytopic type 4 prepilin peptidases (TFPP). Most conserved domains in PSs and IMPAS families are shown in intense gray. The predicted sites of N-glycosylation (marked by asterisks) and endoplasmic reticulum-membrane retention signals (KKXX motif) are shown (NETNLGYC 1.0 and PSORT II predictions). (B) Phylogenetic tree (Cluster algorithm, GENEBEE) for conserved domains (amino acid 269–444) of *C. elegans Ce-IMP-2* and corresponding domains of *C. elegans Ce-IMP-1, Ce-IMP-3*, and human IMP (hIMP1–5) proteins.]
data demonstrate that hIMP1 does not facilitate NotchΔE S3 cleavage. In contrast to PS1wt, ectopic overexpression of hIMP1wt did not restore the S3 cleavage in PS1−/−PS2−/− mouse fibroblasts (Fig. 2B). Together, the data indicate that the substrate-dependent enzymatic function of hIMP1 is not redundant with PSs.

**Ce-imp-2 RNAi Does Not Affect lin-12,glp-1 (Notch)-Signaling in Development in C. elegans.** To gain further insights into the possible functions of IMPAS genes in vivo, we used the C. elegans model. In our preliminary experiments, Ce-imp-1 and Ce-imp-3 dsRNA produced no obvious development abnormalities. Therefore, we focused further upon study of Ce-imp-2, the ortholog of mammalian IMP1/SPP. Wild-type worms were injected with dsRNA or fed with E. coli-expressing dsRNA corresponding to the Ce-imp-2 gene (Fig. 5). The search for the sel-12, lin-12, and glp-1 characteristic phenotypes was performed over the course of multiple replicate experiments. There are several major defects caused by single or concomitant reduction of lin-12 and glp-1 (Notch homologs) and their upstream and downstream regulators. The first of these defects involves cell fate changes between the 4-cell and 12-cell stages that result in embryos lacking a portion of the pharynx and failing to enclose and elongate the body (31). Although the earliest defect caused by Ce-imp-2 (RNAi) is embryonic arrest, <3% of Ce-imp-2(RNAi) embryos exhibited a failure to undergo body morphogenesis (Fig. 9A), which is published as supporting information on the PNAS web site. Instead, the majority of Ce-imp-2 RNAi animals died during late embryonic stages or after hatching (Fig. 9B and C). The lack of an anterior pharynx was previously described as a characteristic specific to diminished maternal glp-1 activity, a decrease in the activity of factors required for glp-1, lin-12 signaling (e.g., sel-8, aph-1, and aph-2) or the simultaneous reduction of sel-12 and hop-1 (9, 32, 33). We found that the development of anterior pharynx is normal in arrested larvae of Ce-imp-2-deficient N2 worms. Both Notch homologs glp-1 and lin-12 function together during late embryogenesis in the specification of the rectum (32). However, development of the rectum was unaffected in Ce-imp-2-deficient worms. Reduction of Ce-imp-2 activity by RNAi did not induce defects in vulva formation similar to those described for mutants in lin-12/Notch or the Ce-imp-2 homolog sel-12 (10, 11, 32). Finally, we used sensitized genetic backgrounds to look for interactions between Ce-imp-2 and Notch-pathway components. None of the hypo- or hypermorph glp-1, lin-12, or sel-12 mutant strains analyzed exhibited enhancement or suppression of their corresponding phenotypes upon induction Ce-imp-2 (RNAi) (Fig. 3A and Table 1, which is published as supporting information on the PNAS web site). The data suggest that, unlike its distant homologs sel-12 or hop-1, Ce-imp-2 is not a positive regulator of lin-12,glp-1 (Notch) signaling in cell–cell interaction and cell fate specification during development in C. elegans.

**Ce-imp-2 Inactivation Induces Embryonic Lethality and Molting Defects During Development.** Two major abnormalities induced by Ce-imp-2 RNAi were embryonic/early L1 lethality and L1→L4 larval molting defects leading to larval arrest (Figs. 3B and 9A–G). Studies of the progeny of adult worms injected with Ce-imp-2 dsRNA showed 80–100% lethality of their offspring at late embryonic or early larval stages [number of experiments n = 7; total number of analyzed progeny n = 138 (100%); total number of dead worms on early stages 120 (87%)]). In an effort to avoid maternal germ-line effects and to delineate potential Ce-imp-2 deficiency phenotypes in later development, we used RNAi by feeding.

Staged populations of L1 worms were transferred to Ce-imp-2 dsRNA plates and were analyzed for phenotypes in both initial generation (P0 population) and their progeny (the F1...
generation). Defects in P0 development were observed at the L3–L4 and L4–adult molt stages. At least 20–30% of N2 worms at these stages (n = 9; n > 2 × 10^3) cannot properly shed the cuticle (Fig. 3B). In these cases, shedding of the cuticle was initiated, but the old cuticle remained attached to the body. Henceforth, we shall refer to this phenotype as the incomplete shedding of cuticle (ISC) phenotype. Analysis of individuals exhibiting this phenotype showed that ~80% of these individuals failed to shed their cuticle and died, whereas the remaining 20% escape from the cuticle, which often remained attached to the tail region. These escapers matured to form sterile adults or Egl animals that could produce only a few eggs. The F1 generation of the dsRNA Ce-IMP-2-fed animals exhibited phenotypes similar to those observed after microinjection of dsRNA. Approximately 3% of these embryos died at early stages before morphogenesis, whereas the majority died at late embryonic stages. Among the hatching F1 progeny, the ISC phenotype was observed at all molt stages (Fig. 3 D–G). In experiments using F1 embryos, we found that >60% of eggs died during late embryogenesis with elongated 3-fold embryos inside the egg shell or as early L1 larvae, shortly after hatching (n = 6, n > 10^3). The remaining animals reached later stages, and most of them arrested with the ISC phenotype. Other postembryonic pathologies in Ce-imp-2 RNAi worms included body constrictions (Fig. 3B), dumpy (Dpy), thick cuticle (Fig. 3C), exploded body (Rup, Fig. 9F), small body size (Sma), slow growth (Gro), slow pumping, uncoordinated movement (Unc), and muscle detachment (Fig. 3A).

Comparison of Phenotypes Caused by Depletion of Ce-imp-2, Ce-lrp-1, and Cholesterol. A similar ISC phenotype has been described for strains deprived of exogenous dietary cholesterol (34). However, this phenotype was not observed in another study in which cholesterol was completely removed from media (19). Recessive mutations in C. elegans Ce-lrp-1 also result in defects in shedding the old cuticle during larval development (34). The C. elegans Ce-LRP-1 encoded by Ce-lrp-1 is an epidermal protein homologous to mammalian low density lipoprotein receptors (LDLR), LRP1 (lipoprotein receptor related protein), and, in particular, megalin (termed also as LRPL/np330). The LDLRs endocytose lipoproteins and regulate lipid homeostasis in mammals. We compared phenotypes caused by removal of supplementary cholesterol and by Ce-imp-2 and Ce-lrp-1 RNAi in parallel experiments. Under similar conditions, the Ce-lrp-1 mutant MH210 [lrp-1(ka156)/gld-1(q266) strain] was also tested. Worms growing on plates with no supplementary cholesterol (as compared with standard plates with 5 μg/ml supplementary cholesterol) developed the ISC phenotype patterns similar to those found for Ce-imp-2 RNAi animals (Fig. 3 B–D). As described in the original report (34), the ISC phenotype was not evident in F1 but was displayed in successive generations after extended cultivation in cholesterol-free medium. Only a small amount of sterol is required for growth of insect cells and free-living nematodes (19, 35–37). Traces of cholesterol in agar, E. coli, or maternal supply in the eggs may be sufficient for normal development of F1 (34), but not the following generations. We tested this prediction preparing ether-extracted peptone (19) for cholesterol-free agarose plates. As a result, we were able to detect 3.7% (75/2020) of animals with ISC in the P0 generation in a synchronized population of worms. Many worms growing under these conditions remained small and weak. One possible model from our studies is that the Ce-imp-2 is required for the production of a cholesterol-derived hormone important for molting. For example, the steroid hormone, 20-hydroxyecdys-
one, regulates molting in *Drosophila* (reviewed in ref. 38). However, these hormones have not yet been found in *C. elegans*.

We next compared phenotypes in *Ce-lrp-1* RNAi and *Ce-imp-2* RNAi worms. The effects of *Ce-lrp-1* RNAi were similar to *Ce-imp-2* RNAi, producing the ISC phenotype during larval development. The partially detached cuticle found on all L1–L4 molt stages was seen frequently in the head and less frequently in the tail. Constrictions in the middle part of body were also found in both *Ce-lrp-1* and *Ce-imp-2* RNAi worms (Fig. 3B and Table 2, which is published as supporting information on the PNAS web site). The most noticeable difference was observed during the late embryonic/early L1 stages; death was much less frequent or not detected in F1 progeny of *Ce-lrp-1* RNAi-treated animals. Animals homozygous for *lpr-1(ku156)* showed the same ISC phenotypes observed in *Ce-lrp-1* (RNAi) (Fig. 3B). Arrested growth (with and without visual ISC), unc, sma, and occasional Dpy, Rup phenotypes were also identified in both *Ce-lrp-1* (RNAi) and *Ce-imp-2* (RNAi) animals. These effects may be related to the overly thick cuticle found in cholesterol-, *Ce-imp-2*-, and *Ce-lrp-1*-deficient worms (Fig. 3 C and D) and the inability to shed old cuticle compressing the growing body. The reduced brood size has been described in progeny of worms maintained on a reduced cholesterol diet (19, 39). We examined the brood size of individual worms on *Ce-imp-2* RNAi food. Worms that developed ISC at the L2-adult stage died, some of them having a low number of progeny hatched inside dead mothers. The surviving worms were able to lay eggs over the next 2 or 3 days, but with a mean brood size at least 3-fold less than control RNAi worms (Supporting Text, which is published as supporting information on the PNAS web site). In summary, our data showed that *Ce-imp-2* deficiency phenotype resembles abnormalities induced by cholesterol depletion and closely mimics the *Ce-lrp-1* deficiency phenotype. Because *Ce-lrp-1* regulates endocytosis of cholesterol in mammals, it is conceivable that both *Ce-imp-2* and *Ce-lrp-1* may contribute to cholesterol uptake in *C. elegans*. Filipin is a fluorescent polyene antibiotic capable of forming complexes with cholesterol and other sterols in accumulation sites (19). Consistent with the idea that *Ce-imp-2* and *Ce-lrp-1* functions may contribute to cholesterol uptake or homeostasis pathway, filipin complexes accumulated in the body locations of *Ce-imp-2* RNAi animals where displacement of old cuticle normally is initiated (Fig. 3E).

The Cytoplasmic Terminus of *Ce-LRP-1* Can Suppress the *Ce-imp-2* Defect Induced by RNAi. We tested further whether *Ce-imp-2* may be an upstream regulator of *Ce-lrp-1* or *Ce-lrp-1*-dependent pathway in development. We asked whether the *Ce-imp-2* deficiency can be compensated by expression of *Ce-LRP-1* protein derive. The intracellular fragment of megalin contains FxN-PxY and PxxPxP conserved motifs essential for endocytosis and association with adaptor proteins (e.g., Ce-DAB-1) (Fig. 6). A construct encoding a cytoplasmic fragment of *Ce-LRP-1* truncated in the N-transmembrane domain (*Ce-lrp-1* L-ICD) was designed (Fig. 6 and Supporting Materials and Methods). The transgenic worm strains with extrachromosomal maintenance of *Ce-lrp-1* L-ICD under *Ce-lrp-1* regulatory regions were generated. *Sur-5 GFP* plasmid was used as a coinjection marker. The wild-type N2 and strains with extrachromosomal maintenance of transgenic marker *sur-5 GFP* alone were used as controls. The synchronized population of worms at the L1 stage were transferred to *Ce-imp-2* dsRNA plates, and, on day 3, worms were observed for the ISC phenotype as discussed above. The truncated *Ce-lrp-1* fragment caused profound rescue of the ISC defect caused by *Ce-imp-2* deficiency. This result was found and confirmed in two independent transgenic strains (Fig. 4). The rate of *Ce-imp-2* RNAi-induced ISC molting defect in *Ce-lrp-1* L-ICD strains was reduced 5- to 10-fold. These data suggest that *Ce-imp-2* may be directly involved in the regulation of *Ce-LRP-1* (by means of regulation of processing or trafficking) or may regulate other upstream elements interacting with the *Ce-lrp-1* pathway (Fig. 10, which is published as supporting information on the PNAS web site).

**Discussion**

**Presenilins and IMPs: Two Related Families of Proteins Regulating Distinct Development Pathways.** We reported here a primary in vivo analysis of the functional role of the IMPAS (IMP) family of proteins, which are distinctly homologous to PSs. Both families (IMPs and PSs) share conserved amino acid signatures and structure identities thought to be critical for their intramembrane protease activities. Although a role of PSs in protein trafficking is also postulated, there are many arguments supporting the view that PSs and IMPs are unusual bi-aspartyl polytopic proteases with many structural and functional similarities. Moreover, many amino acid residues in PSs, which are targets for multiple mutations in AD, also occur and are evolutionarily conserved in IMPs (15). Nevertheless, we demonstrate here that the Notch protein regulated by PSs is not a major target for the IMP proteins. *C. elegans* *Ce-imp-2* does not facilitate *sel-12* Hop-1-dependent *lin-12* glp-1 signaling. Our data, instead, indicated that IMPs and PSs are functionally distinct families of proteins regulating two different pathways in development. Phylogenetic analysis demonstrated that IMPs have a more ancient ancestry prototype (found in *Archaea* and yeast) than PSs (found in *Protista*) (12). Thus, beyond the role of IMPs in the development of multicellular organisms, the common cellular functions of IMPs (e.g., in regulation of cell maintenance, proliferation, or cycles) remain be elucidated.

**Ce-imp-2-Regulated Development Pathway.** *Ce-imp-2* is the ortholog of human IMP1/SSP, which is capable of cleaving or, at least, promoting the cleavage of some proteins within hydrophobic domains (14, 15, 17). The proteolytic properties of IMP1/SSP were demonstrated by direct assays in vitro and in cultured cells. The signal peptide peptidase activity of IMP1/SSP is thought to produce short peptide fragments that serve as HLA-E epitopes in mammals (16). However, the major function of human IMP1/SSP or their orthologs or paralogs remained to be uncovered.

The knock-down of *Ce-imp-2* performed herein revealed that *Ce-imp-2* is required for proper embryonic development and
The observations illuminate the intriguing possibility that similar patterns of interacting molecules and pathways are involved in sterol homeostasis and cholesterol-lipoprotein receptor (Ch-LR)-dependent development in *C. elegans* and mammals. The ISC phenotype may be caused by partial deficiency in Ch-LR signaling and may serve as an excellent phenotype marker in the search for other molecular components of the *C. elegans* Ch-LR pathway. Known and yet unknown proteins interacting with the Ce-lrp-1 pathway may be potential targets or downstream elements regulated by Ce-imp-2 (Figs. 6 and 10). The role of sel-12 in Notch-signaling is established (46). The Ce-imp-2 controls distinct pathway. In summary, the data described here imply that Ce-imp-2 and, perhaps, the human ortholog IMP1/SPP are essential regulators of development. Given the ancient origin and diversity of genes of the IMPAS family, their pleotropic functions are anticipated in both vertebrates and invertebrates.

We thank members of our laboratories for technical assistance, Dr. I. Greenwald and Dr. C. Goutte for providing *C. elegans* mutant strains, Dr. Y. Kohara for providing *C. elegans* cDNA clones, Dr. R. Kopan (Washington University School of Medicine, St. Louis) for providing Notch constructs, and Dr. B. De Strooper (Center for Human Genetics, Katholieke Universiteit Leuven, and Flanders Interuniversity Institute for Biotechnology, Leuven, Belgium) for providing PSI and PS2 knockout mouse fibroblasts. This work was supported by Alzheimer’s Association Grant TLL-03-5777 and, in part, by the Howard Hughes Medical Institute, the Russian Foundation for Basic Research, and the National Institute of Neurological Disorders and Stroke.
No rescue of *sel-12/lin-12, glp-1* defects by *Ce-imp-2* RNAi. Deficiency in *sel-12* alone (with a functional *hop-1* paralogous gene) does not induce the *lin-12, glp-1* phenotypes, but certain alleles produce an incompletely (e.g., *sel-12 (ar131)*) or completely (e.g., *sel-12 (ar171) unc-1*) penetrant recessive egg-laying defective phenotype (Egl) and large vulval protrusion. The loss-of-function of certain *lin-12* mutants is also associated with the Egl (egg-laying defect) phenotype and abnormal vulval development. We found no prominent Egl phenotype or protruding vulvae in N2 wild-type animals fed with *Ce-imp-2* dsRNA. However, we occasionally observed eggs that hatched within the mother and reduced brood size, resembling effects in *sel-12* mutants (1). We next used sensitized genetic backgrounds to look for interactions between *Ce-imp-2* and Notch-pathway components. To do this, we used RNAi to knock down *Ce-imp-2* activity in (i) *sel-12(ar171) unc-1(e538)* and *sel-12(ar131)* (presenilin loss-of-function mutants); (ii) *unc-32(e189) lin-12(n676n930) III* strain (temperature sensitive hypomorphic *lin-12* allele at 25°C); (iii) *lin-12(n137)/unc-32(e189)III;him-5(e1467)V* (a *lin-12* hypermorphic strain); (iv) *glp-1(e2142ts)* (temperature-sensitive hypomorphic *glp-1* allele). None of these mutant strains exhibited enhancement or suppression of their corresponding phenotypes upon induction *Ce-imp-2* (RNAi). For example, in the *sel-12(ar171) unc-1* and *sel-12(ar131)* mutant strain, RNAi targeting the *sel-12* homolog *hop-1* induces an embryonic arrest and loss of anterior pharynx (2). No such defect in pharyngeal development was induced by *Ce-imp-2* (RNAi) in the *sel-12* mutants background. We found also that *Ce-imp-2* (RNAi) did not rescue Egl phenotype of *sel-12(ar171) unc-1*. Likewise, inhibition of *Ce-imp-2* in the *lin-12(n137)/unc-32(e189)III;him-5(e1467)V* hypermorphic strain did not reduce or enhance the multivulva phenotype of this strain. Finally, RNAi targeting

<table>
<thead>
<tr>
<th>Strains</th>
<th>RNAi</th>
<th>Total worms</th>
<th>T, °C</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>sel-12(ar171) unc-1</em></td>
<td></td>
<td>136</td>
<td>room</td>
<td>100% Egl</td>
</tr>
<tr>
<td><em>unc-32(e189)</em></td>
<td><em>Ce-imp-2</em></td>
<td>147</td>
<td>25</td>
<td>100% Egl</td>
</tr>
<tr>
<td><em>lin-12(n676n930) III</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>glp-1(e2142ts)</em></td>
<td></td>
<td>P₀, 162</td>
<td>25</td>
<td>F₁, 100% Emb</td>
</tr>
</tbody>
</table>
*Ce-imp-2* in the hypomorphic *glp-1(2142ts)* genetic background did not reduce the rate of embryonic lethality (Emb) in this strain.


Table 2. ISC phenotypes in N2 worms (P₀) fed with dsRNA *Ce-imp-2* or dsRNA *Ce-lrp-1*

<table>
<thead>
<tr>
<th>Strain</th>
<th>RNAi</th>
<th>Total worms</th>
<th>ISC head</th>
<th>ICS tail</th>
<th>Body constrictions</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td><em>Ce-imp-2</em></td>
<td>999 (100%)</td>
<td>240 (24%)</td>
<td>19 (1.9%)</td>
<td>4 (0.4%)</td>
</tr>
<tr>
<td>N2</td>
<td><em>Ce-lrp-1</em></td>
<td>326 (100%)</td>
<td>46 (14.1%)</td>
<td>12 (3.7%)</td>
<td>21 (6.4%)</td>
</tr>
</tbody>
</table>
Supporting Text

Effect of *Ce-imp-2* RNAi on brood size. Because the reduced number of eggs in a common population may merely reflect increased death of worms during the molt stages, we examined the brood size of individual ST2 worms on *Ce-imp-2* RNAi food. The ST2 strain expressing GFP in the CNS was used for easier detection of weak and dead worms at the late embryonic and early L1 stages. Synchronized L1 larvae (n = 10) were placed in separate *Ce-imp-2* dsRNA plates. The hatched larvae and eggs were inspected daily during 5 days; each day, the parent worms were transferred to fresh plates. Worms that developed incomplete shedding of cuticle (ISC) at the L4-adult stage died, some of them having a low number of progeny hatched inside dead mothers. The surviving worms were able to lay eggs over the next 2 or 3 days, but with a mean brood size at least 3-fold less than control RNAi worms. About 80% of these progeny died at the late embryonic-early larvae stages.

Supporting Materials and Methods

RNA Interference (RNAi) by Double-Stranded RNA (dsRNA) Feeding. To conduct RNAi by feeding, we have subcloned the full-length 1.54-kb cDNA *Ce-imp-2* (yk671a5 clone) into the L4440 vector. We also subcloned *Ce-imp-2* cDNA fragments that include the 1329-bp middle region, and nonoverlapping fragments [5'- *Ce-imp-2* sequence (485 bp) and 3'-*Ce-imp-2* sequences (823 bp)] into L4440 (Fig. 5B). We believed that application of the different dsRNAs would exclude theoretically possible nonspecific RNAi effects and might provide a more detailed characterization of *Ce-imp-2*-regulated phenotypes on different stages of worm development. Identical phenotype defects were induced by expression of all these RNAi constructs; these results were reproduced in multiple experiments in three laboratories (Brudnick Neuropsychiatric Research Institute, Howard Hughes Medical Institute, and Laboratory of Molecular Brain Genetics). Subfragments of *Ce-imp-2* cDNA for RNAi by feeding were cloned into L4440 vector using primers 5’- TTTTGAATTTCGACGGCTAGCAATGTCACAG-3’, 5’- TTTTCTCGAGTCCACATGTCTTGATTCATC-3’ (1329 bp); 5’- TTTTGAATTTC
CTTTCGCGACATGGCTGA-3', 5'-TTTTCTCGAGTATAAGCGTATGTAGCCGCATTT -3' (485 bp); and 5'-TTTTGAATTCCCATTTGGTACCGCGTTTCT-3', 5'-TTTTCTCGAG GCCGGAGTCTACTTTCTTTCG-3' (823 bp) (Fig. 5B). cDNA for Ce-lrp-1 gene corresponding to the extracellular part of the protein was subcloned into BamHI-XhoI L4440 using primers 5'-TTTTGGATCCGCCGTACTTGCTCTCCATTC-3' and 5'- TTTTCTCGAGCCATCCAATCGACATTTTCC-3' (Fig. 5B). These constructs were transferred to the Escherichia coli strain HT115. The bacterial cultures were grown in 1 liter of LB-Amp to OD<sub>600</sub> ≈ 1, spun down, and resuspended in 50 ml of LB, containing Amp 60 µg/ml and 7% DMSO, and were frozen and stored in 5-ml aliquots at -70°C. For each experiment, a fresh aliquot was used. For dsRNA induction, 100 µl of isopropyl β-D-thiogalactoside (IPTG) (200 mg/ml) was added to each E.coli stock. Synchronized population of worms was obtained by using the standard hypochlorite method. L<sub>1</sub> stage larvae, hatched in M9 buffer, were placed on dsRNA agarose plates, and the phenotype of worms (P<sub>0</sub>) was analyzed on the second and third days at room temperature. On the third day, eggs were collected using the hypochlorite treatment. Eggs were placed on dsRNA plates, and the phenotype of F<sub>1</sub> progeny was analyzed for the next two days.

**Mammalian Cell Lines and Western Blot Analysis.** Cell lines with stable or transiently transfected constructs were maintained in appropriate media supplemented with 10% FBS (GIBCO/BRL); 1% penicillin/streptomycin, 2 mM L-glutamine at 37°C; and 5% CO₂. Human embryonic kidney (HEK) 293 cells and mouse fibroblasts were cultured in DMEM; PC12 rat pheochromocytoma cells in RPMI medium 1640; Chinese hamster ovary (CHO) cells in F12; and H4 (human neuroglioma) cells in Opti-MEM (GIBCO/BRL). Transfection was performed using LipofectAMINE PLUS Reagent (GIBCO/BRL). Twenty-four to 48 h after transfection, cells were briefly washed twice in cold PBS, lysed in modified RIPA buffer (50 mM Tris•HCl, pH 7.4/1% Nonidet P-40/0.25% sodium deoxycholate Na/150 mM NaCl/1 mM EDTA) supplemented with protease inhibitors (Roche Molecular Biochemicals) for 15 min at 4°C, and centrifuged at 20,800 × g for 10 min at 4°C. For coimmunoprecipitation (co-IP) experiments of hIMP1, HEK293 and H4 cells were cotransfected with hIMP1-c-myc and hIMP1-V5 plasmids.
Ten to 20 µg of protein extracts were mixed with SDS sample buffer (twice) (Invitrogen) containing reducing agent, centrifuged at 12,000 rpm for 5 min with or without prior boiling for 5 min, and loaded onto SDS polyacrylamide gel (PAAG) minigels. Prestained molecular weight marker was loaded into a separate well. Electrophoresis was run in 10–20% Tricine PAAG or 10% SDS PAAG for hIMP1 products and 8% Tris-Glycine SDS PAAG for NotchΔE and NOTCH1 intracellular domain (NICD) fragments in corresponding 1× SDS running buffer at 125 V. Electro-transfer onto poly(vinylidene difluoride) (PVDF) membranes was performed in a Tris-Glycine transfer buffer 12 mM Tris base, 96 mM glycine, 20% methanol. After transfer, membranes were washed in TBS-T buffer (50 mM Tris•HCl, pH 7.4/150 mM NaCl/0.05% Tween 20) three times for 5 min, incubated in blocking buffer (5% milk in TBS-T) at room temperature for 1 h, and hybridized with primary antibodies, 1:1,000–1:5,000 dilution in 10 ml of hybridization buffer (1% milk in TBS-T) at room temperature for 1 h, or 4°C overnight. The primary polyclonal and monoclonal antibodies against N-terminal-PS1 fragments and N-terminal hIMP1 or V5 and c-myc C-terminal targets of hIMP1 or Notch epitopes have been used to control efficiency of expression in each transfection experiment. After incubation with appropriate secondary antibodies, signal visualization was performed using ECL Western blotting detection reagent kit (Amersham Pharmacia) by exposure to an x-ray film.

**Pulse-chase experiments.** HEK293 cells were transfected with Notch, hIMP1, and PS1 constructs using LipofectAMINE PLUS Reagent (GIBCO/BRL). NotchΔE and NICD (obtained from R. Kopan, Washington University School of Medicine, St. Louis) were fused to c-myc epitopes. hIMP1 wild-type or mutant isoforms were cloned into cDNA3 or pcDNA4/c-myc-HisB. Twenty-four hours after transfection, cells were starved for 2 h in methionine and cysteine-free medium without serum, labeled with 0.1 mCi of \(^{[35}S\)methionine and \(^{[35}S\)cysteine for 30 min, chased for 1 h in DMEM medium supplemented with 10% FBS, and lysed in RIPA buffer with 1% Nonidet P-40 and protease inhibitor mixture. Protein extracts were immunoprecipitated with anti-c-myc antibodies to Notch-C-terminal tag-epitopes and subjected to electrophoresis and autoradiography.
A

C36B1.10

Ce-imp-1 chr. I, 8760290-8763725

C36B1.11

C33H5.10

Ce-imp-3 chr. IV, 7789459-7786002

C33H5.10

Ce-imp-3 chr. IV, 7789459-7786002

rsps-6

T05E11.2

Ce-imp-2 chr. IV, 1111859-11116728

T05E11.3

- 5', 3' UTR
- CDS
- intron
- neighbor gene

B

1.57 kb Ce-imp-2 cDNA

subfragments:
← 485 bp
← 823 bp
← 1329 bp

1.75 kb Ce-tp-1 cDNA

Double-T7 vector L4440
Ce-LRP-1

NH2

- LDLR, YWTD repeat
- LDLR, class A domain
- EGF-like domain
- transmembrane domain
- dileucine domain LL

extracellular space
membrane
cytoplasm

COOH

996bp 5' genomic Ce-lrp-1

Ce-lrp-1 CDS

1026 bp 3' genomic Ce-lrp-1

L 4440 vector

500 bp

BamHI

ATG

TAA

PstI

FxNPxY
PxxPxxP

- LDLR, YWTD repeat
- LDLR, class A domain
- EGF-like domain
- transmembrane domain
- dileucine domain LL
B

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80 KD

C

D

% of NICD, normalized to a sum of NotchΔE and NICD

% of NICD normalized to a sum of NotchΔE and NICD

NotchΔE

NICD

NotchΔE

NICD

*
Cholesterol -daf-9/CYP17, CYP21

-cholesterol (sterol) dependent signal and endocytosis regulators

-nuclear receptors nhr-23, nhr-25; daf-12

-DEVELOPMENT

-Ce-imp-2/IMP1

-Ce-lrp-1/megalin (LRP2)

-endocytosis complex/trafficking molecules (Ce-dab-1/Dab1; let-512)