Ambulacrarian insulin-related peptides and their putative receptors suggest how insulin and similar peptides may have evolved from Insulin-like Growth Factor

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Abstract

Background

Some Insulin/IGF-related peptides (irps) stimulate a receptor tyrosine kinase (RTK) that transfers the extracellular hormonal signal into an intracellular response. Other irps, such as relaxin, do not use an

RTK, but a G-protein coupled receptor (GPCR). This is unusual since evolutionarily related hormones typically either use the same or paralogous receptors. In arthropods three different irps, *i.e.* arthropod IGF, gonadulin and *Drosophila* insulin-like peptide 7 (dilp7), likely evolved from a gene triplication, as in several species genes encoding these three peptides are located next to one another on the same chromosomal fragment. These arthropod irps have homologs in vertebrates, which suggests that the initial gene triplication was perhaps already present in the last common ancestor of deuterostomes and protostomes. It would be interesting to know whether this is indeed so and how insulin might be related to this trio of irps.

Methodology

Genes encoding irps as well as their putative receptors were identified in genomes and transcriptomes from echinoderms and hemichordates.

Results

A similar triplet of genes coding for irps is also found in some ambulacrarians. Two of these are orthologs of arthropod IGF and dilp7 and the third is likely a gonadulin ortholog. In echinoderms two novel irps emerged, gonad stimulating substance (GSS) and multinsulin, likely from gene duplications of the IGF and dilp7-like genes respectively. The structures of GSS diverged considerably from IGF, which would suggest they use different receptors than IGF, but no novel irp receptors evolved. If IGF and GSS use different receptors and the evolution of GSS from a gene duplication of IGF is not associated with the appearance of a novel receptor while irps are known to use two different types of receptors, it seems to suggest that the ancestor of GSS and IGF might have acted on both types of receptors while one or both of its descendants act on only one. There are three ambulacrarian GPCRs that have amino acid sequences suggestive of being irp GPCRs, two of these are orthologs of the gonadulin and dilp7 receptors. This suggests that the third might be an IGF receptor, and that by deduction GSS only acts on the RTK. The evolution of GSS from IGF may represent a pattern, where IGF gene duplications lead to novel genes coding for shorter peptides that activate an RTK. It is likely this is how insulin and the insect neuroendocrine irps evolved independently from IGF.

Conclusion

The local gene triplication described from arthropods that yielded three genes encoding irps was already present in the last common ancestor of protostomes and deuterostomes. It seems plausible that

53 irps, such as those produced by neuroendocrine cells in the brain of insects and echinoderm GSS
 54 evolved independently from IGF and thus are not true orthologs, but the result of convergent evolution.
 55
 56
 57 Key words: insulin; relaxin; receptor tyrosine kinase; G-protein coupled receptor; evolution; gonadulin;
 58 octinsulin; multinsulin; dilp7

59 Introduction

60 Many protein hormone and neuropeptide signaling pathways have orthologs in both protostomes
61 and deuterostomes showing that these pathways were already present in their last common bilaterian
62 ancestor. In some cases the orthologs of the peptide ligands show only limited sequence similarity, but
63 their receptors contain protein domains that are sufficiently conserved to establish homology. Virtually
64 all ligands employ either a single receptor or a number of related receptors that evolved by gene
65 duplication. Co-evolution of peptide ligands and receptors insures that related protein hormones or
66 neuropeptides use receptors akin to those of their orthologs (Mirabeau & Joly, 2013; Hsueh & Feng,
67 2020).

68 Insulin/IGF-related peptides (irps) are an exception to this rule. Whereas insulin and IGF act
69 through a receptor tyrosine kinase (RTK), relaxin uses a leucine-rich repeat G-protein coupled receptor
70 (LGR). This raises the interesting question as how this apparent jump from one type of receptor to
71 another may have come about. In cockroaches, termites and stick insects three different irp genes,
72 gonadulin, arthropod insulin-like growth factor (aIGF) and arthropod relaxin, are located next to one
73 another in the genome and thus likely originated from a local gene triplication (Veenstra, 2020b). To
74 avoid confusion with the vertebrate relaxins and related peptides, the arthropod relaxins will be referred
75 to as *Drosophila* ilp7 (dilp7) in this manuscript. One of their irps, aIGF, is known to use an insulin RTK,
76 while gonadulin acts through insect LGR3 (Vallejo et al., 2015; Garelli et al., 2015; Colombani et al.,
77 2015). Bioinformatic evidence suggested that dilp7 must be the ligand for insect LGR4 and this has
78 now been confirmed experimentally in *Drosophila* (Veenstra, Rombauts & Grbić, 2012; Imambocus et
79 al., 2020), but dilp7 may also activate an RTK (Linneweber et al., 2014). This suggests that the
80 archtype arthropod IGF-related peptide acted through both an RTK and an LGR and that after a likely
81 gene triplication, some of the ligands may have lost one of the two original receptors. Although it is
82 possible that the gene triplication of the ancestral insulin gene occurred in an early arthropod or
83 protostomian, it may well have occurred in a bilaterian ancestor, as homologs of both aIGF and dilp7
84 are also present in deuterostomes.

85 Brain neuroendocrine insect irps are more closely related to IGF than either dilp7 or gonadulin and
86 a gene duplication that gave rise to separate genes encoding these peptides is therefore likely to have
87 occurred after the triplication that gave rise to the ancestor genes of gonadulin and dilp7. Yet in insect
88 genomes irp genes are not located near the IGF gene. Thus the particular organization of these genes
89 suggests that whereas the gonadulin and dilp7 genes likely originated by two successive local gene

duplications, the IGF gene duplication that gave rise to an initial arthropod neuroendocrine brain irp must have materialized in a different fashion. If the earlier mentioned gene triplication was already present in the last common ancestor of the deuterostomes then a similar argument can also be made for the evolution of insulin. Given the importance of insulin as a human hormone and the inherent interest of its evolutionary origin, I explored the evolution of bilaterian insulin-related peptides in more detail and here report on the genes coding for such peptides and their receptors in the Ambulacraria that suggest how insulin may have evolved from IGF.

Materials and Methods

Nomenclature

Hormones have often been discovered independently by different groups using different bioassays. The vertebrate insulin-like growth factors are a good example of that. Predicted protostomian peptides and their receptors have sometimes been given names that refer to similar deuterostomian proteins. In some cases this is very confusing, *e.g.* vertebrate LGR-3, -4 and -5 are not the orthologs of arthropod receptors that have been given the same names. A similar problem occurs with arthropod relaxin that is not an ortholog of vertebrate relaxin. This peptide will therefore be called dilp7 (*Drosophila* insulin-like peptide 7). I will refer to arthropod LGR3 as the gonadulin receptor, arthropod LGR4 as the dilp7 receptor and arthropod LGR5 as GRL101, a GPCR initially identified from the pond snail *Lymnaea stagnalis* (Tensen et al., 1994) that is an ortholog of arthropod LGR5 (Veenstra, 2020b).

Another nomenclature problem concerns the terms, insulin-like and insulin-related that are not well defined. Insulin and IGF are related and must share a common evolutionary origin with other peptide ligands like vertebrate relaxin, INSL3, arthropod dilp7 and gonadulin and a large number of other bilaterian peptides. All these peptides are often collectively called insulin-like or insulin-related without any specification as to in which aspects these hormones are similar to insulin. The typical core sequence of six cysteine residues and its use of an RTK are two characters that are shared by vertebrate IGF and insulin. However, several related peptides have eight cysteine residues and others like vertebrate relaxin use an LGR and not an RTK. Insulin and IGF are also different in that IGF is a single chain molecules, while the insulin precursor is processed into a two chain molecule. The term insulin-like seems more appropriate for a subset of the insulin/IGF-related peptides that look similar to insulin and act through an RTK, yet are different from IGF. Calling IGF-related peptides like vertebrate relaxin, INSL3 or arthropod gonadulin for which there is no evidence that they act through an RTK,

insulin-like is confusing. Unfortunately for many bilaterian peptides we can only speculate as to which type of receptor they use. The difference between one or two chain ligands, *i.e.* IGF versus insulin, is also useless as there is good evidence that some insect IGF-related peptides are processed into two-chain molecules when expressed in neuroendocrine cells and produced as single chain ligands when produced by the fat body, yet in both cases stimulate an RTK. It is for these reasons that all these peptides will be referred to as insulin/IGF-related peptides, abbreviated irps.

Sequence analysis

Sequences for insulin related peptides and their likely receptors were identified from a number of Ambulacraria species. This was done using the Artemis program (Rutherford et al., 2000) and the BLAST+ program (<ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/>) on publicly available genome sequences from the feather star *Anneissia japonica*, the sea urchins *Lytechinus variegatus* (Davidson et al., 2000) and *Strongylocentrus purpuratus* (Sea Urchin Genome Sequencing Consortium, 2006), the sea cucumbers *Apostichopus japonicus* (Jo et al., 2017; Zhang et al., 2017) and *Holothuria glaberrima*, the sea stars *Acanthaster planci* (Hall et al., 2017), *Pisaster ochraceus* (Ruiz-Ramos et al., 2020) and *Patiria miniata*, the brittle star *Ophiothrix spiculata* and the hemichordates *Saccoglossus kowalevskii* and *Ptychodera flava* (Simakov et al., 2015). The genomes were downloaded from <https://www.ncbi.nlm.nih.gov/genome>. For many of these species there are also significant amounts of RNAseq data and these were analyzed using the sratoolkit (<https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=software>) in combination with Trinity (Grabherr et al., 2011) using methods described in detail elsewhere (Veenstra, 2020b). Some protein sequences were found in the NCBI database, but several of them contain errors or are incomplete. Where possible these were corrected and/or completed using the methods described above. As there is only a single crinoid genome assembly available, transcriptome data from the three crinoid species *Antedon mediterranea*, *Florometra serratissima* and *Oligometra serripinna* were also included. For the same reason transcriptome data from the brittle star *Amphiura filliformis*, *Ophioderma brevispina* and the hemichordate *Schizocardium californicum* were likewise analyzed. Obviously, transcriptome data can only demonstrate the presence of gene but not its absence and their usefulness depends largely on the variety of tissues sampled and the expression levels of the genes of interest. Nevertheless, such data often provide additional sequences that even if they are incomplete increase the robustness of sequence comparisons. Genomic and transcriptomic RNAseq short read archives (SRAs) were downloaded from

152 NCBI (<https://www.ncbi.nlm.nih.gov/sra/>); a list of the SRAs analyzed is provided in the
153 supplementary data.

154 As queries for the insulin-like peptides a number of such peptides from a variety of species was
155 used. Insulin RTKs are easily identified in genome and transcriptome assemblies, as their kinase
156 domains are very well conserved. The LGRs that could function as insulin receptors are more variable.
157 Vertebrate RXFP1 and RXFP2 are LGRs are known receptors for relaxin and Ins3 and *Drosophila*
158 LGR3 and LGR4 for gonadulin, and dilp7 respectively. Other LGRs function as receptors for the
159 various glycoprotein hormones, GPA2/GPB5, bursicon, TSH, FSH and LH. These GPCRs cluster on
160 phylogenetic trees with another protostomian LGR, GRL101. This GPCR was initially identified from
161 the pond snail *Lymnaea stagnalis* and was the first GPCR discovered to have in addition to six leucine-
162 rich repeats also twelve repeats of a sequence that was known to exist in the low density lipoprotein
163 receptor and are now called LDLa repeats (Tensen et al., 1994). I have suggested previously (Veenstra,
164 2020b) that this receptor might be an IGF receptor.

165 Both the RTK and LGR receptors have large ectodomains. Those of the insulin RTKs are very
166 similar from one receptor to another, while those of the LGRs differ between different types. The latter
167 all contain numerous Leucine-rich repeats (LRRs) and some also have LDL-receptor class A (LDLa)
168 repeats. Both LRRs and LDLa's are present in many other proteins. Initial searches for orthologous
169 receptors were therefore done using the transmembrane regions of various insect and vertebrate LGRs
170 and the protein kinase domain of RTK. Once partial sequences of putative receptors were identified, the
171 coding sequences of these domains were then used to complete the cDNA sequences as best as
172 possible, using either Trinity on RNAseq SRAs or Artemis on genome sequences.

173

174 *Sequence similarity and phylogenetic trees*

175 Both phylogenetic and sequence similarity trees use Clustal omega (Sievers et al., 2011) to produce
176 alignments. Fasttree (Price, Dehal & Arkin, 2010), using the `/FastTreeDbl` command with the `-spr 4, -`
177 `mlacc 2` and `-slownni` options, was used to construct trees and estimate probabilities.

178 In order to identify putative receptors for the various irps, LGRs that show homology to various
179 arthropod and vertebrate LGRs were identified and a phylogenetic tree based exclusively on the
180 transmembrane regions of these receptors was constructed.

181

182 *Precursor processing*

183 Precursors of insulin-like peptides contain signal peptides that are removed on entry into
 184 endoplasmatic reticulum. Signal P 5.0 (Almagro Armenteros et al., 2019) was used online
 185 (<http://www.cbs.dtu.dk/services/SignalP/>) to predict where this cleavage would most likely occur.
 186 Some, but not all precursors are further processed by convertases. Of these furin is ubiquitously present
 187 in all cell types and can thus potentially cleave any secreted protein with appropriate cleavage site. Its
 188 consensus cleavage site is K/R-X-K/R-R, the two human IGF precursors are processed at KSAR and
 189 KSER respectively (Humbel, 1990). Precursors that are produced in cells with a regulated pathway,
 190 such as neuroendocrine and enteroendocrine cells, are also exposed to other convertases like PC1/3 and
 191 PC2. Their consensus cleavages site is KR. However, effective proteolytic processing by convertases is
 192 strongly influenced by amino acid residues surrounding these consensus cleavage sites. For example
 193 bulky residues immediately following the arginine residue, a proline residue before the consensus site
 194 or disulfide bridges nearby can cause sufficient steric hindrance to inhibit cleavage. Using rules
 195 proposed to predict cleavage by PC1/3 and PC2 in both vertebrates and insects (Devi, 1991; Rholam et
 196 al., 1995; Veenstra, 2000) I have tried to indicate in Figs. 3-6 where the various precursors might be
 197 cleaved. It must be noted though that there is no certainty that these site will be cleaved nor can it be
 198 excluded that proteolytic processing occurs at sites that have not been indicated as such.

199 200 *Expression*

201 With a few notable exceptions (*e.g.* Lin et al., 2017), little is known about the expression of the
 202 various insulin-like peptides in either echinoderms or hemichordates and except for the GSS our
 203 knowledge of their functions is also very limited. Expression data may reveal some preliminary clues
 204 as to where and when they are expressed and thus provide a hint as to their function. For this reason the
 205 number of reads corresponding to the various insulin-related peptides and their putative receptors was
 206 determined in a number of SRAs that might provide evidence as to the time and tissue specific
 207 expression of these proteins. The analysis was performed as described previously (Veenstra, 2020b)
 208 and the data are supplied in Spreadsheet S2.

209 210 211 **Results**

212 *Peptides related to insulin and IGF*

213 Some protein sequences were found in the NCBI database, but several of them contain errors or are
 214 incomplete. Where possible these were corrected and/or completed using the methods described above.
 215 As there is only a single crinoid genome assembly available, transcriptome data from the three crinoid
 216 species *Antedon mediterranea*, *Florometra serratissima* and *Oligometra serripinna* were also included.
 217 For the same reason transcriptome data from the brittle star *Amphiura filliformis*, *Ophioderma*
 218 *brevispina* and the hemichordate *Schizocardium californicum* were likewise analyzed. Obviously,
 219 transcriptome data can only demonstrate the presence of gene but not its absence and their usefulness
 220 depends largely on the variety of tissues sampled and the expression levels of the genes of interest.
 221 Nevertheless, such data often provide additional sequences that even if they are incomplete increase the
 222 robustness of sequence comparisons.

223 Insulin-like peptide precursors are typically characterized as having A, B and C domains that
 224 correspond to the A- and B-chains of insulin and the connecting peptide respectively. In IGF D and E
 225 domains are also recognized, in which the D domain refers to the extension of the A chain and the E
 226 domain to part of the precursor after the D domain that is cleaved from IGF in the Golgi apparatus. For
 227 *dilp7* orthologs it is appropriate to add an F (front) domain for the sequence in N-terminal of the B-
 228 chain that in some peptides is not only larger, but also well conserved (Fig. 1).

229 Previous work on insulin-related peptides in in echinoderms have identified two different types of
 230 insulin-like peptides, gonad-stimulating substances (GSS) and insulin-like growth factors (Mita et al.,
 231 2009; Perillo & Arnone, 2014; Semmens et al., 2016; Smith et al., 2019). The insulin-like growth
 232 factors, but not GSS, are also present in hemichordates. While only a single IGF gene was found in the
 233 crinoids and hemichordates, other ambulacrarians have two such genes (Figs. 2, S1, S2; Spreadsheet
 234 S1). These proteins have large C-terminal extension that are rich in charged amino acid residues,
 235 especially arginine and lysine, but also aspartic and glutamic acid residues. A comparison of the protein
 236 sequences and cDNAs from human IGFs identifies the exact separation between the D and E domains
 237 in these proteins (Humbel, 1990). However, although the corresponding sequences of the hemichordate
 238 and echinoderm IGFs contain numerous arginine and lysine residues (Figs. 2, S1, S2), there are no
 239 obvious convertase cleavage sites as many potential arginine residues are succeeded by residues known
 240 to inhibit such enzymes in vertebrates. It is thus not impossible that the D domains of these proteins are
 241 much larger than in the vertebrate IGFs and if so likely contain numerous positively charged amino
 242 acid residues. There are few transcriptome SRAs for specific tissues, the data that is available suggest
 243 that the IGFs are expressed by many tissues, with the ovary showing significant expression. *Patiria*

244 *pectinifera* is the only species with follicle cell specific SRAs and IGF-1 is strongly expressed by these
245 cells and is probably transferred to the oocyte (Spreadsheet S2).

246 The GSS are known to induce oocyte maturation and ovulation in a two step process, where GSS
247 stimulates the follicle cells to produce 1-methyladenine which subsequently induces resumption of
248 meiosis in the oocyte and about 30 minutes later this is followed by ovulation (Chiba, 2020).
249 Interestingly, GSS was not found in either the genome nor the extensive transcriptome data from the
250 feather star *Anneissia japonica* and was similarly not encountered in the transcriptomes of three other
251 crinoids (Suppl data). Transcriptomes may miss expression of some genes and large genome assemblies
252 are never perfect. The short sequence reads in the genomic SRAs from *Anneissia* were therefore also
253 analyzed for the presence of GSS, but again no evidence for such a gene was found. This peptide is
254 thus likely absent from *Anneissia* and perhaps all Crinoidea. In the Holothuroidea and the Asterozoa,
255 but not the Echinoidea, this gene is duplicated with the two paralogous peptides showing significant
256 sequence variability (Figs. 3, S3, S4; Spreadsheet S1). As for all these peptides and their putative
257 receptors expression data is very limited, but in *Apostichopus* the two GSSs are differentially
258 expressed, with GSS-1 being expressed at specific stages during embryonic development as well as by
259 muscle and GSS-2 strongly expressed by both the ovary and the testes. Interestingly, it is the ortholog
260 of GSS-1 that in *Holothuria scabra* has been tested for biological activity and induces ovulation (Chieu
261 et al., 2019). This makes one wonder what the effects of GSS-2 on ovulation might be in this species.
262 However, *Apostichopus* was the only species where a significant GSS expression was found in the
263 gonads (Spreadsheet S2).

264 Two other insulin-like peptides are commonly present in both hemichordates and echinoderms,
265 including the Crinoidea. The first is an ortholog *dilp7* which has a very characteristic F domain while
266 its A chain is also remarkably well conserved (Figs. 4, S5, S6; Spreadsheet S1). The precursors of this
267 peptide contain typical neuroendocrine KR convertase sites and seems to have its highest expression in
268 the nervous system, although it is also found in other tissues. During embryogenesis its expression
269 occurs relatively late (Spreadsheet S2). The second peptide present in all ambulacrarians has been
270 called octinsulin as it has eight cysteine residues and is thus predicted to have four rather than three
271 disulfide bridges. In echinoderms octinsulin is a single copy gene, but hemichordates have several such
272 genes (Fig. 5, S7, S8; Spreadsheet S1). Octinsulin expression levels are the highest in nervous tissue,
273 and significant expression is also found in the gut and stomach of *Strongylocentrotus* and *Patiria*

274 *pectinifera* respectively. Although virtually absent from normal gut in *Apostichopus*, it has significant
275 expression during gut regenerating in this species (Spreadsheet S2).

276 The Asterozoa have genes coding for a fifth type of insulin, that is usually present in multiple
277 copies and that are referred to as multinsulins. The predicted peptides share structural similarity with
278 the *dilp7* orthologs and their genes have typically four coding exons rather than the two or three of the
279 other *irp* genes. The sprawl of these peptides is perhaps best illustrated by a phylogenetic tree that
280 suggest independent multiplication of these genes in several species (Fig. S10). Within a single species
281 the various multinsulins thus often seem more closely related to one another than to their putative
282 orthologs of other Asterozoa. Some of the multinsulins, like the octinsulins, have acquired two
283 additional cysteine residues and are thus predicted to have four disulfide bridges, but the location of
284 these additional cysteine residues differs from that in octinsulins (Figs. 6, 7, S9, S10; Spreadsheet S1).
285 Like *dilp7* the multinsulins have typical neuroendocrine KR convertase cleavage sites and can thus be
286 expected to be expressed in neuroendocrine and/or enteroendocrine cells but expression data on *P.*
287 *pectinifera* suggest a relatively ubiquitous expression in several tissues.

288 The genome assemblies of *A. planci* and *Pisaster ochraceus* shows these genes to be clustered in
289 the genome and some RNAseq sequences suggests that at least on occasion coding exons from different
290 genes may be combined (Fig. S10). This and the large numbers of SNPs typically present in animals
291 caught in nature and used for RNAseq preparation make it impossible to reliably determine their exact
292 numbers.

293 Genome assemblies allow identification of the introns in these genes. All insulin genes have a
294 characteristic phase 1 intron somewhere in the conceptual C domain of these molecules. This is the
295 only intron in the coding sequences of the octinsulin and GSS genes. The IGF genes have a phase 0
296 intron near the end of the coding sequence and at least some of them have another phase 1 intron just
297 after the transcription start site. The genes coding for the *dilp7* orthologs and multinsulins share an
298 additional phase 2 intron and the multinsulin genes have yet another phase 1 intron. All these introns
299 appear perfectly conserved (Fig. 7).

300

301 *Synteny of genes producing insulin-like peptides*

302 In the *Strongylocentrotus* genome all five genes are located on the same chromosome, with the two
303 IGF genes and those encoding octinsulin and *dilp7* orthologs next to one another and GSS at a distance
304 of 6,000,000 bp (base pairs). At least the *Anneissia* octinsulin and IGF genes are likely located next to

one another on the same chromosome also, as in the current genome assembly two of the three coding exons of IGF and one of the two octinsulin coding exons are located within about 10,000 bp. The three missing exons of these two genes are all located on minicontigs of less than 2,000 bp, as is one of the coding exons for the *dilp7* ortholog. The contigs of the *Lytechinus variegatus* genome assembly are smaller and this may explain why in this species the genes are located on three different scaffolds, with the two ILGF-like peptides and the octinsulin together on a single contig. However in the recently published genome of the closely related *L. pictus* (Warner et al., 2021) the *dilp7* ortholog is also closely associated with the other three genes. The GSS gene is on the same chromosome but at a distance of 28,000,000 bp. In the *Apostichus japonicus* genome assembly the genes encoding the octinsulin and the two IGF genes are located on the same contig, and the other genes each on a different one. In the draft *Holothuria glaberrima* genome assembly only the two IGF genes are located on the same contig, however in a single Oxford nanopore read (SRR9125585.2851.1) from *H. scabra* the octinsulin, *dilp7* and two IGF genes are located next to one another as well (Fig. 8).

Whereas the various Echinozoa genome assemblies suggest a certain degree of synteny with regard to the various *irp* genes, the Asterozoa genome shows that such synteny is disintegrating. This is most clearly demonstrated in the genome assemblies from *Pisaster ochraceus* and *Acanthaster planci*, where the scaffolds are much larger than from *Patiria miniata*. In these species synteny is largely lost (Fig. 8). Interestingly the various multinsulin genes are present in small clusters on different chromosomes in those species.

Sequence similarity tree peptides related to insulin

Peptides having the characteristic insulin signature are notoriously variable in their primary amino acid sequences. Although the various residues allows one to align those sequences, such alignments will not always yield reliable phylogenetic trees as the basic tenet of such analyses is often not met. As an alternative I have proposed to use “sequence similarity trees”. Such trees are constructed using the same methods but do not pretend to illustrate phylogenetic relations, rather similarities between the different proteins.

The structures of the multinsulins are most similar to the *dilp7* orthologs (Fig. 6) and so it is not surprising that the sequence similarity tree (Fig. 9) groups the multinsulins with the *dilp7* orthologs. The hypothesis that this structural similarity between these two types of peptides may reflect a close evolutionary relationship is reinforced by the presence of an intron that is present in the genes encoding

these peptides but lacking in the genes encoding octinsulin, IGF and GSS (Fig. 7). The tree also illustrates significant sequence similarity between GSS and the IGF.

338

339 *Orthologs of receptors for irps: Receptor tyrosine kinase*

340 A single insulin RTK gene was found in all species analyzed here. An alternatively spliced form is
341 present in *Acanthaster* and is likely commonly present in echinoderms (Spreadsheet S1). Hundreds of
342 ambulacrarian protein sequences were identified at NCBI using a BLAST search with the *S. kovalevskii*
343 protein kinase domain as a query. After aligning them with Clustal omega the protein kinase domains
344 were used to make a phylogenetic tree. Results revealed no other known or predicted proteins with a
345 similar protein kinase domain (data not shown). The insulin RTK is ubiquitously expressed
346 (Spreadsheet S2).

347

348 *Orthologs of receptors for irps peptides: LGRs*

349 LGR sequences were obtained using the combination of genomic sequences and, where available,
350 transcriptome shotgun sequences and RNAseq SRAs. The latter were used to produce contigs using
351 Trinity (Spreadsheet S1). Short read assemblers are good in combining sequences into larger
352 continuous ones, but they do produce artifacts, which are more easily obtained when very similar
353 sequences are present in multiple copies, such as the multinsulins, or the numerous LDLa and LRR
354 repeats. These repeats are usually individually coded by single exons that are sometimes skipped and
355 when such skipped individual reads enter in the RNAseq SRA, incorrect constructs are obtained.
356 Furthermore, these repeats are present in numerous proteins, and from time to time this leads to
357 assembled sequences that are from mRNA species from different genes. It is therefore to be expected
358 that not all assembled transcripts, neither those in the databank nor those produced here, will be correct.
359 Some errors were corrected by challenging divergent sequences that were discovered on comparing
360 putative orthologs with one another. Other differences could be confirmed as true differences, but it is
361 not impossible that some errors remain, particularly for those sequences that are incomplete. LGRs that
362 might function as receptors for the various irps were identified by their homology with such receptors
363 from vertebrates and arthropods. The transmembrane regions of the GPCRs don't have the assembly
364 problems of the LDLa and LRR repeats and are the most characteristic domain of the GPCRs. This
365 makes it easier to construct a phylogenetic trees for these receptors based on their transmembrane
366 regions than that it is to produce complete LGR transcripts.

Results show a surprisingly similar distribution of LGRs in the species studied. The tree resolves two major branches, one for the glycoprotein hormone receptors, which itself is divided in two subbranches, one for orthologs of the GPA2/GPB5 receptor - containing the receptors for human TSH, FSH and LH - and a second one for the bursicon receptor orthologs. All species studied are represented by one member on each of these two subbranches, except for *Ophiothrix*, where the draft genome reveals two orthologs each for the bursicon and GPA2/GPB5 receptors (Fig. 10). These are likely receptors for the bursicon and GPA2/GPB5 orthologs identified from various echinoderm species (Semmens et al., 2016). It is interesting to see that whereas vertebrates have different receptors for TSH, FSH and LH, most echinoderms have only one GPA2/GPB5-receptor ortholog (Fig. S11), even though *A. rubens* has two GPA2 and three GPB5 orthologs (Semmens et al., 2016). The LGRs for the glycoproteins were included in the search for putative receptors for the ambulacrarian irp LGRs in order to be sure that no such receptors would be missed.

The lower branches of the LGR phylogenetic tree are the ones of interest as they contain receptors with irp ligands. It consists of three subbranches, that are characterized by *Drosophila* LGR3 and LGR4 – the receptors for gonadulin and dilp7 respectively - and *Periplaneta* LGR5, an ortholog of *Lymnaea* GRL101. Here in all ambulacrarian species studied only one ortholog was found for each of them, despite extensive attempts to find additional LGRs in the various genomes and transcriptomes.

The GRL101 transmembrane regions puts it very close to vertebrate glycoprotein hormone and relaxin LGRs. LRRs are present in many different proteins, but when the LRR part of the *Anneissia* GRL101 (amino acid residues 576-717) is used as query in a protein BLAST against human proteins, the glycoprotein hormone and relaxin receptors are identified as most similar to this ectodomain of GRL101, suggesting that similarity of the GRL101 receptors with vertebrate LGRs is not limited to the transmembrane region of this GPCR.

Sequence alignments of these GPCRs show strong sequence similarity (Figs. S12-S14), however the dilp7 receptor ortholog varies more between species. A schematic representation of the the ectodomains of the LGRs on this second branch is drawn in Fig. 11. The orthologs of the dilp7 and gonadulin receptors each have a single LDLa repeat, except for the *Patiria* and *Acanthaster* orthologs of the dilp7 receptor which both have two LDLa repeats (Fig. S13). This additional LDLa is likely due to a relatively recent duplication of the LDLa since the two LDLa repeats have very similar amino acid sequences (Spreadsheet S1). All three receptors are expressed in the nervous system and the gonadulin

397 receptor is well expressed in the gonads, both testis and ovary, and strongly so in the follicle cells of *P.*
 398 *pectinifera* (Spreadsheet 2).
 399

400 Discussion

401 The genomic and transcriptomic data from both the hemichordates and the echinoderms show that
 402 these two groups share three irps, octinsulin, IGF and a dilp7 orthologs, that are present in both
 403 echinoderms and hemichordates. IGF and dilp7 are orthologs of the arthropod peptides that together
 404 with gonadulin originated from a gene triplication. The structure of gonadulin is poorly maintained,
 405 even within insects (Veenstra, 2020b). The variable structure of gonadulin and its loss in many
 406 arthropod lineages suggests that the evolutionary pressure on gonadulin is weak. This may explain why
 407 the amino acid sequence of gonadulin looks significantly different from octinsulin. Nevertheless, there
 408 are two lines of evidence that suggest that these peptides must be orthologs as well. For one, synteny of
 409 the chromosome fragment containing these genes is conserved between the sea urchin
 410 *Strongylocentrotus purpuratus*, the hemichordate *Saccoglossus kowalewski* and the cockroach *Blattella*
 411 *germanica*, suggesting that these peptides are likely orthologs. More importantly, all ambulacrarians
 412 have an ortholog of the gonadulin receptor and the only plausible ligand for such a receptor encoded by
 413 their genomes is octinsulin. Thus the gene triplication previously reported from arthropods must have
 414 occurred in a common bilaterian ancestor of the deuterostomes and protostomes.

415 Crinoids have the simplest irp signaling system, one gene each for IGF, octinsulin and the dilp7
 416 ortholog. Their putative receptors - insulin RTK, GRL101, and the orthologs of the dilp7 and gonadulin
 417 receptors – similarly are also each coded by a single gene. The hemichordates have a very similar
 418 repertoire, except that the octinsulin gene is systematically amplified and in some species the dilp7
 419 ortholog as well. It thus appears likely that the first deuterostome had a single copy of each of these
 420 genes.

421 Within the echinoderms the irp genes evolved considerably, as shown both by an increase in their
 422 numbers and the loss of synteny. Whereas the feather stars appear to have only a single IGF gene, all
 423 other echinoderms have two such genes and two novel irps, GSS and multinsulin, appeared. The GSS
 424 sequences are most similar to those of IGF, suggesting that they evolved from a gene duplication event
 425 from the IGF gene. Although some GSS genes are located on the same chromosome as the other irps,
 426 they are not close to the IGF genes, indicating that the IGF-GSS split was not a local duplication but
 427 may have been the result of an incorrectly repaired chromosome break.

428 In the Asterozoa a fifth type of irp gene emerged, those that code for the multinsulins which share
429 significant sequence similarity with the dilp7 orthologs. The initial multinsulin gene must thus have its
430 origin in a gene duplication of the dilp7 ortholog gene, with which they furthermore share a
431 characteristic intron. Later the multinsulin gene seems to have undergone several additional gene
432 duplications in this respect the multinsulins resemble the insect neuroendocrine irps.

433 The co-evolution of ligands and receptors allows one to assign the putative receptors for gonadulin,
434 the dilp7 ortholog and IGF as the orthologs of the receptors of their arthropod orthologs. This allows
435 the identification of the ambulacrarian LGRs that are the orthologs of the gonadulin and dilp7 receptors
436 as likely receptors for octinsulin and the dilp7 respectively, as well as the insulin RTK as a receptor for
437 IGF.

438 The appearance of the multinsulins is not accompanied by the evolution of a novel insulin-receptor.
439 Some animals have multiple insulin RTKs, *e.g.* some arthropods have up to four such genes (Veenstra,
440 2020a,b), however, in spite of extensive searches for a second insulin RTK in ambulacrarian genomes,
441 none was found. Searches for an additional LGR that might function as a receptor for the GSS and/or
442 multinsulin were unsuccessful and this raises the question which receptors are activated by these
443 peptides.

444 I have previously argued that the close chromosomal association of the IGF, gonadulin and dilp7
445 ortholog genes in basal insects suggest that they derived from a gene triplication (Veenstra, 2020b).
446 There are three possible scenarios that can explain how IGF and gonadulin came to respectively
447 activate an RTK and an LGR. It is possible that the original irp activated either an RTK and that an
448 LGR was later acquired as a second receptor by gonadulin, alternatively the original irp activated an
449 LGR and IGF acquired an RTK as a second receptor. Given the importance of insulin RTKs for growth
450 in very basal metazoans, it is improbable that the original irp activated an LGR and that an RTK was
451 acquired much later during evolution (see *e.g.* Mortzfeld et al., 2019). This indicates that an irp
452 acquired an LGR as a second receptor and the question is whether this happened before or after the
453 gene triplication that yielded IGF, gonadulin and dilp7. In both *Saccoglossus* and arthropods the IGF
454 gene is in the middle of the three. This suggests that this represents the gene organisation after the gene
455 triplication and that the dilp7 and gonadulin orthologs each evolved independently from the arch irp
456 rather than that dilp7 evolved from gonadulin or *vice versa*. Had dilp7 originated from a gene
457 duplication of gonadulin or the other way round, they also might have been more similar to one another
458 than they are. The acquisition of a second receptor must be an extremely rare event. Since both

gonadulin and dilp7 use an LGR this would mean that such an extremely rare event of the acquisition of a second receptor would have occurred not only twice, but even with a very similar receptor. Furthermore, some metazoans have an LGR that is closely related to the dilp7 and gonadulin LGRs suggesting that it could be an IGF receptor (see below). It is for these reasons that the author favors the hypothesis that the arch irp already acted on both an LRG and an RTK, but, clearly, this remains a hypothesis.

The binding of insulin and relaxin to their respective receptors has gotten resolved in much detail in the last couple of years. The effective binding and stimulation of insulin RTK by the small irp from the snail *Conus* to the RTK shows that a small irp can be an effective ligand for this receptor (Menting et al., 2015). On the other hand the complex interaction of relaxin to its LGR makes it more difficult to imagine a smaller peptide as an effective ligand (Hoare et al., 2019). Furthermore, considering the well conserved F-domain of the dilp7 receptor orthologs it is likely that it is necessary for interaction with its LGR receptor. The loss of this structure in multinsulin suggests that it is unlikely to be a dilp7 receptor agonist. On the other hand, the poor sequence conservation in the various *Drosophila* irps that activate a single RTK is reminiscent of the large structural variability of the multinsulins. This seems to suggest that the multinsulins are RTK ligands rather than that they activate the LGR.

The emergence of the GSS is neither accompanied with the evolution of a novel receptor for these irps. This can also be explained by assuming that IGF acts on both the RTK and an LGR and that the GSS have lost their affinity for the LGR. This raises the question whether an IGF LGR might exist.

If there were an IGF LGR, one would expect it to be related to the gonadulin and dilp7 receptors. GRL101 appears a plausible candidate as its transmembrane regions are closely related to the receptors for gonadulin and dilp7. The ectodomain of GRL101 consists of two parts, a series of LRRs and a second series of LDLa's. In the related GPCRs the LRRs are expected to bind with the insulin core of gonadulin and dilp7 orthologs, just like the human relaxin receptors (Hoare et al., 2019). When the LRR part of the *Anneissia* GRL101, the most basal echinoderm, was used as query for similar human proteins in a BLAST search, the glycoprotein hormone and relaxin receptors were identified as the most similar proteins. This shows that the resemblance of GRL101 to the other LGRs is not limited to the transmembrane regions and reinforces the hypothesis that the ligand of GRL101 has an insulin-like structure. GRL101 has a large number of LDLa's, the ligands of which are typically positively charged surfaces, which in the case of proteins consist of Lys and Arg residues (Daly et al., 1995; Prévost & Raussens, 2004; Fisher, Beglova & Blacklow, 2006; Yasui, Nogi & Takagi, 2010; Dagil et al., 2013).

490 Thus the ligand of GRL101 may consist of two parts, an insulin-like structure and a piece with several
 491 positive charges that interact with the LDLa's. The C-terminal tails of the IGFs, whether from
 492 arthropods, echinoderms or hemichordates, are all rich in charged amino acid residues. The C-terminal
 493 tail of IGF with its numerous positively charged amino acid residue might interact with the LDLa's of
 494 GRL101. I therefore posit that in those species that have a GRL101 it functions as the second receptor
 495 for IGF. The absence of such a tail in GSS would make it likely that it acts on the RTK rather than an
 496 IGF GPCR.

497 The suggestion that GSS activates the RTK goes against the hypothesis that these peptides act
 498 through GPCRs. Indeed it has recently been proposed that it is the ortholog of the dilp7 receptor that
 499 would be activated by the gonad stimulator in *P. miniata* (Mita et al., 2020). Given the clear orthology
 500 of both the dilp7 echinoderm orthologs with the *Drosophila* peptide and the similar orthology between
 501 the dilp7 receptor and the echinoderm receptor, the conclusion that the two constitute a functional
 502 ligand receptor combination seems inescapable. It was impossible to find a GSS in either the genome
 503 assembly or the individual reads of all the genomic SRAs of *Anneissia japonica*, yet it does have a
 504 dilp7 receptor ortholog, thus if the dilp7 receptor were to function as a GSS receptor, it most likely
 505 would not be an exclusive receptor. *A priori* this does not exclude the possibility that GSS could
 506 function as a ligand for the same receptor. As mentioned above, since the dilp7 orthologs have well
 507 conserved F domains, one has to assume that it is important for binding to its receptor. Since this
 508 domain is absent from RTK ligands, it is difficult to understand how GSS that similarly lacks this
 509 domain would be able to bind the dilp7 receptor. It would thus seem unlikely that peptides as different
 510 as GSS and dilp7 would be effective ligands of the same LGR. Furthermore, the GSS genes have been
 511 duplicated and their structures have diverged considerably. Those duplicate gonad stimulators are
 512 present in many species and have not been selected against. Hence they must be physiologically
 513 relevant and able to interact with a receptor. Sharing a common evolutionary origin the two gonad
 514 stimulators should be expected to act either on the same or paralogous receptors, but the number of
 515 putative echinoderm receptors for irps is limited, so it must be the same one. The same arguments that
 516 were used to argue that the multinsulins are likely RTK agonists but not LGR ligands, are therefore
 517 equally valid here and suggest that GSS is an RTK ligand.

518 Furthermore, the experimental evidence that GSS stimulates the ortholog of the dilp7 receptor is not
 519 convincing. The reported response to the dilp7 receptor when expressed in Sf9 cells is very weak and
 520 does not represent a typical response seen in this type of assay. Although the authors have shown high

521 affinity binding of GSS to the follicle cells, such high affinity binding should also have been present in
522 the Sf9 cells expressing the putative GSS receptor, but this was not reported. The follicle cell SRAs
523 from which the putative GSS receptor was identified contains large amounts of RNAseq reads for the
524 gonadulin receptor, a receptor that is more closely related to the vertebrate relaxin receptors than the
525 dilp7 receptor, but surprisingly the authors do not mention this receptor, which they must have found
526 (Mita et al., 2020).

527 I suggest that initially there was an IGF-like hormone that activated both a GPCR and an RTK,
528 after two gene duplications some of the descendant ligands either lost their C-terminal tails or one
529 acquired a larger one and this allowed all three ligands to activate, at least initially, the RTK while each
530 acquired its own LGR. Later, some of the ligands may have lost their affinity for one receptor. Since
531 the primary amino acid sequence of gonadulin is very different from that of the other irps, it likely lost
532 its capacity to activate the RTK (Fig. 12). Holometabolous insect species have lost GRL101 and hence
533 in those species IGF can only act on the RTK. Under this hypothesis the arginine-rich C-terminal tail
534 would be useless in such insect species and in higher flies, such as *Drosophila*, it was indeed lost
535 (Veenstra, 2020b). In vertebrates, there is no GRL101 and so IGF can only activate the two RTKs,
536 while the relaxin related peptides are not known to interact with RTK. The presence of a similar
537 arginine-rich E domain of the vertebrate IGF precursors might thus be an evolutionary relict.

538 This scheme raises the question as to how the functions of these two receptors activated by IGF
539 might differ. IGF and the drosophila irps stimulate growth, the echinoderm GSS stimulates oocyte
540 maturation and ovulation (Mita et al., 2009), relaxin and INSL3 affect various developmental and
541 reproductive processes (Ivell et al., 2020; Esteban-Lopez & AgoulNIK, 2020), gonadulin is expressed by
542 the gonads as well as the imaginal in flies (Garelli et al., 2012; Liao & Nässel, 2020; Veenstra, 2020b;
543 Veenstra et al., 2021) and dilp7 is expressed in a sex specific manner (Miguel-Aliaga, Thor & Gould,
544 2008; Yang et al., 2008; Castellanos, Tang & Allan, 2013). These hormones stimulate growth,
545 development and reproduction, processes that are intimately linked; without growth and development
546 reproduction is impossible and growth without reproduction is useless in sexually reproducing species.
547 On the other hand, resources used for growth and development can not be used for reproduction or *vice*
548 *versa*.

549 Growth is rarely a linear process independent of development; animals are not only getting bigger,
550 but they also mature into adults. Metamorphosis is markedly different between hemi- and holo-
551 metabolous insect species. Every time a cockroach nymph molts, it becomes a little more adult,

552 however during the first molts of a caterpillar the insects mainly become bigger, it is only when it molts
 553 into a pupa that it significantly changes its morphology. Cockroaches have GRL101, caterpillars don't.
 554 This suggests that the RTK might be more directed toward linear growth, or allowing growth by
 555 increasing uptake of resources, such as glucose and amino acids, while the LGRs might be more
 556 important for insuring that the animal develops into an adult and becomes sexually competent. Both
 557 holometabolous insects and vertebrates have lost GRL101 and use steroid hormones to induce sexual
 558 maturation. Interestingly, in vertebrates the production of steroid hormones is controlled by
 559 glycoprotein hormones, the second group of ligands for LGRs.

560 It is plausible that IGF in an early bilaterian was produced by the tissue that stored energy and
 561 perhaps even protein as insects do in the form of storage proteins (Haunerland, 1996). Production and
 562 release of IGF might have happened when the animal had sufficient resources to allow for growth
 563 and/or reproduction. In arthropods growth has become a discontinuous process in which a new cuticle
 564 needs to be made before molting can take place. In those species IGF produced by the fat body may
 565 well be the essential growth hormone. However, if the animal is suddenly starved, IGF would no longer
 566 be released. If formation of a new cuticle is too advanced to be interrupted, this become problematic. It
 567 may have obliged the brain to take at least partial control of growth away from the fat body by
 568 releasing one or more of the neuroendocrine irps to force growth and molting to proceed. It is possible
 569 that this achieved by simultaneously reducing growth of organs that are needed for (sexual) maturation
 570 but not essential for immediate survival, like the gonads. This could be how the neuroendocrine insect
 571 irps initially evolved. In echinoderms IGF probably stimulates growth of the follicles and oocytes, but
 572 the final growth spurt, the one that permits resumption of meiosis in the oocytes and subsequent
 573 ovulation, is delayed until optimal conditions to do so prevail. When the time and place are right the
 574 nervous system releases GSS likely in large amounts to finish the maturation process and induce
 575 ovulation. In vertebrates, growth and the release of IGF has also been brought under control of the
 576 brain but more forcefully by bringing IGF secretion by the liver under control of growth hormone.
 577 Whereas in an early ancestor high plasma concentrations of insulin might have led to secretion of IGF,
 578 this is no longer the case. Here insulin may have evolved to insure that plasma concentrations of
 579 glucose are kept sufficiently low by insuring its absorption by tissues in order to avoid its loss by
 580 excretion. In the three cases these peptides have very different functions, ovulation in echinoderms,
 581 sparing glucose in vertebrates and rescuing interrupted growth in insects. It is plausible then that these
 582 hormones each evolved from a non-local IGF gene duplication and that they are thus not proper

orthologs but evolved by convergent evolution. This hypothesis would explain, why there is no insulin gene located near the IGF, octinsulin/gonadulin and dilp7 triplet in cockroaches, echinoderms and hemichordates, even though insulin – and other peptides such as the insect neuroendocrine insulin-like peptides and GSS - almost certainly evolved from IGF much later.

Conclusions

The gene triplication previously reported from arthropods must have occurred in a common bilaterian ancestor of the deuterostomes and protostomes. The hypothesis that IGF in an ancestral bilaterian used both a GPCR and an RTK may explain the combination of echinoderm irps and putative insulin receptors. This hypothesis implies that insulin is not a hormone that evolved before the split between protostomes and deuterostomes, but that insulin-like peptides evolved independently in different metazoan clades as miniature copies of IGF capable to activate the RTK but unable to stimulate the LGR.

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Figure 1

Domains of insulin/IGF-related peptides.

Human insulin and IGF and *Drosophila* dilp7 are aligned and the different domains that are recognized in the precursors of these peptides are indicated. In insulin the domain borders are the convertase cleavage sites that are highlighted in red. The A- and B-domains of insulin correspond to the A- and B-chains of insulina and the C-domain to the connecting peptide. Although IGF consists of a single protein chain due to its strong sequences similarity to insulin the A- and B-domains correspond to homologous regions of those domains in insulin, while the C-domain is the sequence between the A- and B-domains. In insulin there is only a single amino acid residues after the last cysteine residue, but in IGF there is a longer sequence, that has been called the D-domain. The IGF precursor is cleaved by furin in the Golgi apparatus and the sequence that is removed has been called the E-domain. Dilp7 is only known from nucleotide sequences, it is unknown how the precursor is exactly processed. Nevertheless, the presence of putative convertase cleavage sites, highlighted in red, suggests the presence of A-, B- and C-domains quite similar to those in insulin. However, unlike insulin or IGF, the putative B-chain of dilp7 has a long N-terminal extension that I propose to call the F-domain. The latter is well conserved in dilp7 orthologs from other bilaterians (Fig. 4).

Figure 2

Sequences of selected ambulacrarian IGF.

Partial IGF sequences from selected ambulacrarians are illustrated to show their sequence similarity. The A-, B- and C-domains of the insulin core are aligned, but not the putative D- and E- domains, as their amino acid sequence is only conserved in closely related species (Fig. S1). Not aligning D- and E- domains allows the visualization the context of putative convertase cleavage sites. None of the arginine or lysine residues conform to a typical arthropod or vertebrate convertase cleavage site. Although the sequence of the latter part of the IGF precursors is not well conserved, all of them are rich in positively charged amino acid residues. Conserved cysteine residues are indicated in red, conserved amino acid residues are highlighted in black and conserved substitutions in grey. The arginine and lysine residues in the D- and E- domains are highlighted in blue.

Anneissia QPNHVVRYCGSELTSELEERRCALKGGFNSPKKQESP-----
Strongylocentrotus-1 ---SFPLLCGQELVKAVAAVNCNDR-GYYGQPS-----
Strongylocentrotus-2 ---SFRLCGRELADALAVVCKGR-GYYIDDSEI-----
Apostichopus-1 ---SGQRYCGEALLEAMAYVCADR-GYFGVTSIGIRGRS-----
Apostichopus-2 ---AQRYCGTNLADALRIVCADR-GYYTQ---KGAF-----
Acanthaster-1 ---AVIQVCGNDLLDALKSVCGDR-GFYSPPPGYS-----
Acanthaster-2 ---WQRI CGEQLVETVSVICNTR-GFYSHR-E-----
Ophiothrix-1 ---WQRLCGTFLVDVVSQVCGER-GTYADDSTHDLRKRLDNFVDRGKMYSGISK
Ophiothrix-2 ---SYLCGSQIVQMMAVVCEGR-GYYYTEGSTGQICN-----
Saccoglossus ---WDKLCGRRLVDVLAALICNDR-GYNSGSPKK-----
Ptychodera ---WDRLCGRSLADMLALVCHGR-GYYTDVSRQQ-----
Schizocardium ---WDKLCGRRLADMLSLVCHGR-GYYTDVSKHR-IR-----

Anneissia -----YLWNQEKRIEATS-----TERAKEINIVYECHSACTEAFIDS
Strongylocentrotus-1 ----KRSAGIELETRAKTEFKSGISRGETRRSKRGARTGLIVTECCLNRCVSHLES
Strongylocentrotus-2 ----AQKDSPIVPHHVASSFLGSSS-ASAHSRQRRRVRTGOIVNECCDKECSNNIMES
Apostichopus-1 ----VSRSPFLTEERANSFELTNE-----RTRKTRRTGRIVTECCDNP CSQRNLES
Apostichopus-2 ----PEVIPRQRASSFELTDS---ENHHPRLRLRGTVTECCDREKACDREVLET
Acanthaster-1 ----KREAELEODERTAKSEFLGTHIG-----SRQRRRTGRIVTECCDREKACDREVLET
Acanthaster-2 ----KREAELEODERTAKSEFLGTHIG-----SRQRRRTGRIVTECCDREKACDREVLET
Ophiothrix-1 SDTNNAESNFERGESEAKSEFLGL-----SRQRRRTGRIVTECCDREKACDREVLET
Ophiothrix-2 IVYSFLTDPELTERANSFELTNE-----RTRKTRRTGRIVTECCDREKACDREVLET
Saccoglossus ---KVKRESPEERSGMEANDEFGNIS-SKEKRQRRRSGSGKIVDECHQA CDYTTLES
Ptychodera ---KSRRARETVEATQEEANGFEGVG---S---GRTRRRRSGSLIVVECCDKICDYSTIES
Schizocardium ----LPRETIEQTQEDAHKEFGASV-FG---ERTQRRRSGSLIVAECCDKICDYATIES

Anneissia FCLSSNKDEDTTVESDTVETTTITGKKRKPTRKPKKNPKLSRKKPKGSSEINSEQSASN
Strongylocentrotus-1 YCNPLPPDAVHDAEVHIRLEKSAEEDADEGRPDGQPSQLDTATGTVPETEMSETRGRV
Strongylocentrotus-2 YCNRRTPPEVPESAISENPFSEITEDSTLRDGDGSTEIRDTNPNATNLEVPSPDANTP
Apostichopus-1 YCNVATTQTTEIPTELTTEGTTTEPAASPRRNSRNIEADGTAAAGGGSGQNGRGRKG
Apostichopus-2 YCNPHVTPPTLALASLVTSMTKSPPTPSSEPSSSSSSSSSSSSRNEDKFFMTDNALGEDY
Acanthaster-1 YCNPPSTSQSQTAAAPPRIITTPDERANEIVVDETGTGNTNSQMLRGGNAMGAGSR
Acanthaster-2 YCNPWVPPVEDRDDPMLAPVAPGRVRODKSADADLLLRPDIAEISEDKSSLLRQAAEKD
Ophiothrix-1 YCNPWPTTTTTTATTTQSPEPLPNEQEGGYLTDEIQMKHITRQGEDNSVDLLSEGSDIR
Ophiothrix-2 YCAPLQEGQVKFTSRNLDVFNVEANNVVEEPSVRVPQQVEENKIVEETVPYVSOAIVGE
Saccoglossus YCAPLPEGVVDADSLKRFLLSQSFGNDFKDTANEDKLEIVTVVRPSHDEMMDGTIRIED
Ptychodera YCAPWPKDIDPAKKIEGFKEGTWEEDYHRYHPESVEQPANPEEPTPTTTDLDK
Schizocardium YCAPWPKMDPALRFAGFKYGSWEDEDYRYKYPHEEFTQPDLTWTIASTDSANHDEHF

Anneissia TELPSQTEPTKDKNGRGDKNKKDKCNKKSRLKNNKPCRRKS RKDSRRKNRKNKKNKPK
Strongylocentrotus-1 RIDAVEKVLSERLIPTSTTGSSPSRKKPRKDKSERNNSSREAKQARREERRNRER
Strongylocentrotus-2 DATATSDVEQPRSDNTTAVEKPRKKDNGKGNSSLESSTKKNRTSKGMSKEDRRRIAS
Apostichopus-1 KGRHGKGNRRREQDQTVDVTS AETEGNTEPPRPNNQENEDNRGTPDQSEERPRDRCRG
Apostichopus-2 EGPTNEGPLTSGEPTPTENRISNGPRGPSSNASSLELPTRTSTATTNSSRIVTEGAHL
Acanthaster-1 ANGTKAPPTTEVVDGRSDDDDDAAGEINTSERVGSLEPDEETGRDVATNRPHKTHSKE
Acanthaster-2 EPIDDLDTLENEYADGGNVMLQAREGVKEEGAELGKEMEEGGEKMPFPEVVP TKKRRR
Ophiothrix-1 DETPMTPTGRATGGRSPQLGHDQSDVDVSGFHRFGDIGAEVEESDSTIDNPSSDR---
Ophiothrix-2 TTSDGFDWSDVSETHNINLGGGDMYINDQPRDDPSENDEEHIPKESLIKQINVTES
Saccoglossus NEHVTPPTKPDVITETSSILDDINVNQIISNTSVEVKS KAGNTKPKREKKDRDNS
Ptychodera SLESERDNDVIEESRSKDKSAESESVTENDDLKSEETNDNQDEYSREYLGDRDKGP
Schizocardium TTATPSEETTSSENGHEIKTVNKEDDTQVTGTHELEKDHKFAEYWMNEDKMSRKHKKA

Anneissia TRRPKRWDSETTTLSDFLFSQRFLLQRAYDSIDVDEITTEFGVVPPEEDISSSLSEEL
Strongylocentrotus-1 SSGGRSRNGRRKDKDNDRASRAKRHGLNLWRNMFSDKFFSDIPGLENQPNLHPVNGRA
Strongylocentrotus-2 DERRASREKKELSRERRKRLKLQQRDKKKKKRLESABERNRGTDHMGLESDSTLLAR
Apostichopus-1 SKKKGKCRNQDRVEEPTQDRIEEPTTSREVSSTDGLSRERGS GGGRNRGNGRCK
Apostichopus-2 AESSDQSSLEDTDESEAPT KASHERTRKKQKKQRTTRPPKKPKKLSRERKERKKKKKE
Acanthaster-1 RSKNRTSKSERRRRTNRRSSSERRMLSSERKREDATRLRRKEQRLSRKQPHSNKR
Acanthaster-2 VEGRRSRENSRDRNGKSEKSKSRKSGSREGGRSFRRRGKNSRRKKGRDGRERSKRWEA
Ophiothrix-1 QTDEILDEDTDSENSESKLSIGATKGNSSGRTPKPHRPTSRHSRRKNSREKKKNKTKTR
Ophiothrix-2 SSKKRSHPKPKKSRRKQRLQIRKKARGTRKTCLRHVKVKSSTPIQOIETTTTMMKPF
Saccoglossus ERKHKKAPTKKLFKEKKLSEDNKKKKSRKSKNSTKVKPTYVSSMTTSDEETLTTRPQG
Ptychodera KRLMKERRARKSRTKKGNNTKSKSILGKLVENMETESPTNWQRDGTDERWWTVEDPHF
Schizocardium

Figure 3

Sequences of selected echinoderm GSS.

Sequences of selected echinoderm GSS. Sequence alignment of a few echinoderm GSS showing relatively conserved A- and B- domains of the insulin core sequence and likely KR convertase cleavage sites that can be expected to be cleaved by neuroendocrine convertase as well as a few potential furin sites. Conserved cysteine residues are indicated in red, conserved amino acid residues are highlighted in black and conserved substitutions in grey. The arginine and lysine residues that form likely - or possibly in the case of *Apostichopus* GSS-2 - part of a convertase site are highlighted in blue. For the alignment of a larger number of echinoderm GSS sequences see Fig. S3.

Strongylocentrotus	--QQGPRNRY	CGLEFARAVE	TQCSMANKRSDPGAVAESASAARYLA
Apostichopus-1	-----IRL	CGPDLRAVYQI	CSHG-KRGYIPPTFNS--E-----
Apostichopus-2	----WSHQRL	CGPDLVHALSLV	CGERG---YFGGSRLVER---D-V
Acanthaster-1	-----EKF	CDNDFHLAVYQT	CSTH-KRGDGEFVLSSL--K-----
Acanthaster-2	----DSSSKH	CGSAFPQFVWTAC	SMA-KRS-NRSPRSL--D-----
Ophiothrix-1	--DSARYQPL	CGREFTRAVMEI	CATQVKRTEPLFQRFYNAN-----
Ophiothrix-2	----QDSYKS	CGREFTRRVMEV	CATHVKRTEHF-----
Strongylocentrotus	DTGYEQAE	DMPLEWYDVARQGAERLRP	-----SL---TDIIF
Apostichopus-1	DDQLNQEF	GTDL-----EEYLAETIKEYLKPN	SLYDDVERELYPSL
Apostichopus-2	DDGLDEE	ITTLV-----VGAERT-----	SILECLK--AWSPF
Acanthaster-1	D--VLTGS	-----RLRG-NI	KRSFGSTLE--DEAFF
Acanthaster-2	D--LLET	F-----KSAR-----	HLDIS--Y--RT
Ophiothrix-1	--LVKRS	IDPAFWNNLLEANP	-----DLMD
Ophiothrix-2	---MVKRS	IDDEFWNDLMESGL	-----GL--
Strongylocentrotus	SRFRRSI	HNRGQLPMGQL	CCVYGCTLVELASVCT-----
Apostichopus-1	RG---FRRV	TRTGGIARR	CCSTGCSSSDIAKLC-----
Apostichopus-2	-----RRR	TRGIVEE	CCFRRCTWENLESYCSKTTAYKKADNMI
Acanthaster-1	SR---LVKR	SEYDGIASY	CCIIGCTPSELAVVC-----
Acanthaster-2	IR---LSKR	QDYDGMADY	CCIIGCTSTNELIASGIC-----
Ophiothrix-1	----KRQSS	AGVGMATH	CCQSGCSQQEISMVC-----
Ophiothrix-2	----DKR	SETGMAEH	CCQNGCTDQEISMVC-----

Figure 4

Sequences of selected dilp7 orthologs.

Sequences of selected dilp7 orthologs. Sequences of *Drosophila* dilp7 and several ambulacrarian orthologs illustrating well conserved sequences, not only in typical insulin core of the peptides, but also in the F-domain (underlined in blue). Note that the sequence conservation of these peptides is stronger than in the IGFs or GSSs (Figs. 1,2). Conserved cysteine residues are indicated in red, conserved amino acid residues are highlighted in black and conserved substitutions in grey. Likely convertase cleavage sites have been highlighted in blue. Sequences are from Spreadsheet S1 and Veenstra 2020b, a comparison of a larger number of sequences is presented in Figs. S5 and S6.

<i>Drosophila</i>	-----LQHTEEGLEMLFRERSQSDWENVWHQETHSR--	C-RDKLVRQLYWAC
<i>Anneissia</i>	-----LRDYSDRSHNDWARVWTVESMRQ--	C-HENLREMVHVS
<i>Strongylocentrotus</i>	-EKFCNCMVLPELTMEDYEDRTPEEWRESWNMDTLRT--	CVGPQLQRVGELAC
<i>Apostichopus</i>	-----TLQELNSRTQPSWEQLWIVENVPTVD	CTV-DAVQLHIIAC
<i>Acanthaster</i>	-----APHLPVEQWNSRSKADWVKLWNTERTHVNT-	C-NEALLPVWDVAC
<i>Ophiothrix</i>	-----KITDYSSRTKADWQRLWLTESHQK--	C-NEDLLPLWKIAC
<i>Saccoglossus-1</i>	LTVENEETITFSELNTMYGTRTLTDWQGWKTSETIHA--	C-GSNLYRISEYV
<i>Ptychodera</i>	----AKDYDTVEDLILKYGTRTEDDWRNVWHTESHNK--	C-RNSLYSYVIFAC
<i>Schizocardium</i>	----ENAYETVESLKAKYSGRTFNDWRAAWNTEETHNK--	C-RNSLYSYVIFAC

<i>Drosophila</i>	EKDIYRLTRRNK	KRTGNDEAWIKKTTTEPDGSTWLHVNYANMFL	RSRR	----
<i>Anneissia</i>	RNDPRK-ISS	KRS-----	IFIPRNEATGFLSRFL	----
<i>Strongylocentrotus</i>	INDPRKTIVV	KRSN-----	SDRDLELPAKLAKAFLHYRHRKD	--
<i>Apostichopus</i>	SNDVYKDHEGS	RRRRF-----	SDKRRRS--	IFLNFSEANNELA-KTKRTHS
<i>Acanthaster</i>	QNDIRK-IT-	KRM-----	GREFLNEWTAKNFLAG	-----
<i>Ophiothrix</i>	TYDIRK-IN-	KRA-----	PEFVEDSEAKAFLIGP	-----
<i>Saccoglossus-1</i>	YVDIHKSPDR	KRTD-----	DAFVDSAVAHDFLRGLMEKRTL	
<i>Ptychodera</i>	MVDIYATGDK	KRAE-----	PPEVEHSIANSELAGHRAEKRM	
<i>Schizocardium</i>	MKDIHASGDR	KRGQ-----	FPELDHEVADKFLSGSL-GKRM	

<i>Drosophila</i>	SDGNTPSISNE	CCT-KAGCTWEEYA	EY-CP	SNKRRNHY-----
<i>Anneissia</i>	RTRRPSELHED	CCLD	SRGCTWEEVA	EIAACINNRRRMHRPGSPVGR-----
<i>Strongylocentrotus</i>	SRRRRVGKDEE	CCAE	AQGCRWEE	LGEY-CTLHTRAYHQSGEQP-----
<i>Apostichopus</i>	RVRRTTTFSTE	CCD--KL	CIWEEV	GEY-CWH-SRVYH-----
<i>Acanthaster</i>	-KRRKRG	LNEECC	HEDLGCWEEV	AEY-CVMHGREGKHEDGSPVRGKPGRRR
<i>Ophiothrix</i>	-RRHKR	GLNEECC	HESKGCWEEI	GEY-CRMHSRASHVDGRIDSR-----
<i>Saccoglossus-1</i>	RRYRRTSATSE	CCADD	GGCWEE	LAEY-CTHQREVRTMDE-----
<i>Ptychodera</i>	TRFRRASPT	EECC	SNAHGCNWE	LAEY-CSHQRDDTRRR-----
<i>Schizocardium</i>	ARFRANP	SEECC	SNRNGCTWEE	LAEY-CNHQRDDIDGR-----

Figure 5

Sequences of selected ambulacrarian octinsulins.

Sequences of selected ambulacrarian octinsulins. Sequence alignment of a number of octinsulin sequences show that these sequences all have typical neuroendocrine convertase KR cleavage sites, suggesting these precursors are processed by enteroendocrine and/or neuroendocrine cells. Conserved cysteine residues are indicated in red, conserved amino acid residues are highlighted in black and conserved substitutions in grey. Likely convertase cleavage sites have been highlighted in blue. Sequences are from Spreadsheet S1, a comparison of a larger number of sequences is presented in Figs. S7 and S8.

```

Anneissia      -----ARDWYC--GNAADTLKEFCQS CYASKRAHN--ALSLP
Strongylocentrotus -----QSWHC--GRAAQTIMGM CNS CYASHDKRS-----
Apostichopus  -----SWYC--GSAPETVRAICDGCYAGGIHTR--AFKRS
Acanthaster   -----DSWYC--SDVYSTVQSLCDS CYAGFDKRT-----
Ophiothrix    -----NQWFC--SPVFTMLQSM CGS CYAGVDKRS-----
Saccoglossus-1 -----MSRNWHC--GRPVE TMHEVCQG CYAGHVRPR-----
Ptychodera-1  -----LRREWHC--GRTVETMQGICRG CYAQPSERS-----
Schizocardium-1 -----FTKHWHC--GRIVDTMRAICDGCYASPTARD-----
Saccoglossus-2 RPSGSVD-----DVLTC--KRKLLLV DQICAG CYAPPDIINN VNF DLT
Ptychodera-2  RPRNQGD-----D--VFC--SRTYSMVESVCDGCYATTQDSSPKSESAM
Schizocardium-2 RPNRPSE-----D--VRC--RKT IHMVLKLCNG CLAPIESEVENN--TI
Saccoglossus-3 ---LPVDDTTTG VQVDVRDSRLWLC--GRLVEDLRALCRG CYAG--P-----
Ptychodera-3  -DLLPGNGDY---SNSAKGRRDLD C--THLVESMSLICRG CYATDQGV-----
Schizocardium-3 -----QTSADRP RKWHCRKSVPEILSGVCRG CYAEPLQPP-----

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Anneissia      SIKA-----KKDGMFLTKEGASGYLEAKRTRL-----FSS-LHLNHRQHETT
Strongylocentrotus ISKPSY---TPAKPFLHKRNAVHFLRTTKREIESRPSMGDTAIEVAVERRSTGNR
Apostichopus  SSDIIS---LYKDPFLKKSNA LNFLLPRSHTP-----S-----SLIKRGIRRS
Acanthaster   NSITRP---I-E E P FVERKNAVDFFKRGTAR-----G-----TRR
Ophiothrix    DNSDTLSQKQSLDAFIQKEVAYSFIKRTSVG-----DT-----FLRNARNTHH
Saccoglossus-1 NTRS---VDGVQAFISRRDANMFTKGMSPD-----V-----KRAIDG
Ptychodera-1  T--N---EAERQAFIGKEEASSETKSV-----TIVKR
Schizocardium-1 V--G---SVKGLPELKKHEASTFTRTRS-----SVQKR
Saccoglossus-2 GK-----GPGY-----EEEIVKN
Ptychodera-2  NE-----SVQI-----QDEDLRE
Schizocardium-2 SN-----ETPP-----DQDVVTA
Saccoglossus-3 -----DISKREASKFMQFNA-----HTIRQSR
Ptychodera-3  -----TVNKRLLASSFIPTTTTP-----R-----NKIQR
Schizocardium-3 G-----KRDILNKQEASLFLRSP-----NGGDE

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Anneissia      NFVTECCYNPCSS FEMIKYCCPTRQIE LHN RNPNS SEDK-----
Strongylocentrotus GFIHECCNKFCDPGEMVLYCC EKQIEWAQFHNLLKA-----
Apostichopus  GFIHECC EKNCEIREMVFYCCAEKQREYASEFFEIFRNRI RHT-----
Acanthaster   GIVDECCHRQCAVSEMMLYCC EQKQREY YTFVGWLKRR-----
Ophiothrix    GLIDECC TQCDTGEMILYCCQERQREWHIMMGLYNK-----
Saccoglossus-1 GLIEECCYSQCSLTHMITYCCAEVQNEFQVEINILGNTDESSENDGDDGESSSV
Ptychodera-1  GLLEDCCYRRCNLQKMMTYCCAERQRELNNEFFSLN QKDN GST-----
Schizocardium-1 GIIEHCCNHHCSFTELLIYCCERSEEFYSEFGLLRMDDED TDASLEKGN GDVEA
Saccoglossus-2 QIKEACCKEYCPLPKIIEFC DERQQEFHQFMSSFASTEE-----
Ptychodera-2  KIRDKCCNRRCTIHKMMQFCCEARRNEFHKFLALMGNTDN-----
Schizocardium-2 KVREVCCDNYCSLDKIIEFCCEDLQQEFQFMSFVSNSK-----
Saccoglossus-3 GIIEDCCYHTCPTERKIQYCCFEVQAQYRLFMESAI-----
Ptychodera-3  GIIDECCRNRC SVERKIQYCCDEIQKEFAFFFSLFGS-----
Schizocardium-3 LIINECCLRCTVIEKIH YCCREKQIELYII IQSAPWLVDNQR-----

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```

Anneissia      -----
Strongylocentrotus -----
Apostichopus  -----
Acanthaster   -----
Ophiothrix    -----
Saccoglossus-1 HED-----
Ptychodera-1  -----
Schizocardium-1 EDNTDL DQGDDDDNTQNNEGGSIDVVVERDVGDVLLKTDKSKP
Saccoglossus-2 -----
Ptychodera-2  -----
Schizocardium-2 -----
Saccoglossus-3 -----
Ptychodera-3  -----
Schizocardium-3 -----

```

Figure 6

Sequence comparison of selected ambulacrarian multinsulins and dilp7 orthologs.

Sequence comparison of selected ambulacrarian multinsulins and dilp7 orthologs. Three different sets of sequences are compared. The top five sequences are dilp7 orthologs, the next five are multinsulins having three disulfide bridges and the last five multinsulins having four disulfide bridges. Note that although the multinsulins and the dilp7 orthologs share some sequences similarity this does not include the F-domain. Like the octinsulins these sequences all have typical neuroendocrine convertase KR cleavage sites, suggesting they are processed by enteroendocrine and/or neuroendocrine cells. Conserved cysteine residues are indicated in red, conserved amino acid residues are highlighted in black and conserved substitutions in grey. Likely convertase cleavage sites have been highlighted in blue. Sequences are from Spreadsheet S1, a comparison of a larger number of sequences is presented in Figs. S9 and S10.


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Anneissia      -----LRDYSDRSHNDWARVWTV
Antedon        -----LQDYSDRSHHDWARVWTV
Strongylocentrotus -----EKFCNCMVLPELTMEDYEDRTPEEWRESWMD
Apostichopus   -----TLQELNSRTQPSWEQLWIVE
Acanthaster    -----APHLPVEQWNSRSKADWVKLWNT
Ophiothrix     -----KITDYSRTKADWQRLWLTE

Amphiura-1     -----D-----WVG-
Ophioderma-1   -----DEIGAMKSQNEEPDEHDS-----HIGTTQQPVINQSRGTMEKR-----WRG-
Pisaster-2     -----KLNMKPDEVLSVE-SEHCSAVKEFRQVLADNPGVV-----KRS-
Asterias-1     -----ELRS-----QDGLMREVRQALARNGRHF-----RAA-
Patiria-1      -----APPANDGIEVLTGGKIE-----EDDFEGDDFDVKNKNQYYS-----RSG-
Acanthaster-1  -----HPDADAKH-GGNL-----A-

Ophioderma-5   NSLTRDADDAPVAMDDKPTSPRPIDIIKRSRHLRCAQFKDMALE-DHFRR-LVGKER-
Asterias-3     -----ELN-----VASCQQLADQVKS-EGKKR-----WDG-
Pisaster-1     -----TELD-----EASCQQLADQVKS-EGKKR-----WTG-
Acanthaster-3  -----DKTPS-----TASCEALAAQIST-EGKKR-----WDG-
Patiria-7      -----DKAPS-----AATCEALAATVKA-EGKKR-----WDG-

Anneissia      SM--RQCHENIRE--MVHVS CRNDPRKISS-----KR-SIFIPRNEATGFSLR
Antedon        SM--RQCHEDLRE--MVHIS CHNDPRKITS-----KR-SIFIPRNEATGFSLR
Strongylocentrotus TLR-TFVGPQLQR--VGELA CINDPRKTIVVKR-----SNSDRDLFLPAKLAKAFLHY
Apostichopus   NVPTVDCTVDAVQ--LHIIA CSNDVYKDHEGSRRRRFSDKRRRSIFLNFSEANNEFLAK
Acanthaster    RHV-NTCNEAILP--VWDVA QNDIRKITK-----RMGREFLNEWTAKNFLAG
Ophiothrix     SH--QKCNEDILP--LWKIA CTYDIRKIN -----KRA-PEFVEDSEAKAFLIG

Amphiura-1     -FRT-ICDPPFTPSLYIEDFCGVTV--KR-----E-----YEATDPLGFLKM
Ophioderma-1   -YVY-ICEPOLTR--LKNVICNPASVKRS-----DTAGLEFLTEHQAKRFLMQ
Pisaster-2     -PRNYWNTALSQ--RKTCALCGCT--HH-----TLRDDDFMEEEKKDATNLFLE
Asterias-1     -EDHIYCGVVLLEQ--NRESVCGV--IPG-----SKRNLFVRKEAASEFLE-
Patiria-1      -PRFRVCGTTTHS--WSSFVCHPSGLI-HH-----KRDNDEFSLSAGEANTFLMS
Acanthaster-1  -YRANYCGATTIYE--KVRETCHA-----VR-----GVSNOEFELDSKDASTELFG

Ophioderma-5   -VFAKYCSPTPQT--VMDNYCQCDVVPRSI-----DDKRAFVDKSSAKSFLNH
Asterias-3     -PSHKFCGETINE--KRYAYCTCGLVPRKR-----ELELSEFLNRGKANGFLSA
Pisaster-1     -SWHTFCGETINE--KKYAYCTCGLVPRKR-----ELDLEFLNRGKANGFLSA
Acanthaster-3  -PSHTFCGEDINE--RRNAYCNCQVVPRKR-----ELDLEFLPSGKANAFLSG
Patiria-7      -PAHTFCGETITE--KANAYCHCQVVPRKR-----ELELSEFLTPVKANSFLSG

Anneissia      FL--R-T-RRPSELHEDCCLD SRGCTWEEVAEIA CINNRRRMHRPGSPVGR-----
Antedon        FL--R-T-RRPSELHEDCCLD SRGCTWEEVAEIA CINNRRRMHRPGSPVGR-----
Strongylocentrotus RHRKDS-R-RRRVGKDEECCAEAQGRWEELGEYCTLH-TRAYHQSGEQP-----
Apostichopus   TKRTHS-RVRRTTTFSTECCD--KLCIWEEVGEYCWHSR-VYH-----
Acanthaster    S-----K-RRKRGLNEECCHEDLGCWEEVAEYCVMH-GREKHEDGSPVRGKPGRRR
Ophiothrix     P-----R-RHKRGLNEECCHESKGCVWEEIGEYCRMH-SRASHVDGRIDSR-----

Amphiura-1     RGMVKRLNPNWRELMSEECY--ESCTSEEIKELC-----
Ophioderma-1   NKR--IWGW-GGLSEECN--EGCSVEEIDEVC-----
Pisaster-2     RGIEKR-----SLSEECCH--EGCYEEVYEV-----
Asterias-1     R--GVQ-----GLAECCG--EGCTIEEISES-----
Patiria-1      EGVGKR-----GMHEECCH--EGCWWEEMLEHC-----
Acanthaster-1  RGIEKR-----GLHHECCT--EGCSIEEIIYES-----

Ophioderma-5   RAS-TR-----SLDEECN--EGCNLEEIVELSKTMCSSS-----
Asterias-3     RNVQKR-----SLSEECCH--EGCYWEEIEEV-----
Pisaster-1     RNLQKR-----SLSEECCH--EGCYWEEIEEV-----
Acanthaster-3  RRITKR-----SLSEECCH--EGCYWEEIEEV-----
Patiria-7      RNIAKR-----SLTEECCH--EGCYWEEIEEV-----

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Figure 7

Position of introns in ambulacrarian irp genes

Schematic representation of the location of the cysteine residues, indicated as purple rectangles, and introns, represented by green T's, in the coding sequences of the various types of ambulacrarian insulin-like genes. Numbers indicate the phase of each intron. All genes share the typical phase 1 intron present in insulin-like genes, whereas dilp7 and multinsulin genes also share a phase 2 intron. Signal peptides indicated as interrupted bars.

Figure 8

Synteny of ambulacrarian irp genes.

Schematic representation of the relative localization of different irp genes in several arthropod and ambulacrarian genomes. Arrow heads indicate transcription direction of the various genes, the numbers below the line indicate the number of nucleotides between the coding regions of adjacent genes in kilo base pairs. Note that the relative organization of the two insects – the cockroach *Blattella germanica* and the stick insect *Timema cristinae* – is the same as in the hemichordate *Saccoglossus kowalewskii* and remarkably similar to that of the sea urchin *Strongylocentrotus purpuratus* and the sea cucumber *Holothuria scabra*. In the spider *Pardosa pseudoannulata* and the sea cucumber *Apostichopus japonicus* some of the genes are also next to one another. However, in the sea stars *Acanthaster planci* and *Pisaster ochraceus* synteny has been lost. Arthropod data from Veenstra, 2020b.

Figure 9

Radial sequence similarity tree of ambulacrarian irps.

The five different types of irps are clearly separated from one another. Note that the GSSs are similar to IGFs and seem to be related to them, while the multinsulins are most similar to the dilp7 orthologs. Echinoderm branches are in black, hemichordate branches in red. More extensive sequence comparisons and sequence trees are the supplementary data (Figs. S1-S10). All sequences are from Spreadsheet S1.

Figure 10

Phylogenetic tree of LGRs.

Phylogenetic tree constructed from the transmembrane regions of ambulacrarian LGRs that are putative receptors for irps. A few human and insect sequences have been added for comparison. The insert at the top shows the same data to which the glycoprotein LGRs have been added and where characteristic ligands for each branch have been identified. Numbers in blue indicate the apparent probabilities as determined by Fasttree. For details of the glycoprotein LGRs see Fig. S11.

Figure 11

Ectodomains of ambulacrarian LGRs.

Ectodomains of ambulacrarian LGRs. Schematic representation of the various domains of the putative receptors for ambulacrarian insulin-related peptides. Each green circle symbolizes an LDLa repeat and each purple rectangle an LRR repeat, while the yellow oval indicates the seven transmembrane regions. The top representation corresponds to the gonadulin and dilp7 receptors (Figs. S11,S12). Note though, that the latter are somewhat variable, notably in the sea stars of two species of the *Patiria* genus and *Acanthaster planci* those receptors have two LDLa repeats (for details see Fig. S12). The bottom representation corresponds to the GRL101 receptors (Fig. S13).

Figure 12

How echinoderm irps may have evolved.

A represent an early metazoan in which an arch irp is a ligand for both an LGR and an RTK. B represents an early protostome or deuterostome that has three irps, an IGF and a dilp7 ortholog as well as gonadulin/octinsulin ortholog that evolved from local gene duplication from the arch irp. All three of these ligands each each their own LGR and at least two of them, IGF and the dilp7 ortholog, can also activate the RTK. C represents the Asterozoa where the dilp7 gene got duplicated and yielded several multinsulin genes which are represented here as one. The Asterozoa also have one or two GSS's that evolved earlier during echinoderm evolution. Both multinsulins and GSS's act exclusively through the RTK. Closed arrows indicate gene duplication events and interrupted arrows show ligand-receptor interactions. The question mark conveys uncertainty with regard to whether or not the gonadulin/octinsulin peptides are able to activate the RTK.