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Review

The biology and evolution of the Dilp8-Lgr3 pathway: A relaxin-like pathway coupling tissue growth and developmental timing control

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Review

ABSTRACT

Many insects, like cockroaches, moths, and flies, can regenerate tissues by extending the growth-competent phases of their life cycle. The molecular and cellular players mediating this coordination between tissue growth and developmental timing have been recently discovered in *Drosophila*. The insulin/relaxin-like peptide, Dilp8, was identified as a factor communicating abnormal growth status of *Drosophila* larval imaginal discs to the neuroendocrine centers that control the timing of the onset of metamorphosis. Dilp8 requires a neuronal relaxin receptor for this function, the Leucine rich repeat containing G protein coupled receptor, Lgr3. A review of current data supports a model where imaginal disc-derived Dilp8 acts on four central nervous system Lgr3-positive neurons to activate cyclic-AMP signaling in an Lgr3-dependent manner. This causes a reduction in ecdysone hormone production by the larval endocrine prothoracic gland, which leads to a delay in the onset of metamorphosis and a simultaneous slowing down in the growth rates of healthy imaginal tissues, promoting the generation of proportionate individuals. We discuss reports indicating that the Dilp8-Lgr3 pathway might have other functions at different life history stages, which remain to be elucidated, and review molecular evolution data on invertebrate genes related to the relaxin-pathway. The strong conservation of the relaxin pathway throughout animal evolution contrasts with instances of its complete loss in some clades, such as lepidopterans, which must coordinate growth and developmental timing using another mechanism. Research into these areas should generate exciting new insights into the biology of growth coordination, the evolution of the relaxin signaling pathway, and likely reveal unforeseen functions in other developmental stages.

1. Regenerative potential is limited by the exoskeleton in arthropods

From the extraordinary capacity of planarians to replace missing structures after amputation, to the more limited ability of the human liver to recover size following resection, regeneration after injury is a frequently observed phenomenon in metazoans. However, the regenerative capacity of different tissues can vary significantly between animal groups and their life history stages.

The precursors of adult external structures of arthropods are able to regenerate during the growth phases of their life cycles. Regeneration is tightly coordinated with molting, due to growth restrictions imposed by the hard exoskeleton, so that crabs and other crustaceans, which continue growing and molting after reaching adulthood, can accordingly regenerate appendages throughout their life (Hopkins, 1993). In contrast, this capacity is lost in insects after the cessation of the growth period. This temporal limitation is partially circumvented in insects by tissue-damage triggered mechanisms that have evolved to extend the growth-competent period and provide extra time for regeneration. The existence of such regeneration-promoting mechanisms, which ensure developmental stability at the cost of some plasticity in the timing of major life history stage transitions, has been known for decades in cockroaches, moths, and flies (Stock and O'Farrell, 1954; Madhavan and Schneiderman, 1969; Dewes, 1973; Kunkel, 1977; Simpson et al., 1980; Poodry and Woods, 1990; Smith-Bolton et al., 2009), but only recently the first molecular players involved have started to be revealed.

Moths and flies are holometabolous insects whose development

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includes two morphologically distinct postembryonic stages: the feeding larva and the reproductive adult or imago. Larvae have a specialized set of tissues, the imaginal discs, which give rise to most adult external structures during pupal metamorphosis. On the other hand, hemimetabolous insects, such as cockroaches, lack both the larval and pupal stages. Instead, a nymph that usually resembles the adult, but is devoid of functional reproductive organs, emerges after embryonic development and goes through successive molts into larger nymphal stages that eventually develop into an adult. In both groups, progression between stages is directed by pulses of the steroid hormone 20-hydroxyecdysone released from the prothoracic gland, in response to the brain-derived prothoracicotropic hormone (PTTH) (McBrayer et al., 2007; Ou et al., 2016). Despite the differences in life cycles of holometabolous and hemimetabolous insects, transition from juvenile to reproductive stage can be delayed in response to tissue damage to accommodate regenerative growth in both groups. Numerous studies have shown that the magnitude of the developmental delay is directly proportional to the amount of affected tissue. Surgical removal of one leg at the first instar stage of the cockroach *Blattella germanica* results in the appearance of a differentiated new leg after a delayed molt, while simultaneous amputation of a second leg postpones even more the transition to the second instar (Stock and O'Farrell, 1954). The same happens in the moths *Ephestia kuehniella* and *Galleria mellonella* (Madhavan and Schneiderman, 1969; Dewes, 1973), and in the fly *Drosophila melanogaster* in response to a variety of growth alterations. Increasing doses of radiation (Bourgin et al., 1956; Poodry and Woods, 1990; Halme et al., 2010), chemical mutagens (Garelli et al., 2012), or surgically- or genetically-induced damage (Simpson et al., 1980; Smith-Bolton et al., 2009; Colombani et al., 2012) all result in greater developmental delays. Independently of the triggering mechanism, the delay is a consequence of the inhibition of the biosynthesis of the molting hormone ecdysone, indicating that regenerating peripheral tissues somehow communicate their abnormal growth status to the endocrine centres that govern developmental transitions. While this communication requires an intact nerve connection between the injured leg and the brain in cockroaches (Kunkel, 1977), it is mediated by humoral signals, namely retinoids (Halme et al., 2010) and the recently discovered Drosophila insulin-like peptide 8 (Dilp8) in flies (Colombani et al., 2012; Garelli et al., 2012).

2. Regeneration constraints can be relaxed: Dilp8 and Lgr3

The *dilp8* gene encodes a 150-aminoacid prepropeptide with a predicted molecular weight of ~18 kDa. It has the 6-Cysteine motif typical of members of the insulin-IGF-relaxin family of peptides and a long C-peptide that is predicted to be cleaved (Garelli et al., 2012). Dilp8 is cell-autonomously upregulated in response to different imaginal disc growth perturbations, e.g., slow growth, tumoral growth, and regenerative growth in response to physically-, genetically-, and chemically- induced apoptosis (Klebes et al., 2005; Colombani et al., 2012; Garelli et al., 2012) (Fig. 1). When larvae are fed the DNA-damaging agent *ethyl methanesulfonate* (EMS), which induces apoptosis in mitotically-cycling tissues, *dilp8* transcript induction is readily detectable in imaginal discs, but not in other larval tissues. This occurs even though the other tissues have been equally damaged by EMS. As other larval tissues have either no or relatively less regenerative capacity, these results indicate that *dilp8* regulation is tightly linked to the regenerative potential of the tissue (Garelli et al., 2012).

Dilp8 expression in regenerating tissues is regulated by Hippo and Jun N-terminal Kinase (JNK) signaling pathways acting in parallel (Colombani et al., 2012; Boone et al., 2016) and JAK-STAT activated downstream of JNK (Katsuyama et al., 2015) (Fig. 1). They stimulate the expression of Dilp8, which is sufficient to prolong the duration of the larval stage by delaying PTTH production and the expression of ecdysone biosynthetic enzymes including *disembodied* and *phantom* (Colombani et al., 2012; Garelli et al., 2012). Animals lacking *dilp8* fail to properly extend the larval period upon tissue damage, and consequently show incomplete regeneration of damaged imaginal-disc-derived structures (Garelli et al., 2012; Demay et al., 2014; Kashio et al., 2016)

Even though this growth-coordinating mechanism has been unveiled through the experimental induction of massive imaginal disc damage, a similar mechanism has been proposed to take place upon small-scale spontaneous growth alterations due to random developmental errors. Consistent with this, *dilp8* mutant adult wings present increased fluctuating asymmetry relative to control populations. The model is that the inability to communicate small deviations from normal growth in *dilp8* mutants emerges as the appearance of size differences between otherwise symmetrical body parts (Garelli et al., 2012). For instance, if some cells are lost in part of the left wing imaginal disc as a result of stochastic developmental errors during the growth stage of a Drosophila larva, it would normally respond by producing and secreting Dilp8. This Dilp8 would slow down the growth of the right wing imaginal disc (and all other discs) and concomitantly delay the onset of metamorphosis, allowing the extra time for the left wing imaginal disc to catch up with the right one. As a result, both wing discs will achieve the same final size. In the absence of *dilp8,* there is no signal to generate this extra time, therefore the left wing imaginal disc, which has lost cells and does not have time to catch up, will end up smaller than the right wing imaginal disc, which has not lost any cells. The Hippo pathway has been proposed to contribute to this endogenous growth sensing mechanism by directly regulating *dilp8* expression through binding of Yki to Hippo Response Elements located upstream of the *dilp8* gene (Boone et al., 2016). These findings indicate that Dilp8 is a fundamental homeostatic factor required to adjust developmental timing to buffer deleterious effects of growth perturbations, coordinate growth of body parts, and ensure the production of a robust phenotype.

The Dilp8-dependent developmental stability pathway requires the function of Lgr3 in the larval brain (Colombani et al., 2015; Garelli et al., 2015a; Vallejo et al., 2015; Jaszczak et al., 2016). *Lgr3* is a type C1 Leucine-rich repeat containing G protein-coupled receptor (LGR, see Box 1), whose activity is coupled to an increase in cAMP levels (Van Hiel et al., 2015). *Lgr3* and *Lgr4*, the other type C1 LGR encoded in the *Drosophila* genome, are both homologous to human RXFP1 and RXFP2 (Van Hiel et al., 2012). Transcripts of both genes are weakly expressed in many larval and adult tissues, with a slight relative enrichment of *Lgr3* in the larval brain and *Lgr4* in the larval midgut (Van Hiel et al., 2015). They have long been considered orphan relaxin receptors and their roles in *Drosophila* biology remained unknown until recent strong genetic evidence placed Lgr3 downstream of Dilp8 in the pathway that coordinates organ growth with developmental timing (Colombani et al., 2015; Garelli et al., 2015a; Vallejo et al., 2015; Jaszczak et al., 2016). Similarly to *dilp8* mutants, *Lgr3* homozygous mutant larvae developed asymmetrically-sized wings and were unable to delay development in response to Dilp8 (Garelli et al., 2015a). *Lgr3* was shown to be expressed at detectable levels in the Central Nervous System (CNS) of larvae by tagging the endogenous Lgr3 protein with a superfolder GFP (sfGFP-Lgr3) (Garelli et al., 2015a), and by placing a GAL4 transcriptional reporter under the control of *Lgr3* regulatory sequences (Colombani et al., 2015). Accordingly, neuronal-specific down-regulation of *Lgr3* using RNA interference (RNAi) proved to be sufficient to prevent the Dilp8 induced delay, indicating that the message encoded by the fly relaxin is relayed to the prothoracic gland by Lgr3-positive neurons (Colombani et al., 2015; Garelli et al., 2015a; Vallejo et al., 2015; Jaszczak et al., 2016).

Analyses of the CNS of sfGFP-Lgr3 larvae revealed detectable expression of Lgr3 protein in approximately 180 cells, most strongly in four neurons in the ventral nerve cord (VNC), two in its posterior tip and two in the thoracic segment of the VNC (Midline Internal Lgr3-positive (MIL) neurons), and a pair of bilateral neurons in the *pars intercerebralis* of the brain (Garelli et al., 2015a). The notion that the latter are the neurons that require Lgr3 to mediate the Dilp8 peripheral

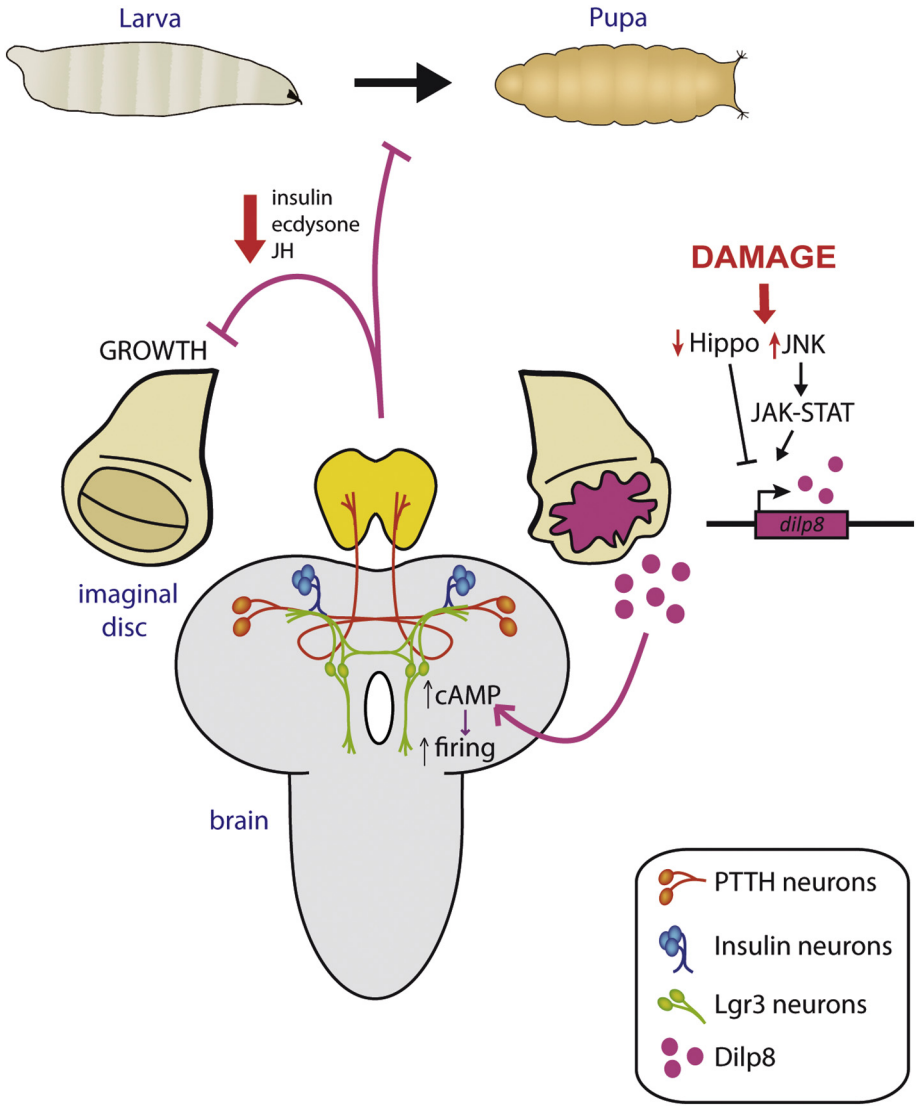


Fig. 1. A neuroendocrine circuit coordinates imaginal tissue growth with developmental transitions. A variety of growth alterations induce the production and release from imaginal discs of Dilp8, a member of the insulin/relaxin/IGF family of peptides. Dilp8 reaches the larval brain and induces an increase in cAMP levels in a subset of Lgr3-expressing neurons in the *Pars Intercerebralis* which make contact with insulin- and PTTH-producing neurons located in close vicinity. The current model proposes that the Dilp8-dependent activation of Lgr3 neurons delays PTTH production and prevents the surge of the molting hormone ecdysone, postponing the initiation of metamorphosis. The extended larval growth period allows regeneration of damaged tissues but does not result in oversize of healthy imaginal discs. A decrease in insulin, juvenile hormone and ecdysone levels could account for the reduction in the growth rate of unaffected organs. Together, these mechanisms contribute to the generation of symmetrical and proportionate individuals. Yellow: prothoracic gland.

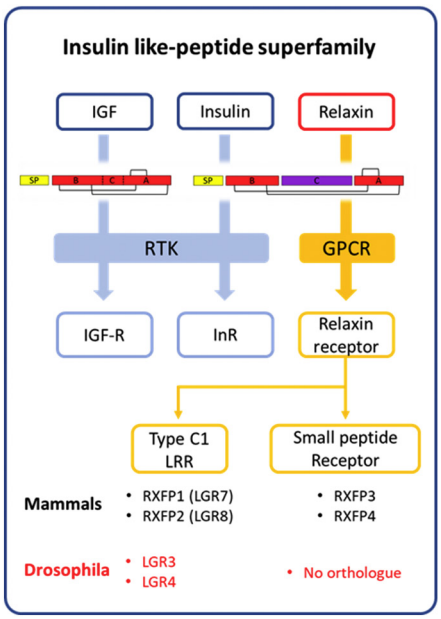
stress signal is supported by two pieces of evidence. First, these are the only cells in the CNS that show a detectable consistent response to ectopic Dilp8 expression by increasing cAMP levels (Garelli et al., 2015a; Vallejo et al., 2015). Second, knocking down Lgr3 with RNAi in a series of different cell populations of the CNS suppressed the Dilp8-induced delay only when this pair of bilateral neurons was included in the population (Colombani et al., 2015; Garelli et al., 2015a; Vallejo et al., 2015). These cells are referred to as PIL (*Pars Intercerebralis* Lgr3-positive) neurons (Garelli et al., 2015a) and GCL (Growth Coordinating Lgr3) neurons (Colombani et al., 2015). They send descending projections to the subesophageal zone and highly arborized ipsilateral and contralateral projections, which make contact with insulin producing cells and PTTH neurons, as revealed by the GRASP technique (Fig. 1) (Colombani et al., 2015; Vallejo et al., 2015). Thus, the anatomical and functional data is consistent with the proposed role of PIL/GCL cells as intermediate neurons relaying the Dilp8 signal to neuronal populations that regulate developmental timing. However, this assumption has not been formally assessed. Validation of this hypothesis will require the generation of constructs that drive expression exclusively in PIL/GCL neurons to perform cell-type specific RNAi against Lgr3.

Two experimental observations suggest that the Dilp8 and Lgr3-dependent cAMP-level increase in PIL/GCL neurons leads to increased neuronal activity: first, silencing of *R19B09*-expressing neurons (hereafter, *R19B09* neurons) by Kir2.1-dependent hyperpolarization partially reduces the EMS-dependent delay in the onset of metamorphosis (Garelli et al., 2015a). *R19B09* is a 3-kb enhancer fragment corresponding mostly to the 7th intron of the *Lgr3* gene (Jenett et al., 2012), which drives gene expression in cells where Lgr3 is required to transduce the Dilp8-dependent developmental delay (Colombani et al., 2015; Garelli et al., 2015a; Vallejo et al., 2015; Jaszczak et al., 2016). Second, activation of *R19B09* neurons by expression of the NaChBac ion channel produces a delay (Vallejo et al., 2015). However, the PIL/GCL neurons represent only four of ~270 *R19B09* neurons (Li et al., 2014; Garelli et al., 2015a). Hence, the phenotypes observed following these manipulations represent the output of collective manipulation of *R19B09* neurons, not of the PIL/GCL neurons themselves. Again, it will be important to generate PIL/GCL-neuron specific drivers to confirm these findings.

The possibility that Dilp8 acts as a ligand for Lgr3 is supported by their strong genetic interaction and biochemical data. Namely, a synthetic Dilp8 peptide specifically stimulates cAMP production in Lgr3-transfected *Drosophila* Kc cells with an EC50 of 6.3 nM, and a tagged Dilp8 co-precipitates with the extracellular domain of Lgr3 (Vallejo et al., 2015). Together, these results indicate that Dilp8 binds to Lgr3 and activates the intracellular pathways that will eventually delay the accumulation of Ecdysone required for the entry into metamorphosis.

If we consider that Dilp8 travels from the discs to the CNS, crosses the Blood-Brain Barrier and reaches Lgr3 in the surface of the PIL/GCL

Box 1
Insulin-like peptides and their receptors.



HUMAN		
IGF	IGF-I IGF-II	IGF-R
Insulin	Insulin	InR
Relaxin	Relaxin-1/2	RXFP1
	INSL3	RXFP2
	Relaxin-3	RXFP3
	INSL5	RXFP4
	INSL4	?
	INSL6	?

Drosophila		
IGF	**Dilp6**	d-InR
Insulin	**Dilp1-4** Dilp5	
Relaxin	**Dilp7** (?) Dilp8	Lgr4 Lgr3

Bold: binding to its putative receptor has not been experimentally confirmed
(?): The relaxin identity of Dilp7 is not confirmed

The insulin-like peptide (ILP) superfamily is diverse and is subdivided into insulins, insulin-like growth factors (IGFs), and relaxins on the basis of primary structure, processing and receptor binding preferences (Blundell and Humbel, 1980; Wilkinson and Bathgate, 2007).

All ILPs have six conserved cysteines that form three disulfide bonds. They are synthesized as a pre-propeptide that encodes an N-terminal signal peptide and a propeptide consisting of two chains (B and A) separated by a C-peptide. While the short C-peptide of IGFs is retained in the mature hormone, that of insulins and relaxins is proteolytically released, leaving the B and A chains linked by two intermolecular disulfide bonds (the third disulfide bond is intramolecular, between two Cysteines of the A chain).

Functionally, while the insulin/IGF-signaling (IIS) pathway regulates growth, metabolism and ageing (Blundell and Humbel, 1980; Wilkinson and Bathgate, 2007; Gronke et al., 2010), members of the vertebrate relaxin family have antifibrotic, vasodilator and cardiac stimulatory functions, affecting many processes, including -but not limited to- female and male reproduction, such as menstruation, sperm motility, and pregnancy (reviewed in (Bathgate et al., 2013)). Insulin binds to a receptor tyrosine kinase [the insulin receptor (InR)] and stimulates a signaling pathway that includes phosphoinositide 3-kinase (PI3K) and serine/threonine kinase (AKT). IGFs preferentially bind a related RTK [IGF receptor (IGFR), which shares an evolutionary history with the InR], and activate the MAPK pathway. On the other hand, relaxins can bind G-protein-coupled receptors (GPCRs) that belong to two divergent groups: one is similar to the type 1 small peptide receptor family that modulates cAMP production and activate MAPK and the other group belongs to the type C Leucine-rich repeat-containing family (LGR) which influence cAMP and NO levels, as well as MAPK and tyrosine kinases activity.

LGRs have seven transmembrane domains typical of GPCRs and a large extracellular domain containing a varying number of leucine-rich repeats (LRR) required for ligand binding. Based on structural features, LGRs are classified as type A, B or C. Only type C LGRs are relaxin receptors. They have 7 to 10 LRRs and are differentiated by the presence of an N-terminal low-density lipoprotein receptor class A domain (LDLa) essential for signaling (Van Hiel et al., 2012; Bathgate et al., 2013). Type C LGRs can be further divided into type C1 or C2, based on features such as their number of LDLa domains (one and multiple, respectively) and specific amino-acid signature in the “hinge” region that connects the extracellular domain to the 7 transmembrane domains. (Van Hiel et al., 2012).

Whereas humans have ten ILPs (1 insulin, 2 IGFs and 7 relaxins), one InR, two IGFRs, and 4 relaxin receptors (RXFP1–4) (Bathgate et al., 2013), *D. melanogaster* has eight ILPs (Dilp1–8; (Brogiolo et al., 2001; Garelli et al., 2012), a single insulin/IGF-like receptor, and two homologues of the relaxin receptors of the LGR family (Lgr3 and Lgr4) (Van Hiel et al., 2012). Some Dilps (e.g., Dilp2 and Dilp5) are strongly expressed in the so-called insulin-producing neurons in the brain, and are the major source of IIS (Miron et al., 2001; Junger et al., 2003; Puig et al., 2003; Colombani et al., 2005). Others, like Dilp6–8 have strikingly different expression patterns (Yang et al., 2008; Okamoto et al., 2009; Slaidina et al., 2009; Colombani et al., 2012; Garelli et al., 2012). The only IGF-like Ilp in the fly genome is Dilp6 (Okamoto et al., 2009; Delanoue et al., 2010). All other Dilps are predicted to encode a C-peptide, suggesting they are either insulin or relaxin-like (Gronke et al., 2010; Garelli et al., 2012).

The binding of insect Ilps to insect InRs has only been demonstrated for mosquito Ilp3 (Brown et al., 2008; Wen et al., 2010) and the *Drosophila* Dilp5 (Sajid et al., 2011). Furthermore, Dilp2 and 5 can induce tyrosine autophosphorylation of the *Drosophila* InR (Rulifson et al., 2002). Other Ilps, such as Dilp1, 3, 4, 6 and 7, are usually assumed to activate IIS based either on sequence information indicating appurtenance to the Ilp family and/or their ability to modulate InR-dependent growth in gain and loss-of-function studies (e.g., Refs. (Brogiolo et al., 2001; Ikeya et al., 2002; Rulifson et al., 2002; Yang et al., 2008; Okamoto et al., 2009; Slaidina et al., 2009; Zhang et al., 2009; Gronke et al., 2010).

Dilp8, similarly to Dilp7 [a relaxin-like candidate peptide (Yang et al., 2008)], however, shares properties at the sequence and regulatory level with members of the relaxin subfamily of Ilps (Garelli et al., 2012). For instance, both Dilp8 and many vertebrate relaxins have similarly positioned basic amino-acids between the two B-chain Cysteines, both abruptly end on Cysteine C6, and both have a long C-peptide. At the regulatory level, both are endogenously enriched in reproductive tissue [apart from the abnormally-growing imaginal discs, Dilp8 is also strongly expressed in the adult ovary (Garelli et al., 2012)]. Finally, Dilp8 and the relaxin receptor-like GPCR, Lgr3, act in the same genetic pathway, consistent with a ligand x receptor interaction (Colombani et al., 2015; Garelli et al., 2015a; Vallejo et al., 2015; Jaszczak et al., 2016), and Dilp8 can stimulate Lgr3 activity in vitro (Vallejo et al., 2015), and in vivo (Garelli et al., 2015a; Vallejo et al., 2015), which is also consistent with Lgr3 being a Dilp8 receptor. Together, these properties suggest that Dilp8 is an invertebrate relaxin.

neurons to trigger a cAMP-dependent response in them, this raises the possibility that other anatomical or molecular factors could be required to confer specificity for the Dilp8 affinity towards these neurons. This is because PIL/GCL-neurons express similar amounts of Lgr3 as other neurons, such as the MIL neurons, which, in contrast to PIL/GCL-neurons, do not seem to respond to imaginal-disc-derived Dilp8 by activating cAMP signaling (Garelli et al., 2015a). The fact that not even Dilp8 expressed under the control of the ubiquitous driver *Tubulin* triggers cAMP signaling in MIL neurons, suggests that this is not an anatomical problem of accessibility. One possibility is that Lgr3 signals through different G protein signaling pathways in different neurons. For instance, in MIL neurons, Lgr3 could couple to G proteins that stimulate Ca + 2 signaling, such as Gαq, or couple to an inhibitory Gα protein, instead of a stimulatory one as is assumed to be the case in PIL/GCL-neurons, so that no increase in cAMP signaling levels would be detected. Another possibility is that this specificity could come from alternative receptors and/or co-receptors which are present in a subset of Lgr3-positive neurons. Insight towards this possibility comes from an unbiased biochemical assay aimed at discovering binding partners of Dilp8 in *Drosophila* S2 cells. This analysis identified the *Drosophila* insulin receptor (InR) and the neuronal protein Derailed (Drl) among other four Dilp8 candidate binding proteins that, unexpectedly, did not include Lgr3 (Garelli et al., 2015a). Further studies will tell if any of these proteins acts as a receptor or co-receptor and participates in PIL/GCL neuron selectivity.

3. Just the right size

The developmental delay that ensues upon imaginal disc damage does not result in overgrowth of undamaged organs, despite the extended time that these animals spend feeding (Simpson et al., 1980; Stieper et al., 2008). This occurs because unaffected tissues respond to the presence of an aberrantly-growing disc by reducing their growth rate, thereby maintaining body parts proportions throughout development (Martin and Morata, 2006; Parker and Shingleton, 2011). The Dilp8-Lgr3 molecular pathway is the critical signaling pathway mediating this growth compensation mechanism. Overexpression of Dilp8 in otherwise unperturbed larvae leads to downregulation of insulin signaling in imaginal discs and a reduction in growth rate (Colombani et al., 2012; Garelli et al., 2012). The systemic negative effect on imaginal disc growth does not seem to be exerted by the direct action of Dilp8 on imaginal tissues, but is rather considered to be mediated by PIL/GCL neurons activity, as it requires neuronal expression of Lgr3 (Colombani et al., 2015; Vallejo et al., 2015; Jaszczak et al., 2016). Once neuronal Lgr3 is activated and a Dilp8-dependent delay is triggered, Lgr3 is also required in the prothoracic gland to inhibit the growth of imaginal discs via a nitric oxide synthetase-dependent pathway (Jaszczak et al., 2016).

The growth-coordination mechanism downstream of Dilp8 and Lgr3 has been proposed to be mediated by modulation of insulin, ecdysone, and juvenile hormone pathway (Colombani et al., 2015; Garelli et al., 2015a; Vallejo et al., 2015; Jaszczak et al., 2016). The mutually-regulated effects of these three hormones on systemic growth control have been intensively studied (Colombani et al., 2005; Delanoue et al., 2010; Mirth et al., 2014), but a clear mechanistic model in this new context is still lacking (Fig. 1). Neuronal Lgr3-dependent modulation of ecdysone biosynthesis seems to be the key molecular pathway affected by Dilp8 that coordinates organ growth rate, because feeding larvae with ecdysone is sufficient to prevent both the developmental delay and the growth restriction induced by localized growth perturbation (Parker and Shingleton, 2011), tissue damage, and *dilp8* overexpression (Jaszczak et al., 2015).

The findings discussed above allow us to devise a current model of how the Dilp8-Lgr3 pathway works to coordinate imaginal disc growth with the timing of the onset of metamorphosis (Fig.1). Upon aberrant imaginal disc tissue growth, the *dilp8* gene is transcriptionally activated, leading to Dilp8 production and secretion into the hemolymph. Dilp8 enters the CNS and specifically binds to Lgr3, which leads to an increase in cAMP levels and PIL/GCL neuronal function, likely increasing their firing rates. PIL/GCL neurons then relay the signal to the prothoracic gland via an unknown mechanism, which might involve direct inhibition of PTTH and/or insulin producing neuron activity. This ultimately influences ecdysone biosynthesis in the prothoracic gland in order to negatively affect the timing of the onset of metamorphosis while simultaneously restricting growth of unaffected tissues.

4. Evolution of the growth coordination molecular pathway

The relaxin pathway is very ancient and probably existed in the most basal metazoans (Van Hiel et al., 2012; Roch and Sherwood, 2014). However, as clear Dilp8 homologues are difficult to find outside of flies due to low conservation at the sequence level (Garelli et al., 2012), it is difficult to determine how well conserved is the role of Dilp8 in coordinating regeneration with developmental timing. The recent discovery that Dilp8 and Lgr3 are in the same pathway nevertheless allows us to revise a series of studies that addressed the evolutionary history of arthropod GPCRs, such as Lgr3 (Van Hiel et al., 2012; Veenstra et al., 2012; Veenstra, 2014, 2016), to generate a tentative cladogram depicting the evolution of the Dilp8-Lgr3 pathway (Fig. 2). This cladogram generates many interesting questions that could be addressed in future research. First, if we consider that all clades that have a clear Lgr3-like homologue should also have a Dilp8-like ligand, as has been previously hypothesized (Veenstra, 2014, 2016), we expect to find Dilp8-like ligands in many non-dipteran insect clades, such as apocritan hymenopterans (bees, wasps, and ants), and even other arthropods, such as decapod crustaceans (Van Hiel et al., 2012; Veenstra et al., 2012; Veenstra, 2015, 2016). Using this rationale, for instance, the insulin-like androgenic gland (IAG) hormone of crustaceans, has been hypothesized to be a Dilp8-like ligand for the crustacean Lgr3-like receptor (Veenstra, 2016).

Another interesting piece of information that we can extract from currently available data is that the Dilp8-Lgr3 pathway seems to have originated when the last common ancestor to all arthropods lived (Fig. 2). One possibility is that the Dilp8-Lgr3 pathway arose via duplication events involving an ancestral relaxin-like pathway consisting of homologues of the other relaxin-like peptide of *Drosophila*, the Drosophila insulin-like peptide 7, Dilp7, and homologues of the orphan *Drosophila* relaxin receptor-like protein Lgr4. The co-evolution of *dilp7* and *Lgr4* genes offers strong support for this candidate ligand-receptor relationship (Van Hiel et al., 2012; Veenstra et al., 2012; Veenstra, 2014, 2016) (Fig. 2). Addressing this relationship experimentally is a promising area for future research, which apart from shedding new light on relaxin-like pathway biology, could help understand the evolution of the Dilp8-Lgr3 pathway. This is critical because the ability to coordinate regeneration with developmental transitions seems to be a common feature among arthropods, but there is evidence that not all arthropods have a Dilp8-Lgr3 pathway (Fig. 2). First of all, lepidopterans (a group containing moths and butterflies), such as *Galleria* and *Ephestia,* which clearly coordinate growth with developmental timing via humoral factors (Madhavan and Schneiderman, 1969; Dewes, 1973), are thought to completely lack a Dilp8-Lgr3 pathway or in fact any other relaxin-like pathway (Garelli et al., 2012; Van Hiel et al., 2012; Veenstra, 2014) (Fig. 2). The same seems to be true for the body louse, *Pediculus humanus corporis* (Fig. 2). How these groups of insects couple growth and maturation timing remains an open question. Second, many other non-dipteran insects such as the beetle *Tribolium castaneum* or even some dipterans as the *Culicidae Aedes*, *Anopheles*, and *Culex* seem to have lost both *dilp8* and *Lgr3* homologues (Garelli et al., 2012; Veenstra, 2016). How animals that lack the Dilp8-Lgr3 pathway couple abnormal peripheral tissue growth with maturation time remains to be defined. It is intriguing to note that *Culicidae* mosquitoes

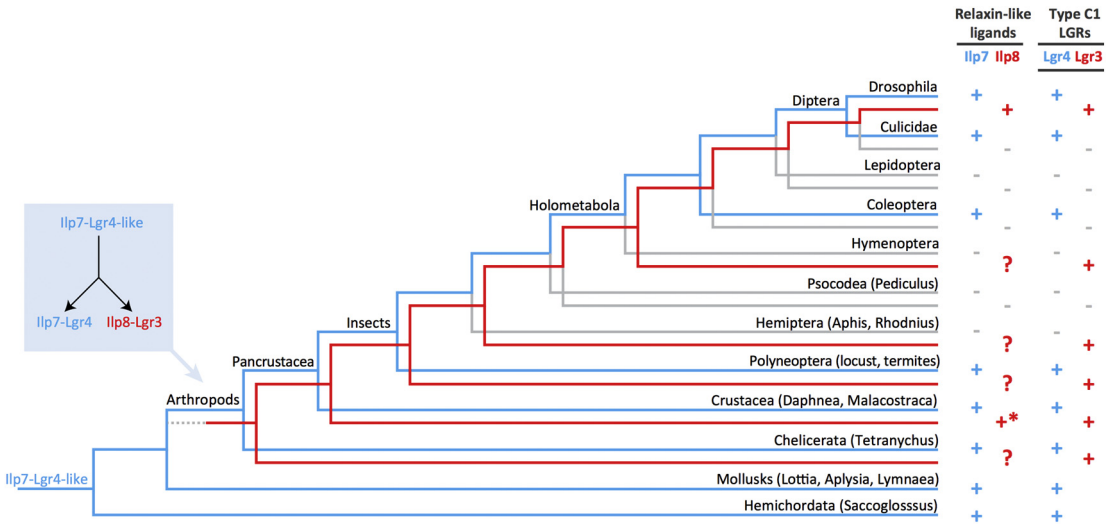


Fig. 2. Cladogram showing the presence ("+", red or blue) or absence ("−", light gray) of the genes encoding homologues of the relaxin-like ligand x receptor pairs Dilp8 and Lgr3 (red), and the candidate relaxin-like ligand x receptor pair Dilp7 and Lgr4 (blue). The cladogram suggests the Dilp8-Lgr3 pathway originated early during Arthropod evolution, derived from a Dilp7-Lgr4-like pathway. The presence of a Dilp8-like ligand is some clades, labelled with "?", has not been demonstrated, but is expected due to the presence of candidate Lgr3-like receptors. The asterisk (*) indicates that the crustacean IAG peptides have been hypothesized to be the Dilp8-like ligands of the crustacean Lgr3-like receptor. Note that both lepidopterans (moths and butterflies) and Psocodea (lice) seem to have lost both relaxin-like pathways. Data was collected from the following references: (Veenstra, 2010; Garelli et al., 2012; Van Hiel et al., 2012; Veenstra et al., 2012; Veenstra, 2014, 2016).

and coleopterans have genes encoding orthologs of *dilp7* and *Lgr4* (Veenstra et al., 2012). It will be interesting to test whether the Dilp7-Lgr4 pathway performs the functions of the Dilp8-Lgr3 pathway in these organisms. Clearly, more work is required to understand the full extent of the conservation between the presence of a Dilp8-Lgr3 pathway and the ability of the organism to coordinate its growth with maturation timing.

5. Dilp8 beyond growth coordination

Dilp8 has been identified as a growth-coordinating hormone released from imaginal discs. However, the analysis of its spatio-temporal expression pattern suggests that it might have novel functions in other life history stages. For instance, Dilp8 is highly expressed in the ovary (Tootle et al., 2011; Garelli et al., 2012; Brown et al., 2014), where it could be acting similarly to vertebrate relaxins controlling some aspects of reproductive biology such as egg laying or in a feedback loop with oogenesis-promoting hormones like ecdysone, insulin, and juvenile hormone (Bownes, 1989; Ables et al., 2016). Multiple pulses of *dilp8* expression also appear to take place during development and some of them occur closely to the ecdysone surges that trigger major developmental transitions. Genomewide expression profiling of synchronous animals indicate that there is a peak at the end of embryonic development, L2 to L3 molt, larva to prepupa transition, head eversion and the end of pupal stage (Colombani et al., 2012; Brown et al., 2014) (Fig. 3). The highest expression is reached at the moment of puparium formation. When the larva shortens and hardens its cuticle to acquire the typical barrel shape, *dilp8* is found among the genes that peak in the larval carcass under the control of the Ecdysone Receptor (Li and White, 2003; Beckstead et al., 2005). The biological message being sent by the ovary- and carcass-derived Dilp8 signal and whether the hypothetical target tissues receiving the message require the Lgr3 receptor to transduce it remains to be determined. Notably, *Lgr3* mutants show defective puparium contraction, as evidenced by an increase in the length/width ratio (Garelli et al., 2015b), indicating that the interaction between Lgr3 and Dilp8 could transcend growth coordination and these molecules could act together multiple times in *Drosophila* life cycle.

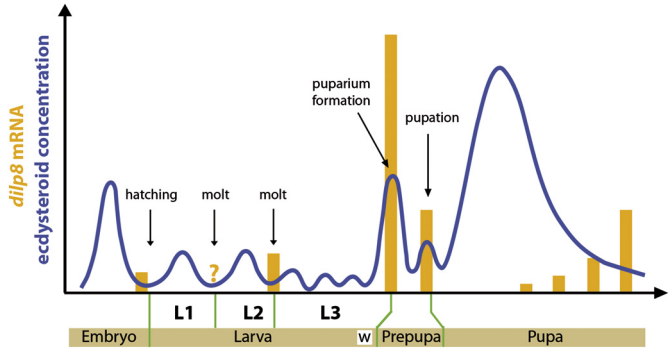


Fig. 3. Dilp8 expression during *Drosophila* development
Dilp8 and ecdysone levels appear to be coordinated during major developmental transitions. Ecdyseroid profile and *dilp8* transcriptional levels adapted from Ou et al., 2016 and Brown et al., 2014 respectively.

6. Final remarks

Dilp8 and Lgr3 were initially identified due to their role in controlling the timing of the onset of metamorphosis in animals with abnormally growing imaginal discs. Many interesting questions remain to be answered regarding how this pathway works, including how the Dilp8 signal reaches the CNS and signals specifically to the candidate PIL/GCL neurons, what are the second messages elicited by Lgr3 in these neurons, and what exactly are the critical signals emitted by the PIL/GCL neurons upon Dilp8 stimulation. The fact that Dilp8 and Lgr3 are expressed in other tissues and neurons, respectively, including in other developmental stages, indicates that the pathway might have other functions at different life history stages, which remain to be elucidated. Finally, there are many unanswered questions regarding the evolution of the Dilp8-Lgr3 pathway. Some insects that clearly coordinate growth and developmental timing lack Dilp8 and Lgr3-encoding genes, and some clades have even lost the relaxin pathway completely. These findings suggest that these animals must coordinate growth and developmental timing using other mechanisms, which might not necessarily involve humoral factors. Research into these areas should generate exciting new insights into the biology of growth

coordination, the evolution of the relaxin signaling pathway, and likely reveal unforeseen functions in other developmental stages.

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