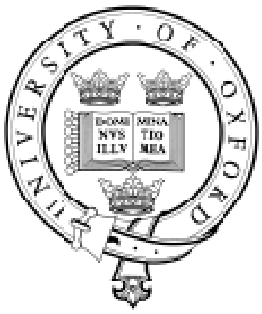
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Submitted for the degree of Doctor of Philosophy, Michaelmas 2010

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Oxford

Phenotypic characterisation of the *C. elegans* latrophilin homolog, *lat-1*

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ABSTRACT

G proteins coupled receptors (GPCRs) play essential developmental roles with functions in all of the immune, olfactory sensory systems amongst other systems as well as exhibiting essential roles in the central and peripheral nervous system. GPCRs are also major targets of pharmaceutical drugs currently used to treat a vast number of conditions. Despite their clear importance, the function of many GPCRs is still obscure. Identifying the physiological role of more GPCRs provides a niche for more drugs to be developed and thus more conditions to be treated. The *C.elegans lat-1* gene encodes the latrophilin vertebrate homolog; it is a member of the adhesion GPCR family and is structurally related to the flamingo/CELSR, an essential component of planar cell polarity pathway. This study aims to phenotypically characterise *lat-1* mutants in *C.elegans* to provide insights into the physiological role of this important member of adhesion GPCRs. *lat-1* mutants exhibit several morphological defects throughout development and during vulva development. Analysing the embryonic development of such mutants also identified an anterior-posterior polarity defect. The results implicate a second evolutionary conserved subfamily of adhesion GPCRs in the control of tissue polarity and morphogenesis.

A mes chers parents

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I. Chapter I: Introduction

1. General introduction

1.1. G-proteins coupled receptors (GPCRs)

Throughout evolution, organisms have maintained and developed several communication methods that allow them not only to interact with the external environment but also to enable cells, organs and whole systems to communicate with each other. Inter and intracellular communications are key to the development of any organism as it permits to sustain a stable functional state. Amongst the several cell-cell communication mechanisms are ligand-receptor interactions. Amongst such communication methods is the signaling via G protein coupled receptors (GPCRs). Broadly speaking, upon ligand binding, membrane-bound receptors transmit extracellular information to the inside of the cell by interacting with small G proteins. GPCRs represent the largest family of membrane-bound receptors with fundamental function in the sensory and immune systems and occupy a strategic role in homeostatic feedback cycles controlling a variety of organ and system functions. Additionally, GPCRs are also targets for many drugs such as antihistamines, antacid drugs, cardiovascular drugs and antipsychotics. The primary structure of the first GPCR, rhodopsin was achieved twenty seven years ago (Hargrave, McDowell et al. 1983; Nathans and Hogness 1983), and since then, the number of known GPCRs grew rapidly, with the completed human genome sequence which enabled a more comprehensive view of the human repertoire of GPCRs. Additionally, advances in methods such as radioligand binding, solubilisation and purification of monoamine-binding GPCRs greatly contributed to the growing number of GPCRs.

Throughout the race to identify more GPCRs, several attempts were made to classify GPCRs in order to facilitate their studies according to a combination of factors including structure, conserved functional domains, and ligand preferences (for a detailed GPCRs family classification refer to (Lagerstrom and Schioth 2008)). GPCRs could bind a broad

range of ligands including small organic compounds (Dixon, Kobilka et al. 1986; Kobilka, MacGregor et al. 1987), peptides and hormones (Masu, Nakayama et al. 1987; Parmentier, Libert et al. 1989).

1.1.1 GPCRs as drug targets

So what makes GPCRs good drug targets? G protein coupled receptors are active in about every organ system and present a wide range of opportunities to develop pharmaceutical drugs in conditions such as cancer, immunity, cardiac dysfunctions and diabetes, obesity and nervous system disorders. Cell-based screening assays and structural studies have greatly contributed to the development of the current arsenal of drugs modulating GPCR's functions. Not surprising, initial drugs were not aimed at a particular protein, but were developed based of functional activity as observed in assays, emphasising the importance of understanding the function of GPCRs and their mode of action. Many of the big and small pharmaceutical companies exclusively focus on GPCRs targeting. The screening approaches adopted by the several pharmaceutical companies and research laboratories were both diverse and complimentary. Post Human Genome Project, there was a more comprehensive view of the human repertoire of GPCRs and several technologies were specifically developed to tackle GPCRs. Cell-based high throughput screening assays took advantage of the common feature of GPCRs in that they interact with small G proteins, and the trick for high throughput screening was to find a marker that would be applicable to the diverse GPCRs despite their ligand binding specificity and unique function.

One such target is Ca^{2+}, it is naturally occurring in the cytoplasm and is produced in cells upon activation of GPCRs coupled to G_q regardless of their tissue specificity, ligand binding inclination and cellular function. In combination with aequorin, a jelly fish derived photo-protein that displays a photo activity proportional to Ca^{2+} concentration, a cell based screening assay (AequoScreen) was developed by Euroscreen (a Brussels-based company) in search of compounds that have the ability to modulate GPCR's function(Siehler 2008; Michelini, Cevenini et al. 2010). Genetic expression methods were then developed to widen the use of Ca^{2+} as a marker even in GPCRs that do not signal through G_q. Moreover, several other cell-based screening assays were developed based on the use of different markers such as the use of luminescent tags that bind cyclic adenosine monophosphate (cAMP) a common second messenger in the cascade of GPCR signaling pathways. These and other cell-based screening assays bypass the requirement to know a GPCR's biological ligand. However, the importance of ligands and GPCRs deorphanization does not go unnoticed due to the rather uncharted therapeutic potential they represent. In addition to cell-based screening, structural characterization of GPCRs is critically important especially in selective targeting. However, due to the difficulties to crystallize membrane-bound proteins, little has been accomplished since the first published crystal structure of rhodopsin in 2000 (Bourne and Meng 2000), nevertheless, the Rhodopsin family of GPCRs remains the most largely drug targeted family, emphasizing the magnitude of structural studies in drug development. The resemblance in downstream mediators in GPCRs signaling pathways (such as Ca^{2+} and cAMP) and their ubiquitous nature in addition to the sensitive high throughput screening assay render GPCRs highly druggable targets. Thus, the common features shared between GPCRs signaling such as the downstream effectors and the availability of a structural template (crystal structure of Rhodopsin) in combination with the different developed high throughput screening assays without the need to know the endogenous ligand, made GPCRs excellent drug targets. Further characterising the

molecular function of GPCRs provides more stringent drug-mediated modulation of GPCRs for better drugs and treatments.

1.2. Members of the G proteins coupled receptors family

The recent phylogenetic classification of GPCRs divides this class of receptors into five families based on two main factors: sequence similarity (and consequently structure) and ligand-binding inclination, into: ***G****lutamate*, ***R****hodopsin*, ***A****dhesion*, ***F****rizzled/Taste 2* and ***S****ecretin* (together referred to as GRAFS) with members present in most bilateral species (Bockaert and Pin 1999).

1.2.1. The *Rhodopsin* receptor family. The rhodopsin receptor family contains the largest number of receptors targeted by clinically used drugs such as antihistamines, antacid drugs, cardiovascular drugs and antipsychotics, and is the largest family of GPCRs with more than 670 members in humans (Lagerstrom and Schioth 2008). Members of this family have short N-termini without any common conserved domains. The heterogeneity of the rhodopsin family falls within 7-transmembane region (7TM) and the ligand(s) each member binds to. Several of the identified conserved residues form inter-helical structures that have important role in the stabilization and activation of rhodopsin (Palczewski, Kumasaka et al. 2000) and (Lagerstrom and Schioth 2008). The Rhodopsin family members bind a vast variety of ligands such as peptides, amines and purines.

1.2.2. The *Secretin* receptor family. This small family of GPCRs includes members that all have an extra-cellular hormone binding domain and bind peptide hormones (Lagerstrom and Schioth 2008). As opposed to the *Rhodopsin* family, most of the diversity within the *Secretin* family lies within the N-terminal regions. Conserved cysteine residues within the first and second extracellular loops of the TM regions help stabilise the N-terminal structure (Grace, Perrin et al. 2004). The Secretin receptors are important drug targets owing to their crucial role in central homeostatic functions. Targets aimed at

secretin GPCRs include calcitonin and glucagon used for the treatment of hypocalcaemia and hypoglycaemia respectively (Tyndall and Sandilya 2005).

1.2.3. The *Glutamate* receptor family. Also referred to as class C, most members of this family bind their endogenous ligand within their N-terminal regions in a fashion compared to a "Venus Flytrap" mechanism where two lobes of the ligand-binding region form a cavity to which the ligand binds and activates the receptor. Many endogenous ligands have been identified to bind to this receptor family. Although this family of receptor is well characterised, new orphans (proposed members whose endogenous ligand is still unknown) are being added to the list such as the human GABABR-like GABABRL receptor (Galvez, Parmentier et al. 1999) and GPRC5A/5B/5C (Brauner-Osborne and Krogsgaard-Larsen 2000; Robbins, Michalovich et al. 2000; Brauner-Osborne, Jensen et al. 2001). Several of the identified orphan glutamate receptors show no conserved domains within their N-termini (e.g. GPR158 and GPR158L (Ota, Suzuki et al. 2004; Bjarnadottir, Fredriksson et al. 2005)) further illustrating the need for a more stringent classification approach extending far beyond phylogenetic attributes.

1.2.4. The *Frizzled/Taste2* receptor family. The human homologs were identified following the discovery of the first frizzled receptor in *Drosophila* in 1989 (Vinson, Conover et al. 1989). Members of this receptor family bind the family of Wnt glycoproteins (although other types of ligands were also reported such as secreted proteins, (Xu, Wang et al. 2004)). Signaling through G proteins was evidenced in several species including *Xenopus* and rat ((Lagerstrom and Schioth 2008) and references therein).

1.2.5. The *Adhesion* receptor family. This family represents the second largest class of GPCRs and by 2003, 33 and 31 members were found in humans and mouse respectively. It is also known as LNB7TM whereby LN stands for Long N termini and B for the sequence similarity between adhesion and secretin GPCRs (excluding N termini

architecture, which marks the difference between secretin and adhesion GPCRs receptors, and which actually distinguishes adhesion GPCRs from the rest of receptor families).

1.2.5.1. Characterizations of adhesion GPCRs.

1.2.5.1.1. Long-N termini with adhesive properties. Members of this receptor class are hallmarked by long and diverse N termini which can extend to a few thousand amino acids (reviewed in (Bjarnadottir, Fredriksson et al. 2007)). Long N-termini also harbour different domains that are also found in other proteins such as cadherin, lectin, laminin, olfactomedin, immunoglobulin and thrombospondin, tyrosine kinases and integrins. Studies conducted by Lin and colleagues suggest that these domains have a role in receptor-ligand interactions (Lin, Stacey et al. 2001) and the nature of the domains contained within the N-termini suggest that these proteins interact with other cells or the extracellular matrix. In fact, de-orphanized adhesion GPCRs were shown to bind extracellular matrix molecules, for instance, glycosaminoglycan chondroitin sulphate was shown to interact with epidermal growth factor (EGF)-like module containing mucin-like receptor protein 2 (EMR2) and EMR3 can interact with molecules expressed at the cell surface of neutrophils and macrophages (Stacey, Lin et al. 2001; Stacey, Chang et al. 2003). The adhesive description adopted by the adhesion GPCRs owes to the different functional domains found within the N-termini (14 different types of functional domains have been counted for the 33 human adhesion GPCRs) many of which are implicated in protein-protein interactions in other non-GPCRs (for instance the leucine-rich repeats-LRR), cell-cell adhesion or cell-extracellular matrix interactions (such as the CalxBeta) and galactose-binding lectin domains (Schwarz and Benzer 1997; Rabinovich 1999; Kobe and Kajava 2001). Binding to glycosaminoglycans in the extracellular matrix was shown to be linked to biological processes such as cell adhesion (Stacey, Chang et al. 2003). Additionally, most of the domains within the N-termini contain conserved sequences that were previously linked with protein-protein interactions. For instance, Ca^{2+} binding sites found in the EGF domains

(Epidermal Growth Factor) were linked to stabilization of protein-protein interactions even outside the GPCRs kingdom. Although these domains have been implicated in adhesion in other receptors, their adhesive function in adhesion GPCRs is still to be further characterised. However, growing evidence is emerging to strengthen the adhesive property/function of members of this family, for example, these Ca^{2+} binding sites in the CD97 receptor (belonging to the EGF clan of adhesive GPCRs (for an introduction to subclasses of adhesion GPCRs refer to (Bjarnadottir, Fredriksson et al. 2007)) contribute to the binding of CD97 to the decay-accelerating factor (DAF/CD55). The DAF is found on the cell surface of lymphocytes and erythrocytes (Downing, Knott et al. 1996; Hamann, Vogel et al. 1996; Lin, Stacey et al. 2001), suggesting that adhesion GPCRs may function to provide adhesion to the cells on which they are expressed in order to recruit other signaling molecules. So far, most of the reported adhesive characterization of this receptor family is related to immune system cells/molecules, for instance, CD97 recognizes DAF/CD55 on the cell surface of lymphocytes and erythrocytes; and human EMR3 (hEMR3- EGF module-containing receptor 3) recognizes a ligand on the cell surface of monocyte-derived macrophages and activated neutrophils (Stacey, Lin et al. 2001). It would be worthy to demonstrate adhesion-related property with molecules outside the immune system to generalize the adhesive character of adhesion GPCRs.

1.2.5.1.2. Complex genomic structure. Splice variants results in a different combination of N-terminal functional domains that give the receptor different ligand-binding properties illustrating the possibility that one receptor can bind to more than one ligand and also illustrating one of the reasons why most of adhesion GPCRs are still orphan. Furthermore, sequence analysis suggests that this class of receptor are likely subjected to post translational modifications as indicated by their relatively high content of Ser/Thr residues in their N-termini. These residues are candidates for *O* or *N*-glycosylation which is one of the most common forms of post-translational modifications and which also affects the

binding affinity to ligands as shown in the case of CD97 (Wobus, Vogel et al. 2004; Bjarnadottir, Fredriksson et al. 2007).

1.2.5.1.3. Unique 7TM regions. Sequence similarity analysis between members of adhesion GPCRs and other GPCRs reveal very little conserved sequences and the present common features over the entire 7TM region are to be judged as very minute. Furthermore, membrane-spanning regions seem diverse even within members of adhesion GPCRs themselves. However there is growing evidence on the importance of the 7TM integrity for the function of GPCRs, for instance, the 7TM region of EMR2 is crucially important in the formation of homo and hetero oligomers with other closely related EGF-7TM proteins. This homo and hetero oligomerization plays important roles in regulating the expression and function of this leukocyte adhesion GPCR EMR2 (Davies, Chang et al. 2007). Similarly, the 7TM region was also proved essential for the function of another adhesion GPCR BAI-1 (Park, Tosello-Trampont et al. 2007).

1.2.5.1.4. Conserved proteolytic site. Also, adhesion GPCRs display a proteolytic site termed GPS (for GPCR Proteolytic Site) which is not found in *Secretin* class of receptors. It is found in a single copy and is conserved in all adhesion GPCRs (apart from GPR123). The GPS site functions as an internal autocatalytic process that generates two non-covalently bound subunits (Krasnoperov, Bittner et al. 1997). It is thought that the cleavage of the protein happens within the endoplasmic reticulum (ER) or in early compartments of the Golgi apparatus and that this proteolytic cleavage is required for the expression of the receptor on the cell surface (Krasnoperov, Bittner et al. 1997). Deletions and point mutation phenotypes emphasise the importance of the GPS role. For instance, the hereditary human disease bilateral and frontoparietal polymicrogyria is caused by mutation in the GPS domain of GPR56. The GPS site is also important outside the GPCR kingdom, as mutations in the GPS site of the *polycystin-1* gene cause polycystic kidney disease and

autosomal dominant disease (Piao, Hill et al. 2004; Jin, Tietjen et al. 2007; Yu, Hackmann et al. 2007).

1.2.5.2. Functions of adhesion GPCRs. Most of the adhesion GPCR genes are still orphans, meaning that their endogenous ligand(s) is still unknown (although it is not exactly clear whether adhesion GPCRs have endogenous or exogenous ligand(s). Note that the "orphan" description is also used to point out that the receptor's physiological function is unknown). There is a great urge to better characterise this family of receptors and to pin-point their physiological role for several good reasons: adhesion GPCRs represent the second largest family of 7TM-membrane bound receptors after *Rhodopsin* GPCRs which are major drug targets. Accordingly, there is great potential for adhesion GPCRs to be the next major target of pharmacological drugs. In view of this, adhesion GPCRs play fundamental roles in several developmental processes mainly cellular adhesion, signaling, immune and nervous systems further emphasizing their importance as drug targets. The current knowledge regarding the physiological role of adhesion GPCRs is derived from studies of tissue specific expression, ligand binding, EST and reverse genetics. For example, the EGF clan of adhesion GPCRs are mainly expressed in cells of the immune system and smooth muscle cells with evidenced physiological function in the immune system (Bjarnadottir, Fredriksson et al. 2007). An example would include the role of CD97 in induced localised inflammatory responses and migration of neutrophils (Gray, Haino et al. 1996; Leemans, te Velde et al. 2004). Adhesion GPCRs especially those linked to function in the nervous system (central nervous system and peripheral nervous system) are also characterised by a strict expression pattern and mutations have been linked to dysfunction in the nervous system of several species. For example, the *Drosophila* adhesion-GPCR receptor flamingo (*fmi*) mutants exhibit damaging developmental malfunction including defects in axonal pathfinding and targeting in the visual system (Senti, Usui et al. 2003). Similarly, mutations in the mammalian *fmi* ortholog (CELSR) cause thalamocortical and

cortocofugal axonal fasciculation defects (Tissir, Bar et al. 2005). Furthermore, the human Usher syndrome which manifests as severe deafness and blindness from birth is connected to mutations in the human VLGR1 (another human adhesion-GPCR) (Willson, Amliwala et al. 2004).

For most adhesion GPCRs, genetic studies are restricted to vertebrate species since most members of this receptor family do not have orthologs in non-vertebrate species. For instance, it appears that the adhesion GPCR GPR56 is only present in higher vertebrates processing a cerebral cortex (Piao, Hill et al. 2004) and the *Drosophila* METHUSELAH (Mth) has no known orthologs (Lin, Seroude et al. 1998). Another factor adding to the complexity of studying adhesion GPCRs is the degree of redundancy within species usually expressing several homologs.

There is a clear indication implicating adhesion GPCRs in fundamental neurodevelopment mechanisms and neuronal functions. However, the scarce information about their physiological function, endogenous ligand and under developed functional assays hampers the analysis of their mechanisms of signaling and renders them unapproachable for drug targeting. Accordingly, shedding light on the biological role of this class of receptors would enable scientists to better understand the pathology of associated conditions and move towards developing clinical drugs.

1.2.5.3 The need to functionally characterise adhesion GPCRs

The importance of studying adhesion GPCRs is highlighted by the growing number of scientific evidence linking human pathologies to defects in the function of adhesion GPCRs. A representative summary of some human conditions is presented in table i. The table was constructed by searching current OMIM (*Online Mendelian Inheritance in Man)* release accessible at: http://www.ncbi.nlm.nih.gov/omim.

The *Adhesion* GPCR family is the second largest family of 7TM membrane bound receptors. They play major roles in the immune system, central nervous system, hormone signaling and development. Studies on adhesion GPCR mutants in several species highlighted the impact of this group of family of receptor on the function f the nervous system. For instance, mutations in the *Drosophila* CELSR/FLAMINGO adhesion GPCR cause defects in planar cell polarity in both neuronal and non-neuronal tissue (Gao, Brenman et al. 1999; Lu, Usui et al. 1999; Usui, Shima et al. 1999). Additionally, *Drosophila* flamingo (fmi) mutants exhibit defects in axonal pathfinding in their visual system (Lee, Clandinin et al. 2003) in addition to dendritic branching defects in the peripheral nervous system (Gao, Kohwi et al. 2000). Similarly, mutations in the murine CELSR (the mammalian fmi ortholog) cause major fasciculation and migration defects in the thalamocortical and corticofugal axons (Tissir, Bar et al. 2005) in addition to dysfunctional inner hair cells and defective neuronal tube closure (Curtin, Quint et al. 2003). In mice also, mutations in another adhesion GPCRs VLGR1/MASS1 results in audiogenic seizures (Skradski, Clark et al. 2001; Nakayama, Fu et al. 2002; McMillan and White 2004) just like the human VLGR1 locus in implicated in the etiology of Usher syndrome (Weston, Luijendijk et al. 2004) which is a hereditary condition characterized by severe deafness, progressive retinitis pigmentosa leading to blindness in addition to vestibular dysfunction. Human bilateral frontoparietal polymicrogyria, a neurodevelopmental disease caused by defective cortical lamination was linked to mutations in GPR56, another adhesion GPCR gene (Piao, Hill et al. 2004). These examples fortify the impact of adhesion GPCRs function in the nervous system. In addition to functions in the nervous system, other clans of adhesion GPCRs are heavily implicated to function in the immune system. For instance, CD97 and members of EMR class of adhesion GPCRs are expressed on the surface of different cells of the immune system, and the binding of CD97 to its ligands (CD55, dacay accelerating factor and extracellular matrix components chondroitin sulfate glycosaminoglycans and dermatane sulfate) is implicated in the

activation, migration and proliferation of cells of the immune system (Stacey, Lin et al. 2001; Stacey, Chang et al. 2003). Additionally, BAI1, brain-specific angiogenesis inhibitor binds phosphatidylserine on the surface of apoptotic cells and is required for their engulfment during the resolution phase in the inflammatory response (Park, Tosello-Trampont et al. 2007) . Moreover, BAI1, a p53-inducible gene, has been implicated in the regulation of vascularization of malignancies in several cancers such as colorectal cancer, pulmonary adenocarcinoma, gastric cancer, and glioblastomas (Fukushima, Oshika et al. 1998; Hatanaka, Oshika et al. 2000).

Human adhesion GPCR ortholog	Gene name and accession number/Entez gene ID	Phenotypes associated with mutations in human (when available) or across species	Reference
BRAIN-SPECIFIC ANGIOGENESIS INHIBITOR 1	BAI1 - AB005297	Transcriptionally regulated by p53. Associated with glioblastoma. BAI1 functions as an engulfment receptor in both the recognition and subsequent internalization of apoptotic cells.	(Shiratsuchi, Nishimori et al. 1997; Park, Tosello-Trampont et al. 2007)
BRAIN-SPECIFIC ANGIOGENESIS INHIBITOR 2	BAI2 -	Expression reduced in glioblastoma cell lines.	(Shiratsuchi, Nishimori et al. 1997)
CD97	CD97 - 976	CD97 is present on the surface of most activated leukocytes. Involved in cell adhesion and signaling.	(Hamann, Eichler et al. 1995)
CADHERIN EGF LAG SEVEN-PASS G-TYPE RECEPTOR 1	CELSR1 - AF231024	CELSR1 point mutations results in abnormal cell shape and cytoskeletal polarization in embryonic mouse skin.	(Devenport and Fuchs 2008)
CADHERIN EGF LAG SEVEN-PASS G-TYPE RECEPTOR 2	CELSR2 - D87469	Neuronal branch retraction leading to prominent simplification of dendritic arbors of cortical pyramidal neurons and Purkinje neurons in knockdown Celsr2 rat pups by RNAi. The neuronal migration function is conserved in mouse as well.	(Shima, Kengaku et al. 2004; Qu, Glasco et al. 2010)
LATROPHILIN HOMOLOG 2	LPHN2 - AJ131581	gene product variability was higher in the breast tumour cells. The *C. elegans* homolog of human latrophilin *lat-1* is required for correct anterior-posterior cell division in early embryos and the *Droshophila* homolog Cirl was pulled in a genome wide screen for synaptic vesicle cycle proteins.	(White, Varley et al. 1998; Lloyd, Verstreken et al. 2000; Langenhan, Promel et al. 2009)
LATROPHILIN HOMOLOG 3	LPHN3 - AB018311	Contributed to the susceptibility of attention deficit/hyperactive disorder (ADHD)	(Arcos-Burgos, Jain et al. 2010; Ribases, Ramos-Quiroga et al. 2011)

Table i. The table illustrates some of the aberrations associated with mutation in genes encoding members of adhesion GPCRs. Whenever available, human phenotypes are indicated, otherwise, phenotype(s) of gene orthologs in other species (such as mice and *C. elegans*) is indicated. Adhesion GPCRs are heavily implicated in the function of the nervous system, immune system and cell adhesion. The recent link between human psychological disorder and human latrophilin homolog LPHN3 further reinforces the importance to characterise this class of receptor.

Despite their clear importance in the function in the nervous and immune systems, most of adhesion GPCRs are still functionally uncharacterised. Understanding the function of more adhesion GPCRs is critical to move forward and develop drugs targeting this subfamily. Accordingly, in order to understand the function of this class of GPCRs we have opted to study one particular member of adhesion GPCRs: latrophilins.

1.3. Latrophilins.

Latrophilins are members of the *Adhesion* GPCR family. They are also known as CIRL (**C**alcium-**I**ndependent **R**eceptor of α-**L**atrotoxin), lectomedin and CL. Like other adhesion GPCRs, latrophilins have a GPS domain located within the juxtamembrane domain of the N-terminal extracellular region (Krasnoperov, Lu et al. 2002). Now that sequence similarity comparison has become available, latrophilins are regarded as the most highly conserved members of the adhesion GPCRs subfamily, thus making latrophilin a prototype of adhesion GPCRs.

Latrophilins were first discovered as binding partners of α -Latrotoxin (α -LTX) *Lactrodectus mactans* the main component of the black widow spider venom. Over expression of CIRL in chromaffin cells suggested a role of CIRL in the regulation of secretion (Bittner, Krasnoperov et al. 1998), and the binding of the receptor to its exogenous ligand α –LTX implicated latrophilins in the regulation of transmitter release (for more details, refer to section **1.1.1** of Chapter IV), while expression of rat latrophilin in COS cells associate signaling trough G-proteins as the two proteins co-purify in affinity chromatography column (Lelianova, Davletov et al. 1997). On the other hand, there is also evidence that coupling with G protein is not required for the action of latrophilins in the regulation of neurotransmitter release in the presence of α –LTX (please refer to Chapter IV, section **1.1.1** and (Sugita, Ichtchenko et al.

1998)). Latrophilin signaling through G protein is still to be proven, however signaling through G protein has been demonstrated in other adhesion GPCRS including GPR56 (Iguchi, Sakata et al. 2008) and BAI-1 (Park, Tosello-Trampont et al. 2007) .

The available low resolution data of expression profiles of latrophilins suggests a tight control over expression during development (Matsushita, Lelianova et al. 1999). For example, the human genome contains three latrophilin homologs *Lphn*1-3, two of which as expressed in the brain. The *Caenorhabditis elegans* genome contains two homologs encoded by *lat-1* and *lat-2*. The scarce information available on latrophilins is derived from studies using the neurotoxin α-LTX. However, the complexity of the toxin signaling confines its usefulness as a tool to study the function of latrophilin. Accordingly, the urge to develop a functional assay that addresses the role of latrophilin (and adhesion-GPCRs in general) in its physiological environment is crucial for moving forward in studying this class of receptors.

2. Aim of the study. Considering their importance in development, and owing to the large number of members populating this subfamily, adhesion GPCRs deserve rigorous investigations to shed light on their physiological role and thus enlarge the number of druggable genes (Hopkins and Groom 2002; Russ and Lampel 2005). We have turned to study the function of latrophilin using the model organism *Caenorhabditis elegans* and our strategy is based on a two-fold question: why latrophilin?, and why *Caenorhabditis elegans*?

2.1. Why latrophilin? Comparative genomics of the highly divergent *Adhesion*-GPCR family has shown that in addition to *flamingo/starry night/Celsr* FMI, only the latrophilins domain architecture is strictly conserved across phyla (Nordstrom, Lagerstrom et al. 2009). Accordingly, latrophilin represents a prototype of adhesion GPCRs that overcomes

the complexity associated with previous studies that were limited by the high diversity within members of the adhesion GPCR family. Furthermore, latrophilin is conserved across phyla with members in mammals, rodents and nematodes. Accordingly, it would be possible to assess the function of latrophilin in several species, and observe whether this function is also conserved across phyla. Functional classification is something to consider on top of phylogenetic classification due to the presence of large discrepancy amongst members of the same family based on phylogenetic classification alone.

Next to the Flamingo domain, the latrophilin members of adhesion GPCRs were shown to be the most conserved members amongst the adhesion GPCRs superfamily, they are also conserved across phyla with homologs in human, mouse, and rat, chicken, fly and nematode genomes. Comparative genomics of the highly divergent adhesion GPCR family shows that next to FMI only the domain architecture of latrophilins is strictly conserved across phyla (Nordstrom, Lagerstrom et al. 2009).

Three latrophilin homologs exist in humans LPHN1-3 two of which are expressed in the brain. The rat latrophilin homologs LPHN1 and 3 are expressed in the brain. It is possible that LPHN2 is also expressed in the brain but is quickly degraded and thus difficult to detect. PEST sequences confer susceptibility to quick degradation by proteolysis (Rogers, Wells et al. 1986; Roth, Sullivan et al. 1998; Sekhar and Freeman 1998) and LPHN1 contains three putative PEST sequences, LPHN2 has two PEST sequences and one PEST sequence is found in LPHN3 from rat and bovine origin. The number of PEST sequences does not correlate with the amount of LPHNs in rat tissues, indicating a possible tissue specific regulation of protein turn over. For instance, northern blotting of LPHN RNA from rat tissues showed that although LPHN1 and 3 are most exclusively brain-specific, LPHN3 is much less abundant despite having less PEST sequences than LPHN1, whereas LPHN1, with its three PEST sequences is highly abundant in

the brain while mRNA was also found in very low amounts outside the brain (in the kidneys, lungs and spleen) (Matsushita, Lelianova et al. 1999). In humans, pair wise comparison of gene expression in adult and fetal adrenal glands revealed that both LPHN1 and 3 are expressed in the adrenal glands with higher expression in the fetal versus adult tissues.

The functional and structural information of latrophilin were elucidated from studies using the alpha-LTX. However, although vertebrate latrophilins were linked to function in the excretory machinery (neurotransmitter and hormone release from synaptic terminals), the findings do not allow one to draw firm conclusions about the physiological function of latrophilins where the toxin is not naturally present. Interestingly, a recent study by Arcos-Burgos and colleagues (Arcos-Burgos, Jain et al. 2010; Ribases, Ramos-Quiroga et al. 2011) found that the most brain specific latrophilin isoform LPHN3 confers high susceptibility to the condition ADHD (Attention-deficit/hyperactivity disorder) a psychological condition characterized by a persistent impairing pattern of inattention and/or hyperactivity-impulsivity, and one of the most occurring psychological conditions in children and adolescents. The association of LPHN3 with high risk of developing ADHD, in combination with inverse relationship between the N-acetyl aspartate/creatine (NAA/Cr) ratio (which is used in many studies as a reliable marker of neuronal integrity) and the dosage of the LPHN3 susceptibility haplotype, indicate that LPHN3 might function in neuronal transmission and/or maintenance of neuron viability. Adhesion GPCRs are becoming growing candidates associated with abnormalities in brain function. This is further indicated by the fact that another adhesion GPCR gene is implicated in the etiology of ADHD. Ribase and colleagues in their 2009 study showed that BAIAP2 (a brain-specific angiogenesis inhibitor (BAI1)-binding protein which functions as an adaptor protein linking membrane bound G-proteins to cytoplasmic effector proteins.) and ADHD in Spanish and German populations (Ribases, Bosch et al. 2009). There are thus at least two firmly

identified adhesion GPCRs linked to ADHD, and the scarce evidence of their mode of action in causing the pathology points to a function in the generation/maintenance of the neuronal circuit, further emphasizing the importance of adhesion GPCRs in brain function and stressing the need to further elucidate their function in the hope of developing cures for this and other conditions.

2.2 Why *Caenorhabditis elegans?* The *C.elegans* latrophilin homolog *lat-1* is an excellent candidate for studying the role of latrophilin in its physiological context without the use of its exogenous ligand alpha-latrotoxin. *C. elegans* as a model organism comes with an excellent genetic toolkit where the availability of deletion alleles (resulting in a true null or a strong hypomorphic character) together with the availability of additional reverse genetic methodologies such as RNA interference (RNAi) allows the analysis of the loss of function phenotypes in vivo. Additionally, epistasis analyses can be employed to dissect the latrophilin signaling pathway and its interaction with other molecules/genes, without the need to know its ligand. Additionally, *C. elegans* is an excellent model organism for in vivo studies, it is possible to design tissue-specific rescuing experiments, in addition to conducting in vivo experiments to dissect the structure-function relationship in order to shed light on the requirement of the different segments of the protein, in the hope of better understanding the molecular mode of action of latrophilin in particular and adhesion-GPCRs in general. The *C. elegans* genome contains two adhesion GPCR designs: flamingo-like GPCR and latrophilin, conferring both a reduced functional redundancy, a complication usually associated with using higher model organisms and suggesting that those LPHN and FMI to be prototypes of all other adhesion GPCRs. Furthermore, the GPS site which is considered as the defining structural feature of adhesion GPCRs is well conserved between vertebrate and invertebrates including *C. elegans* and human latrophilin (figure i A). Additionally, the RBL (Rhamnose Binding Domain) which

was previously shown to be essential for the function of latrophilin in *C. elegans*, (Langenhan, Promel et al. 2009) is also conserved in its consensus cysteine residues between *C. elegans* and humans (figure i B). Accordingly, despite the distance relationship between nematodes and humans, the *C. elegans lat-1* gene is well conserved and presents an excellent mean to study the function of latrophilin in a simple model system. The second *C. elegans* design of adhesion GPCR is a Flamingo homolog encoded by *cdh-6*. Mutations affecting *cdh-6/flamingo* disturb cell polarity affecting axon migration defects, a phenotype similar to defects observed in *Drosophila* suggesting a conserved function across species. The second *C. elegans* latrophilin gene is encoded by *lat-2*. *lat-2* loss of function mutation (*tm463* is a 1378bp deletion allele that was used in our lab, it removes the RBL domain (figure i C) and causes a frame shift resulting in a truncated protein) does not cause adverse phenotypes and the worms remain viable. The function of *lat-2* has not been characterised is this study, and all efforts were directed towards studying *lat-1*. However, *lat-1*; *lat-2* double mutants are severely affected and are significantly less viable than either single mutants (T. Langenhan, personal communication), indication that *lat-2* deserves further characterisation.

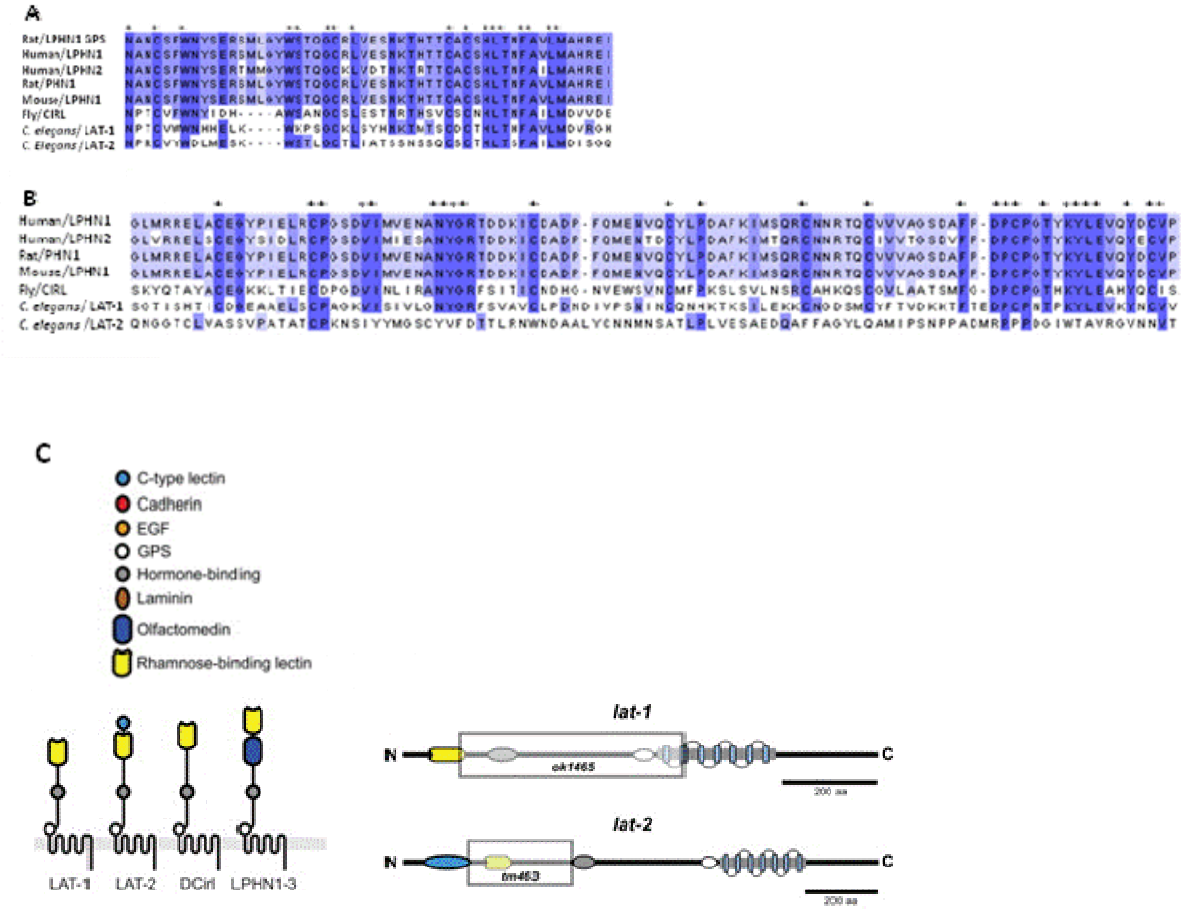


Figure i. Sequence alignment of the GPS (A) and RBL (B) domains of latrophilin. Highly conserved amino acids are indicated on the top of the sequence alignment. Despite the distance relationship between *C. elegans* and vertebrates, cysteine residues are still highly conserved in *C. elegans*. The conserved residues required for the structure and function of the RBLdomain as well as the RBL domain boundaries were taken from (Vakonakis, Langenhan et al. 2008). C), schematic representation of the deleted fragment in the *lat-1(ok1465) and lat-2(tm463)* alleles. Picture was adapted from (Langenhan, Promel et al. 2009). Sequence alignment was performed using ClustalW Multiple Sequence Alignment and the alignment was carried out in Jalview 2.3 accessible at http://www.ebi.ac.uk/Tools/msa/clustalw2/. The rat Lphn1(U72487) GPS sequence was identified using motif scan and was used as a bait for the alignment of the GPS sequence. The following protein accession numbers were used in the alignment: rat LPHN1 (ENSRNOT00000041025), mouse LPHN1 (ENSMUST00000152978), human LPHN1 (ENSP00000340688), human LPHN2 (ENSP00000353006), *C. elegans* LAT-1 (transcript B0457.1a), *C. elegans* LAT-2 (transcript B0286.2a) and fly CIRL (FBtr0290113).

3. Experimental design/Plan of the study. This study aims at providing a starting point/platform for investigating the function of latrophilin in particular and adhesion GPCRs in general. The study is based on reverse genetics whereby *lat-1* and *lat-2* knockout mutant strains are used to first characterize the phenotypes associated with the lack of functional LAT-1 and to a lesser extent LAT-2 gene products. Second, it touches upon verifying the implication of latrophilin in the regulation of neurotransmission from nerve terminals, this time however in an organismal context, mimicking the physiological role of latrophilin. Third, the study focuses on the use of the *C. elegans* benefits as a model organism, and test whether the preliminary data discussed in this study represent functional conservation across the *C. elegans* tissues and organs, i.e. is the function exerted by latrophilin the same in all organs/tissues or it is likely to exhibit a tissue-specific role, a possibility that would open a new field of investigation taking into consideration the previously reported tissue-specific splice variants and the impact of splice variants on ligand-binding specificity (Lagerstrom and Schioth 2008).

4. Material initially available for this study

Knocking out *lat-1* gene product causes embryonic lethality and larval arrest. The *lat-1* knockout worms were generated by the knockout consortium in Oklahoma and the worms were maintained as heterozygous where by the *lat-1* gene deletion was balanced against *mln1* chromosome rearrangement which carries an integrated GFP reporter linked to a morphological Dpy allele (for details on the *mln1* balancer, please refer to chapter II, section 4.1). The *lat-1* heterozygous strain was obtain from the CGC (*C. elegans* Genome Centre) and was out crossed by a former PhD student (Tobias Langenhan) in the lab to generate the XA7500 strain. The XA7500 strain is heterozygous for *lat-1* deletion and the heterozygous worms are GFP-labelled. Selfing of the XA7500 hermaphrodites throws heterozygous GFP

progeny, balancer homozygous GFP-labelled progeny (brighter GFP and look Dpy) and homozygous for the *lat-1* deletion which are not GFP-labelled. *lat-1* homozygous mutants arrest development as embryos and larvae and are thus not viable. However, at a very low frequency (≥ 1%), some of this *lat-1* homozygous progeny survive, proceed to adulthood and produce progeny. A stable line from these "escapers" was isolated (strain named XA7542) and was used as the *lat-1* mutant strain in this study. Why some *lat-1* mutant homozygous from heterozygous mothers survive is not fully understood, and questions the use of this strain as a reliable/correct *lat-1* mutant strain, as it is possible that some kind of compensation mechanism(s) accounts for the survival of those otherwise unviable worms and thus make this strain inappropriate for investigating the function of *lat-1*. Accordingly, in order to check that the XA7542 is a true *lat-1* null strain, Tobias Langenhan tested complementation with deficiencies known to cover the *lat-1* loci, the same and other deficiencies were again repeated by me and none of the tested chromosomal deficiencies complemented *lat-1* deletion (*ok1465*) for the associated *lat-1* larval arrest (please refer to deficiencies and complementation test in chapter III, section 3). Additionally, the *ok1465* allele removes the extracellular RBL, hormone binding, the GPS domain and the first transmembrane sketch of the 7TM region (figure xxxpanel C, adapted from (Langenhan, Promel et al. 2009)), most likely making *lat-1* mutant protein (if translated) a non-functional mutant protein (as transcript was not detected in *lat-1* mutant worms as initially tested by Tobias Langenhan). In view of this, a rescuing construct lacking the RBL domain made my another PhD student in the lab (Simone Promel) fails to rescue lethality associated with loss of *lat-1* in ten independent lines indicating the *ok1465* allele most probably generates a non-functional protein. Furthermore, a mini cosmid rescuing construct expressing *lat-1* gDNA under the control of its endogenous promoter (cosmid containing the entire locus and intergenic regions prior and after the *lat-1* coding region

(pTL2), for details on the cloning procedure please refer to supplement materials and methods in (Langenhan, Promel et al. 2009)) was injected in *lat-1* mutant heterozygous and the stable transgenic lines were scored for the ability of the minicosmid to rescue the lethality in *lat-1* mutant homozygous. The rescuing capacity was only 50% compared to wild type, and we were indeed not expecting a full rescue because of the silencing of transgenes in the germline that would hinders early embryonic expression, knowing now that the embryonic lethality and larval arrest observed in *lat-1* mutants are the cause of a very early embryonic polarity defect. At the beginning of the project, no LAT-1 antibody was available, so we solely relied on the deficiency complementation tests and on the fact that RNAi directed against *lat-1* did not enhance the *lat-1(ok1465)* phenotype. During the progress of the project, an antibody was raised against the N-termini of LAT-1, and antibody staining failed to detect any LAT-1 protein in our XA7542 [*lat-1(ok1465)*] or in the *lat-1;lat-2* double mutants (antibody staining was performed by Tobias. L). However, an antibody against the C termini would also be necessary to fully confirm that *lat-1* mutant worms do not pocess any sort of LAT-1 function, as it is possible that a C-termini moitie can still function independently on its N-terminal fragment, or even interact with N-termini of other proteins (such as LAT-2). It would be interesting to see if a chimera between LAT-2 N-terminus fused to a LAT-1 C-terminus can rescue the *lat-1* phenotype.

The survival of this small fraction of mutants that we initially named "escapers" remains intriguing and unexplained, and there is always a possibility that a rescuing/compensating mechanism(s) arises in these mutants, however, in light of the early embryonic polarity defect that was later identified independently (by Tobias and myself) I would like to put forward a possible explanation for these "escapers": the early polarity defects affecting the ABal blastomer (presented in Chapter II) causes aberrant cell contact, inappropriate cell-cell induction signals

and subsequent gross morphological defects accounting for the embryonic lethality and the larval arrest. My analysis of the ABal division plane shows that in the absence of LAT-1, the division is rather random (Chapter II, table1). The random division planes ultimately include the correct division plane required for viability and that could explain the survival of ≤1% of mutant *lat-1*. What adds to the complexity of the XA7542 strain is the fact that progeny from homozygous mothers seems to do better than progeny from heterozygous mothers, indicating a negative maternal effect. The maternal contribution of a wild type LAT-1 is enough to rescue the early blastomer division defect (i.e. homozygous from heterozygous mothers) but doest not rescue the larval arrest in the homozygous progeny, may be because a truncated LAT-1 is very toxin in the presence of a wild type LAT-1 (although no truncated transcript could be detected- in homozygous mutant worms, work conducted by Tobias. L). On the other hand, a stable line of homozygous mutants from homozygous mothers can be established (with reduced brood size and elevated embryonic lethality and larval arrest ≈ 60%). *lat-1;lat-2* double mutants shows enhanced embryonic lethality and larval arrest but no qualitative change was observed in the double mutants indicating a functional overlap between *lat-1* and *lat-2* and that only *lat-1* is required for development (*lat-2* single mutants have no obvious phenotype).

5. Chronological progress of the project

When I joined the lab in January 2007, the project was already lunched and an important amount of starting material was available. Tobias Lanhenhan had successfully designed a full genomic rescuing construct in addition to a transcriptional constructs to analyse the expression patterns of *lat-1*. Further tissue-specific translational constructs revealed that expression in the early pharyngeal premordium rescues (to some extent the *lat-1* phenotype) whereas epidermal expression is very toxic to the worms even at very low concentrations. In combination with the larval arrest (also a characteristic of several feeding mutants), the observation that the

pharyngeal expression is enough to rescue *lat-1* phenotype led us to think that *lat-1* might function in the pharynx to regulate the feeding process, so I went on to carry on profiling the pharyngeal pumping in *lat-1* mutants (a project started by a former Masters' Student Valeria. P. Who was using homozygous mutant larvae from heterozygous mothers whereas my project consisted at profiling the pharyngeal pumping in non-maternally rescued larvae and adult worms). The previous allegations linking LAT-1 to regulating neurotransmitter release opened a new chapter for the project so I went on test the scope of neurotransmitter release in *lat-1* mutants using a well described and rapid method involving the use of aldicarb and its effects on several tissues/systems (the feeding organ, the egg laying system and the neuromuscular junctions). The pharyngeal pumping profile of *lat-1* mutants and the sensitivity to aldicarb did not seem to account for the severe embryonic and larval arrest in *lat-1* mutants, so the main question remained: what kills the *lat-1* mutants? Accordingly, I started to analyse the embryonic lethality, first by obtaining accurate quantitative analysis of the severity of *lat-1* phenotypes. Then with help from Tobias Langenhan who introduced me to *C. elegans*, I embarked on identifying the exact developmental stages during which the embryos were arrested and found that the majority initiated the elongation step but failed to complete it. In order to further refine the embryonic defect I analysed live recording of developing embryos and found a sticking ventral enclosure defect. All of the embryos that showed a ventral enclosure defect either failed to initiate elongation, or started elongation but failed to complete it leading to gross morphological defect and traval arrest. The process of the ventral enclosure is well studied in *C. elegans* and the Ephrin receptor signaling plays crucial roles in regulating the migration of the specialised hypodermal cells responsible for sealing up the ventral cleft during enclosure, so I tried to further enhance the ventral enclosure defect of *lat-1* mutants by using a genetically sensitized background and found a rather sticking qualitative and

quantitative increase in the ventral clef closure defect in the sensitized genetic background (chapter III, section1.2.4). The gross embryonic morphological defects in *lat-1* mutants were already observed in the lab before I joined the project. The analysis of live developing *lat-1* mutant embryos allowed us thus to link the morphological defects to failure to elongate, and then the elongation defect to an earlier ventral enclosure failure. The detailed molecular faults impairing the leading cells to migrate/adhere during ventral enclosure have not been characterised yet. Thus, the embryonic lethality was continually being refined throughout the progress of my project, from scoring morphologically aberrant embryos to detecting the developmental stages of arrest, and from elongation defects to an earlier ventral enclosure failure, and then from ventral enclosure defect to a much earlier blastomere division abnormality.

The knowledge on the function of latrophilin that was available at the time was based on the latrotoxin experiments that linked latrophilin to the regulation of neurotransmission (chapter IV, section 1). Accordingly, I started the project by setting up experiments that would allow us to investigate the involvement of *lat-1* in either neurotransmitter release (the aldicarb assay) and/or in the feeding system (profiling the pharyngeal pumping). Although I observed a previously unidentified phenotype in *lat-1* mutants (in that they show sensitivity to aldicarb) neither systems (neurotransmission nor feeding) seemed to be the cause of embryonic lethality, so again, the question remained the same: what kills *lat-1* mutant embryos? More detailed analysis on the embryonic development were thus performed and culminated in identifying a polarity defect affecting early blastomer division plane. At the same time I was scoring early balstomer division axis in *lat-1* mutants in our lab in Oxford, Tobias Langenhan was conducting similar experiment in collaboration with Prof. Ralf Schnabel, a leading expert in *C. elegans* embryonic cell lineaging. Although embryonic lineage is fully described in C. *elegans*,

the detection of subtle division defect remains difficult for a beginner, and the fact that the same division defect was also independently observed by Tobias in R. Schnabel's lab further confirms my observations.

II. Chapter II: *lat-1* functions during early embryogenesis

1. Brief introduction

1.1. Asymmetric cell division and the axis of mitotic spindle in *C. elegans* embryos

An important requirement for asymmetric cell division is the coordination between the correct positioning of segregated cytoplasmic determinants and the axis of cell division. Wnt signaling pathways play a pivotal role in both asymmetric cell fate determination and the alignment of mitotic spindle during cell division. In *C. elegans*, asymmetric cell fate determination is largely controlled via a Wnt-β-catenin pathway that requires new gene transcription in responding cells involving several downstream transcription factors such as the TCF/POP-1 transcription factor which is required for the asymmetric cell division of EMS blastomere (reviewed by (Mizumoto and Sawa 2007)), whereas mitotic spindle alignment is controlled by a non-canonical Wnt/Frizzled (Wnt/Fz) pathway independent of gene transcription probably acting directly on influencing the cytoskeleton arrangement of target cells (reviewed by (Walston and Hardin 2006)). The role of the Wnt signaling pathway in polarity is conserved across phyla, and several Wnt signaling pathway components have been identified.

In C. *elegans*, Wnt signaling control over asymmetric cell division has been well documented in early embryos (Cowan and Hyman 2004; Walston and Hardin 2006). Following fertilization, the *C. elegans* embryo is composed of one cell called P0. A few minutes later, following extensive cytoskeleton rearrangement, the P0 zygote divides into two cells of unequal sizes: a smaller P1 and a larger AB blastomeres. The position of the P1 blastomere specifies the posterior side of the embryo whereas the AB blastomere is located in the anterior side of the embryo. Thus the first axis of the *C. elegans* embryo is established early during embryogenesis. The second round of blastomere division establishes the dorsal-ventral axis of the embryo: the

P1 blastomere divides asymmetrically to give rise to a smaller P2 blastomere (which is retained in the posterior location within the embryo) and to a larger EMS blastomere which now specifies the ventral side of the embryo. The AB blastomere divides in an anterior-posterior (a-p) axis although the orientation of the mitotic spindle looks tilted due to space constrains applied by the hard egg shell protecting the embryo. The anterior daughter of AB blastomere (ABa) is more anterior than is sister ABp. The left-right axis of the embryo is the last to be established as the ABa/p blastomeres divide in a left-right (l-r) orientation with the left daughter cells slightly tilted towards the anterior side of the embryo compared to their posterior sister blastomeres.

Therefore, the first rounds of cell divisions are governed by a series of asymmetric cell divisions which are critical for healthy embryonic development as it enables the establishment of founder cells such as EMS, P2 and AB blastomeres that later on will divide to generate tissue specific cells such as neurons, epidermal, muscle and germ line cells. The first rounds of asymmetric cell divisions also plays a major role in the positioning of cells making sure that signaling and responding cells are correctly located.

1.2. Wnt signaling and asymmetric cell division in *C. elegans* embryo.

A Wnt signal originating from the posterior blastomere P2 instructs the adjacent responding blastomere EMS to divide asymmetrically into a posterior Endoderm precursor cell E and anterior Mesoderm precursor cell MS along an anterior-posterior axis (a-p), a division plane that is also controlled by Wnt signaling originating from the signaling cell P2 (Goldstein 1995; Hutter and Schnabel 1995; Bischoff and Schnabel 2006; Goldstein, Takeshita et al. 2006). It is widely accepted that the capacity to generate a polarising Wnt signal is retained in the descendents of the founder blastomere P1, making all of P2, C, E and MS Wnt signaling cells (P2, C, E and MS are descendants of the P2 blastomere) (Goldstein 1995; Hutter and Schnabel

1995). For example, the C blastomere, bearing a Wnt signaling capacity orients the division plane of ABar in an axis that is perpendicular to the site of cell contact between C and ABar blastomeres (Walston, Tuskey et al. 2004; Walston and Hardin 2006). Similarly, laser ablation experiments along with mosaic blastomere recombination provided evidence that the E blastomere also influences the division axis of MS (Park and Priess 2003). The mechanisms controlling asymmetric cell division in the first, second and third round of cell division is well understood (Cowan and Hyman 2004; Walston and Hardin 2006), however, little information is available to answer the question of how such a posteriorly located polarising signal is propagated across the embryo to reach more anterior cells, and more importantly, how it is coordinated in a growingly complex environment as the embryo moves to the tenth division cycle.

lat-1 mutants arrest as embryos or larvae and many of the arrested embryos show morphological defects of different degrees of severity (refer to chapter III, section 1). We therefore asked the question of whether *lat-1* functions during embryogenesis. Accordingly, we made use of the extensively described reproducible cell lineage of the *C. elegans* embryos and analysed the blastomeres division pattern in *lat-1* mutant embryos to differentiate between the role of *lat-1* in morphogenesis and morphological defects being a secondary consequence of earlier embryonic abnormalities.

2. Results and discussion

2.1. LAT-1 is required for the spindle alignment of the ABal blastomere

Microscopic analysis of live developing embryos enabled us to detect a highly penetrant and reproducible cellular phenotype in our *lat-1* mutant embryos. In *lat-1* mutant embryos from homozygous mothers (that is lacking deposition of maternal material) the anterior most blastomere ABal loses its a-p polarity as it divides. In wild type embryos, the division axis of

AB granddaughters occurs along the a-p plane apart from the ABar which divides in transverse to the rest of AB granddaughters (towards the point of cell contact with the C blastomere). The a-p division plane of ABal places the posterior ABalp in contact with the adjacent MS cell, whereas the anterior ABala retires to the anterior side of the embryo with no cellular contact with the signaling cell MS or any other signaling cell (MS is referred to as a signaling cell due to its capacity to send a Notch signal to its neighbouring cell ABalp that specifies the cell fate of ABalp descendents, for more details on Notch signaling refer to Notch signaling chapter in www.wormbook.org). Conversely, in *lat-1* mutants, the lost a-p polarity observed as ABal divides, places both daughter cells ABalp and ABala in contact with MS (Figure 1). Defects in the division planes of early blastomeres have been identified in Wnt mutants affecting the division plane of ABar, EMS, MS, but no reports on ABal division defect have been previously described.

Our results show that one of the most important aspects of development, that is the orientation of cell division, is disturbed in our *lat-1* mutant embryos suggesting a role of the adhesion GPCR *lat-1* in cell polarity, at least as far as a-p axis is concerned. Additionally, our results describe for the first time, a polarity defect affecting *C. elegans* embryos in their anterior side in a blastomere that has no cell contacts with a Wnt-signaling cell: the previously described blastomere division defects were attributed to blastomeres that have direct cell contacts with Wnt-signaling cells such as the MS (in direct contact with the Wnt-signaling P2 blastomere), and ABar (in direct cell contact with the Wnt-signaling C blastomere). Furthermore, it is important to state that our analysis was performed strictly in a *lat-1* mutant background without disturbing any of the Wnt-polarising signals, in other words, the capacity to send a polarising Wnt-signaling was not compromised in our *lat-1* mutant embryos. Accordingly, the

observed a-p defect in ABal division is strictly attributed to the lack of *lat-1* and is not hindered by lack of a Wnt polarising signal.

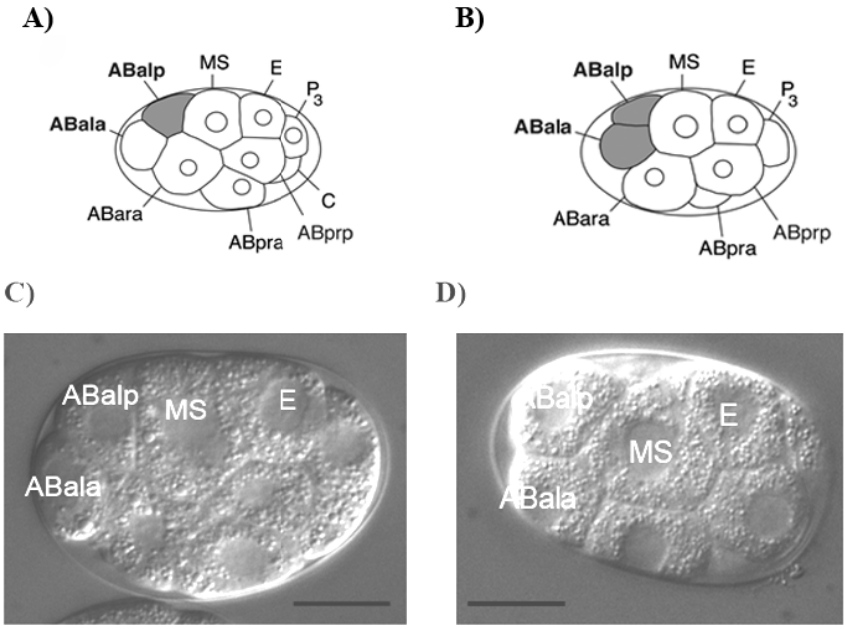


Figure 1: ABal division defect in *lat-1* mutants places both ABala and ABalp in contact with MS. A) and B) schematic diagram representing the position of ABala and ABalp relative to MS in wild type *lat-1* mutant embryos respectively. C) and D) are DIC photographs of wild type and *lat-1* mutant embryos respectively at the AB8 cell stage. In wild type embryos ABal division occurs along an anterior-posterior axis placing the posterior daughter ABalp in contact with MS while the anterior daughter cell ABala retires to the anterior most side of the embryo with no cell contact with MS blastomere(C). Conversely, in *lat-1* mutant embryos ABal loses its anterior-posterior plane of division making both ABala and ABalp in contact with MS (D). Bar= 17um.

To further analyze the defect of the ABal division plane we evaluated the exact division plane of ABal in *lat-1* mutant embryos to detect whether a "preferred" or "default" plane of division is adopted in the absence of LAT-1. Our results indicate that *lat-1* function is required to align the mitotic spindle of ABal along the a-p axis, and that lack of *lat-1* gene product results in a randomized division plane (table 1). This observation suggests that LAT-1 does not function in refining a default division plane and that its function is solely required for the alignment of the ABal spindle along the a-p axis.

Table 1: Percent penetrance of ABal division defect in *lat-1* mutant embryos, and random plane of division observed in ABal blastomere. AP, anterior-posterior; DV, dorsal-ventral; LR, left right.

genotype	Total number of examined embryos (n)	MS contacts n (%)		
		alp	ala	alp and alp
Wild type	12	12(100%)	0	0
lat-1 mutants	48	12(20%)	0	38(80%)
		ABal division axis		
		AP	DV	LR
Wild type	12	12(100%)	0	0
lat-1 mutants	48	3(25%)	4(33%)	5(41%)

2.2. *lat-1* function in ABal is independent of the transcription-independent Wnt/beta-catenin pathway

As with the Wnt signaling-mediated alignment of the mitotic spindle, we asked whether the a-p alignment of ABal spindle is independent of the Wnt-transcription pathway. Similarly, we sought whether the identity of the cell adjacent to ABal, MS is required for the correct alignment of the ABal spindle. To answer those questions we used *pop-1* mutant embryos. *pop-1* encodes an HMG box-containing protein that is the sole *C. elegans* member of the TCF/LEF family of transcription factors. In *pop-1* mutant embryos, the Wnt/B-catenin signaling that is dependent upon transcription is defective due to lack of the downstream transcription factor POP-1. Furthermore, the Wnt/β -catenin pathway polarising the cell fate in EMS daughter cells is also prohibited and both EMS daughter cells (E and MS) adopt an E-like cell fate in *pop-1* mutants (Thorpe, Schlesinger et al. 1997). Accordingly, analysing the ABal spindle alignment in *pop-1* mutants enables us to see whether the MS identity is required for ABal a-p spindle alignment and to find out about the requirement of the Wnt/β -catenin transcription pathway in the ABal division axis. Not surprisingly, in *pop-1* mutants the ABal divides along the correct a-p axis (Figure 2) placing only the posterior ABalp in contact with its neighbour cell (presumably MS).

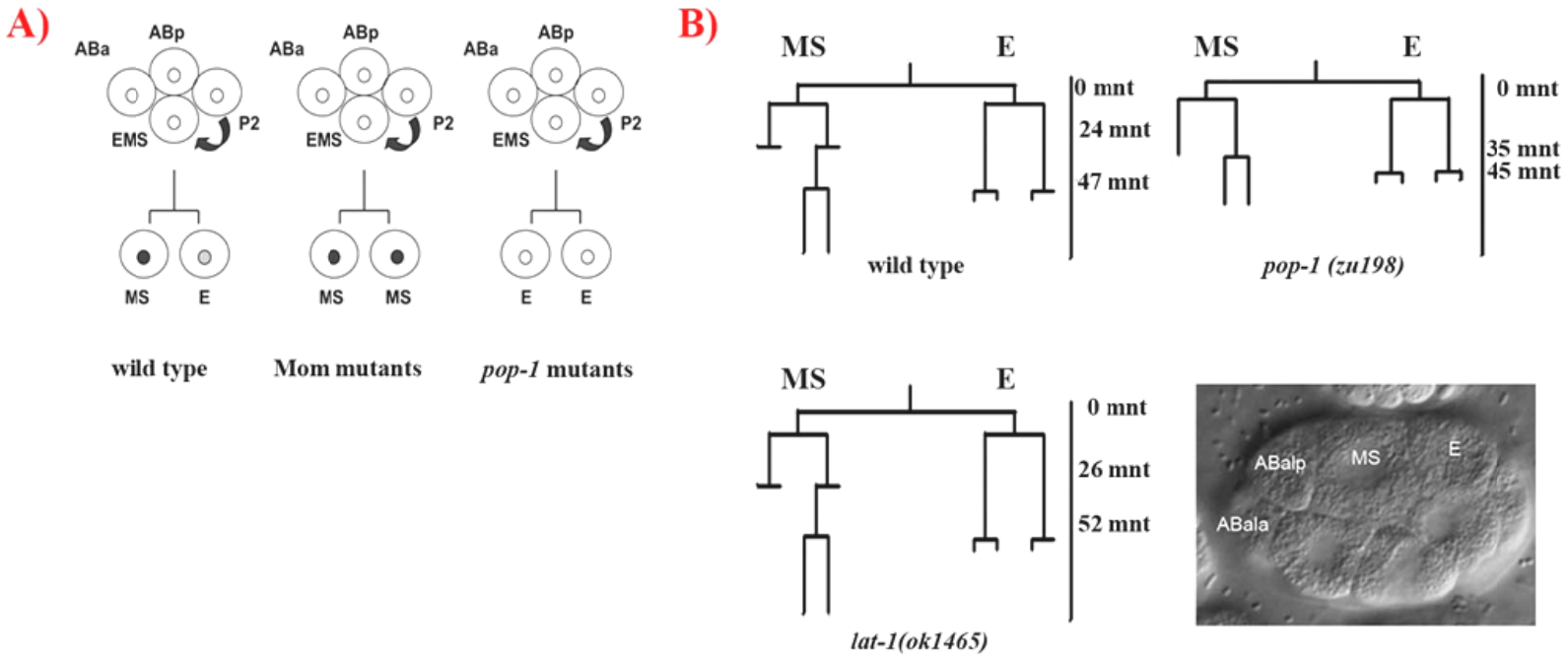


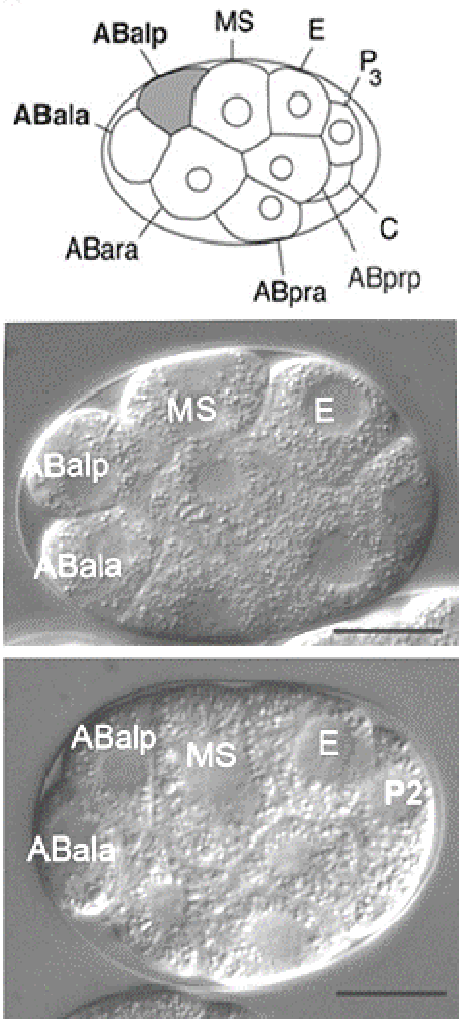
Figure 2. ABal blastomere division in *pop-1* mutants. A) Schematic diagram showing a 4 cell stage embryo and indicating the Wnt-mediated induction signal from P2 to EMS (curved arrow). In wild type embryos EMS divides asymmetrically to produce anterior MS and posterior E blastomeres. In *mom* mutants the Wnt-mediated induction of endodermal cell fate is compromised and consequently EMS divides into two MS-like cells making the embryo void of intestinal cells. In *pop-1* mutants however, the downstream targets of Wnt signaling (TFC/POP-1) is absent and the suppression of MS-cell fate is prohibited resulting in two E-like cells. The Wnt-mediated endordermal cell fate induction is dependent upon the transcription factor TFC/POP-1 through the Wnt/β -catenin signaling pathway (reviewed by (Mizumoto and Sawa 2007)). In *pop-1* mutants while the Wnt/β -catenin pathway is compromised, ABal divides in a wild type like a-p plane placing only ABalp in contact with MS indicating that the LAT-1 requirement for correct a-p division of ABal occurs through a process independent of Wnt/β -catenin and does not require new POP-1-dependent transcription. B) The two E-like phenotype of *pop-1* mutants was verified by following the E lineage. In wild type embryos, E blastomere descendents divide one round of cell division later than MS descendents. *pop-1* mutants having two E-like cells show similar delayed timing of cell division in both daughters of EMS in a lineage pattern resembling the division of E blastomere. DIC micrograph of *pop-1* mutant embryo showing ABalp but non ABala making cell contact with MS.

Accordingly, just like the Wnt/Frizzed spindle alignment pathway, the LAT-1 requirement for the correct ABal division is independent of Wnt-induced transcription at least as far as the known Wnt/β -catenin transcription pathway is concerned.

Furthermore, the mesoderm fate in MS does not seem to be required for ABal a-p division plane, suggesting that the cell fate determinants asymmetrically localized to MS (as a result of the Wnt signaling) are not required for the a-p spindle alignment in ABal. However, taking into account the signal strength emanating from the E blastomere, previously shown to be stronger than the signal emanating from the MS blastomere (Park and Priess 2003), an alternative interpretation of the correct ABal division plane in *pop-1* mutants would be the availability of an even stronger Wnt spindle alignment signal from the E-like blastomere which is responsible for the correct alignment of ABal mitotic spindle in *pop-1* mutants. Therefore, the analysis of ABal division plane in *pop-1* mutants allowed us to rule out the requirement of Wnt/B-catenin pathway in orienting ABal mitotic spindle along the a-p axis. Additionally, the mesoderm cell fate determinants that would normally be asymmetrically segregated into MS cell do not seem to be required for the ABal plane of division (unless a novel polarising signal redundant to Wnt signaling is responsible for the asymmetric localization of cell fate determinants). This observation is important because it provides us with insights regarding the hypothesis of Wnt signaling functioning as positional cues in establishing polarity and its implication in ABal division plane (Gleason, Szyleyko et al. 2006).

Several lines of evidence point to the hypothesis that Wnt signaling establishes polarity through asymmetric distribution of polarizing components in responding cells. In *C. elegans* for instance, changing the site from which the Wnt signal is presented influences the polarity of the Frizzled receptor in the responding cells (e.g. Wnt signals orient axon outgrowth in PLM and

ALM neurons, and it influences the polarisation of its Fz receptor in these two neurons (Hilliard and Bargmann 2006)) and such is the case in the embryonic EMS cell, the post-embryonic T cell and the V5 seam cell (Goldstein, Takeshita et al. 2006; Hilliard and Bargmann 2006). Similarly, such a phenomenon of asymmetric distribution of polarizing components in responding cells was reported in planar cell polarity pathway (PCP) in the *Drosophila melanogaster* (Adler 2002). However, disturbing the Wnt pathway did not perturb the PCP pathway, nor was a clear PCP-like pathway reported in C. *elegans*. The positional cues hypothesis of Wnt mode of action as proposed used to explain polarity defects in other organisms. For example, it was proposed in the axon guidance defect observed in mouse Wnt mutants (Lyuksyutova, Lu et al. 2003; Yoshikawa, McKinnon et al. 2003). Such axon guidance defects were similar in Wnt and in Frizzled (Fz) mouse mutants suggesting a Wnt/Fz signaling pathway in the regulation of axon guidance. The different severity in axon guidance in multiple Fz mutants also suggests the existence of an alternative redundant route through which Wnt signals establish polarity. In view of this Yoshikawa and colleagues showed that mouse Wnt4 functions in axon guidance, but instead of signaling through Fz receptor, it signals through the Derailed receptor. It is plausible thus to suggest that interacting pathways function in parallel or redundantly to provide asymmetric cues not only to tissues but also to individual cell polarization (Yoshikawa, McKinnon et al. 2003). Our analysis of ABal division axis in *pop-1* and in Wnt mutants (*mom-2* and *mom-5*, Figure 2 and 3) suggest that Wnt-dependent asymmetric distribution of polarising components is not required for the correct alignment of ABal mitotic spindle and invites considerations of the existence of an alternative mechanism through which a-p polarity is homogenised across the C. *elegans* embryo.



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2.3. *lat-1* functions in parallel to the Wnt signaling pathway

A posterior Wnt polarizing centre originates from the descendents of the founder blastomere P1, and can orient the division plane of the immediately adjacent cells. A Wnt-dependent relay mechanism was proposed in an attempt to explain how this posterior polarising signal is propagated across the embryo to reach further anterior cells (Bischoff and Schnabel 2006). Our data suggest that *lat-1* might function in propagating the spindle alignment cues to reach the anterior side of the embryo, and ABal would be a "weak spot" easily affected for the following two reasons: first, it only makes cell contact with a "tertiary" signaling cell MS which itself shows delayed spindle alignment in more that 50% of *lat-1* mutant embryos (Langenhan, Promel et al. 2009). Second, the anterior localisation of ABal makes it less spatially constrained and less likely for its division axis to be "rescued" by more diverse cell contacts that would provide compensating signals as is the case with ABar-descendents (Langenhan, Promel et al. 2009).

Analysis of l*at-1;mom-2* and *lat-1;mom-5* double mutants further suggests that *lat-1* functions in parallel to Wnt signaling to orient cell division along the a-p axis. The delayed alignment of MS spindle along the a-p axis is increased in *lat-1;mom-2* and *lat-1;mom-5* double mutants as compared to either single mutants (Langenhan, Promel et al. 2009). However no EMS spindle alignment defect was detected in *lat-1* mutants nor was it made worse in *lat-1;mom-2* or *lat-1;mom-5* double mutants as is the case in mom/scr double mutants (Bei, Hogan et al. 2002). This indicates that LAT-1 is required in ABal and MS but not in EMS spindle alignment bearing in mind that EMS is in contact with a primary signaling cell P2.

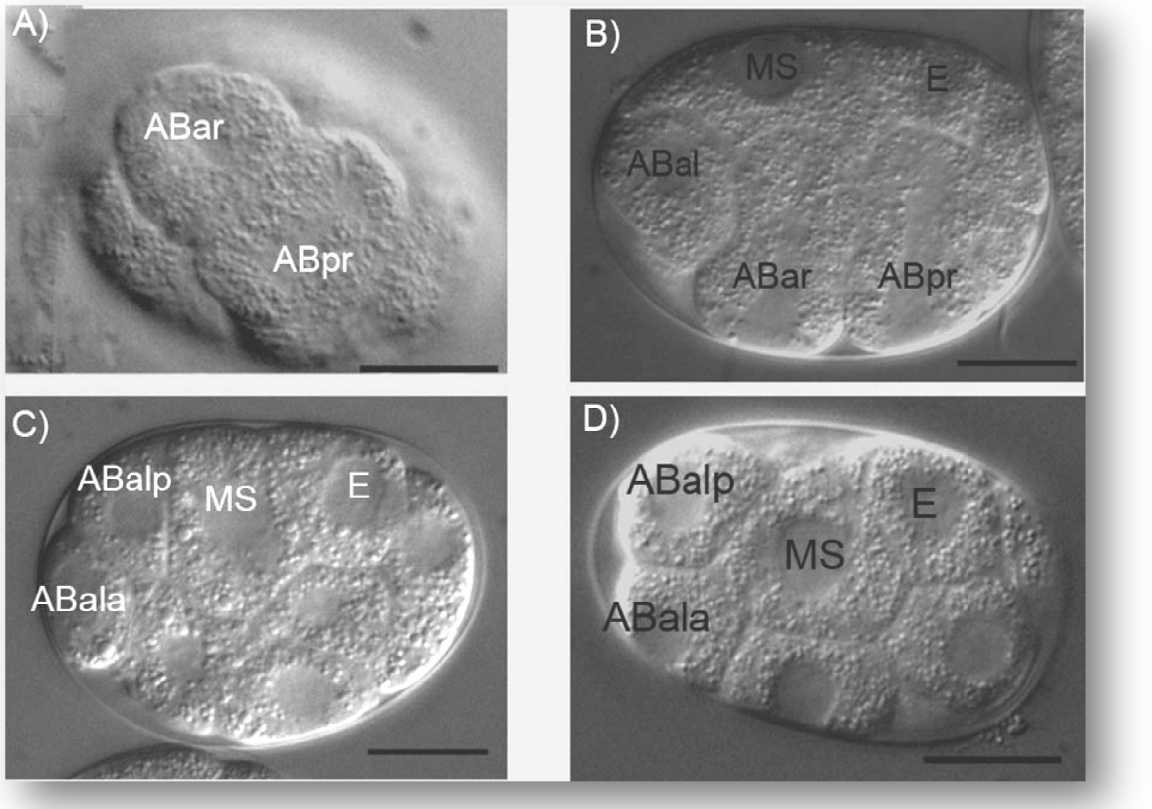


Figure 4. DIC micrograph of AB-8 staged embryos showing the additive cellular phenotypes in *lat-1; mom* double mutants. A, B) ABar and ABpr divide in transverse to each other in wild type embryos (A), while they divide in parallel division planes in *mom* mutants (B) (Park and Priess 2003). C) ABal divides to anterior ABala and posterior ABalp (*mom* mutants). Only ABalp has cell contacts with MS. In *lat-1;mom-2* double mutants, embryos exhibit a *mom-2* like ABar defect (B) and *lat-1* ABal division defect (D). Bars in A and D = 20uM, bars in B and C = 17uM

Further, *lat-1;mom-2* and *lat-1;mom-5* double mutants phenotype was additive, represented by *lat-1* specific ABal division defect and *mom-2* and *mom-5* specific ABar division defect (Figure 4). Interestingly however, the endoderm induction was more defective in *lat-1:mom-5* double mutants when compared to *mom-5* single mutants (36% and 20% respectively, refer to Figure 6). In addition, the ability to maternally rescue *mom-5* mutant embryos was compromised in *lat-1;mom-5* double heterozygous as indicated by the reduced number of UNC worms from the selfing of *mom-5* heterozygous mothers in a *lat-1* mutant background (Figure 5, see materials and methods). This phenomenon was neither observed with *mom-2* mutants nor was the endoderm induction defect increased in *lat-1;mom-2* double mutants (Figure 6). These data, combined with the defect in MS division observed in *lat-1* mutants, bring *lat-1* into close functional relationship with MS blastomere: it is possible to propose that LAT-1 could be required in MS to "prime" its responsiveness to Wnt signaling thus making MS responsive to spindle alignment Wnt signals (hence the MS division defect in *lat-1* mutants and the increased endoderm induction defect in *lat-mom-5* double mutants), and for MS to be able to pass through the Wnt signal to align to ABal mitotic spindle, or for MS to acquire a new polarising capacity which then orients the division plane of ABal blastomere (a proposition that would go in line with the suggestion reported in (Park and Priess 2003) referring to the existence of a Wnt-alternative signaling emanating from MS).

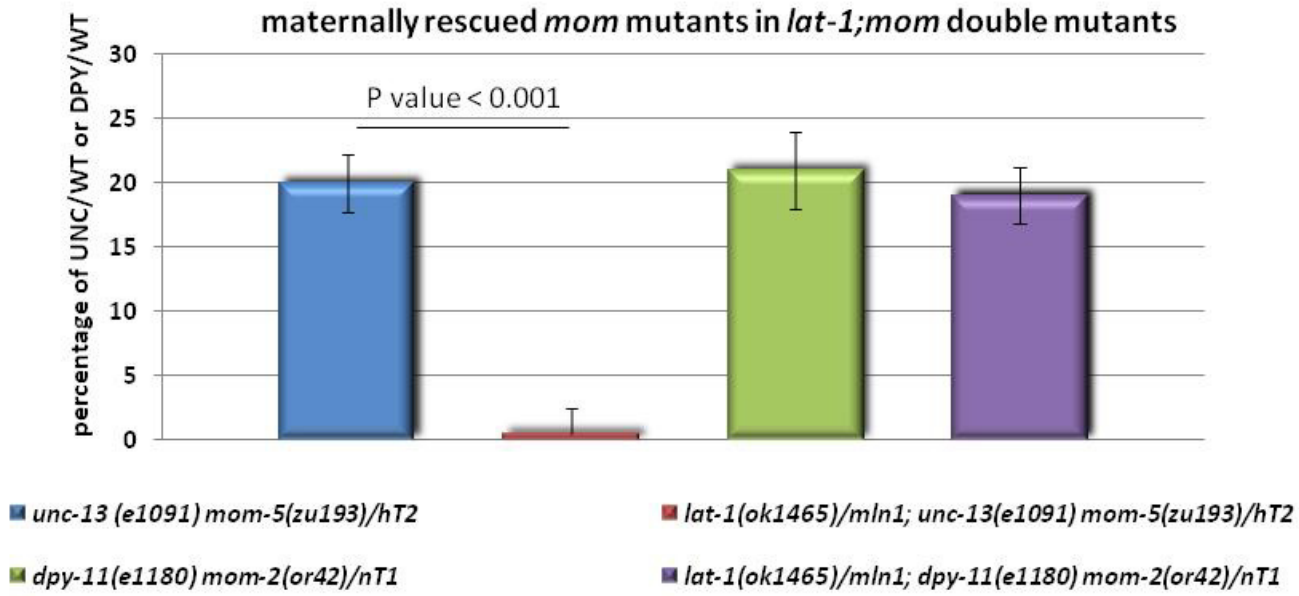
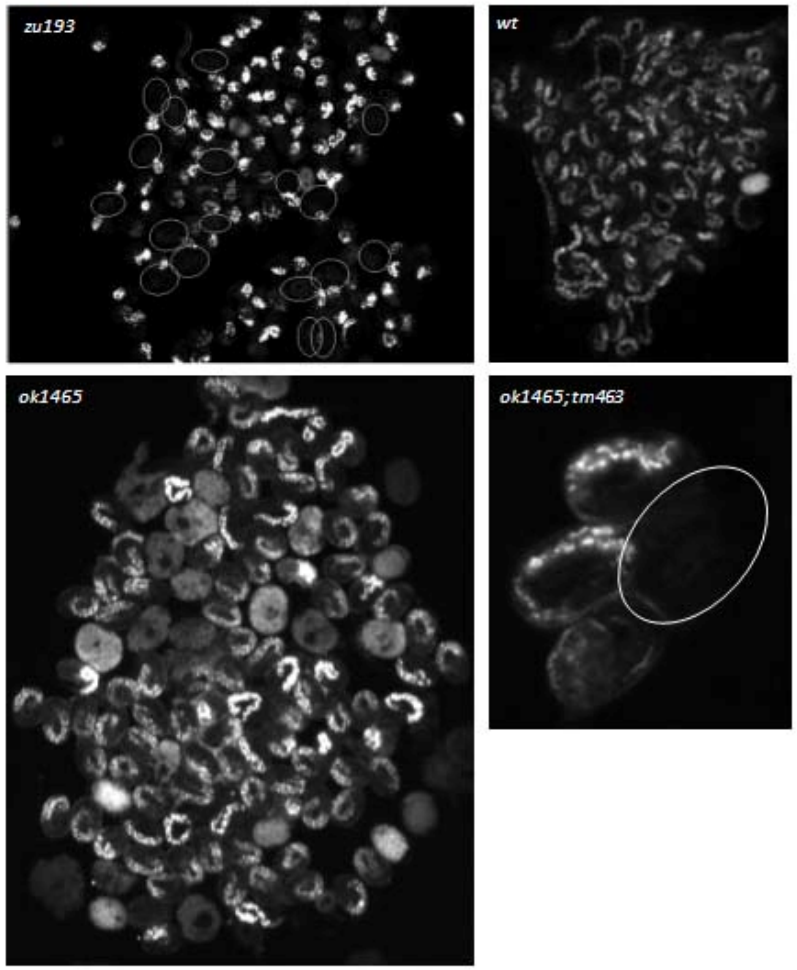


Figure 5. *lat-1* dosage negatively influences the capacity to maternally rescue *mom-5* mutants but not *mom-2* mutants. Column graph representing ratio of maternally rescued *mom* mutants. Y axis represents the percentage of UNC/WT and DPY/WT ratios. UNC morphological mutation is linked to *mom-5(zu193)* and DPY linked to *mom-2(or42)*. The ratio UNC/WT represents the ratio between maternally rescued *mom-5* homozygous (which appear UNC) and *mom-5* heterozygous (which appear WT). Similarly for *mom-2* mutation, DPY/WT represents a ratio between maternally rescued *mom-2* homozygous (which appear DPY) and *mom-2* heterozygotes (which appear WT). 1 in 5 self progeny of heterozygous *mom* mutants are maternally rescued *mom* homozygous: selfing of *mom-5* heterozygous mother (*unc-13(e1091) mom-5(zu193)/hT2*) produces one maternally rescued *mom-5(zu193)* (which appears UNC) in every five *mom-5* heterozygous (which appear WT). Similarly, selfing of *mom-2* heterozygous mother (*dpy-11(e1180) mom-2(or42)/nT1*) produces one maternally rescued *mom-2(or42)* (which appears DPY) in every 5 *mom-2* heterozygous (which appear WT). Accordingly, 20% of viable self progeny is maternally rescued homozygotes. In *lat-1;mom-5* double heterozygotes, the number of maternally rescued *mom-5* homozygous drops down dramatically, while the number of maternally rescued *mom-2* homozygous from *lat-1;mom2* double heterozygotes remains unchanged. The data suggest that the capacity to maternally rescue *mom-5* mutants is sensitive to the dosage of *lat-1*. Error bars= ±SEM. Statistical significance P value,t-test for was for statistics, only significant differences are shown.

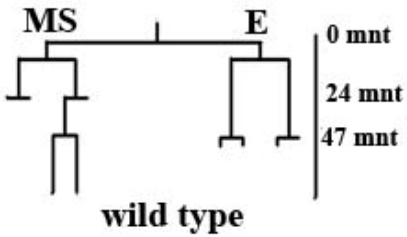
2.4. *lat-1* function is not required in the Wnt-dependent Endodermal fate induction

To further analyze the involvement of *lat-1* in Wnt signaling, we tested for the requirement of LAT-1 in Wnt/B-catenin mediated cell fate induction. We therefore analysed Endoderm induction in *lat-1* mutants and found that endoderm was correctly generated in *lat-1* mutants. In order to make sure that intestinal cells were origination from the E blastomere instead of an alternative ectopic E fate induction, we followed the cell lineage of the E blastomere and found that the posterior daughter of EMS divides in timing typical of E linage. Therefore, *lat-1* function is not required in the Wnt/B-catenin pathway in the induction of the endoderm fate in the posterior daughter of EMS blastomere indicating that *lat-1* does not interfere with the asymmetric division of EMS (Figure 6).

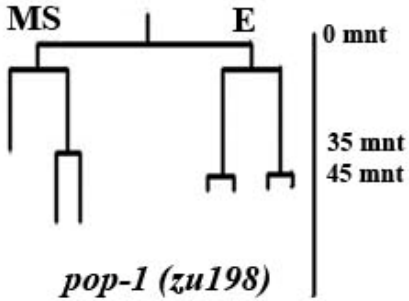
Analysis of mutations disturbing the spindle alignment of EMS led to the identification of an additional spindle alignment pathway suggested to function in parallel to the Wnt/Fz spindle alignment pathway. It was proposed that the Wnt/Fz signaling pathway plays an instructive role in aligning the EMS mitotic spindle whereas the Src tyrosine kinase pathway plays a permissive role in orienting the division plane. *mom-2/-5;src-1* double mutants result in a higher EMS spindle alignment defect than either single mutants, however, neither E nor MS division plane was affected in either genetic backgrounds (Walston and Hardin 2006). These results indicated that both Wnt/Fz and *Src/mes-1* pathways function in EMS spindle alignment. However, the absence of EMS spindle alignment defect in *lat-1* mutants and the absence of "worse" EMS spindle alignment defect in *lat-1;mom-2/-5* double mutants suggest that *lat-1* is not required in orienting the division plane of EMS, instead it is required solely in ABal and to a less extend in MS a-p division axis.



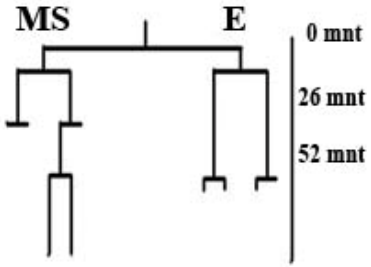
gene	allele	E specification defect
mom-5	*zu193*	20.23% (n=252)
Wild type	/	0% (n=175)
lat-1	*ok1465*	0% (n=152)
lat-1;lat-2	*ok1465;tm463*	2.8% (n=70)
lat-1; mom-5	*ok1465;zu193*	36% (n=58)



wild type



pop-1 (zu198)



lat-1(ok1465)

Figure 6. Descriptive analysis of endodermal induction in *lat-1* mutant embryos. Right panels show Rhabditin granules under polarized light (Rhabditin granules mark differentiated endoderm), embryos that do not look bright exhibit endoderm induction failure and are circled in white. Wild type (Wt) and *lat-1* mutants (*ok1465*) show no endoderm induction problem as all embryos look bright under polarized light. *mom-5* mutants (*zu193)* have a relatively high penetrance endoderm fate induction failure (20%). While *lat-1* and *lat-2* single mutants show no endoderm specification defect, *lat-1:lat-2* double mutants exhibit a low penetrance E specification defect (2.8%). Detailed percent penetrance of E specification defect is summarized in accompanying table. The embryonic cell lineage of MS and E blastomeres was followed to ensure endoderm marked by Rhabditin granules was produced from E blastomere and do not originate from ectopic expression of E-like cell fate.

2.5. *lat-1* functions in propagating the Wnt polarizing signals across the embryo

Based on the previous posterior polarizing signal hypothesis, our results suggest that *lat-1* function is required to propagate the Wnt polarising signal to reach more "remote" anterior embryonic cells. The ABal spindle orientation defect in *lat-1* mutant embryos could thus be due the inability of Wnt polarizing signals to reach to the anterior most ABal blastomere, which is in contact only with a "tertiary" signaling cell MS. MS in its turn exhibits a delay in spindle alignment, a cellular phenotype similar but not identical to the phenotype previously described in EMS blastomere when the Wnt signaling is disturbed (Schlesinger, Shelton et al. 1999; Bei, Hogan et al. 2002; Walston, Tuskey et al. 2004). A role of LAT-1 in cell polarity implicates a second evolutionary conserved subfamily of adhesion GPCRs in the control of cell polarity and morphogenesis (Lawrence, Struhl et al. 2007; Langenhan, Promel et al. 2009).

3. Investigating the potential polarizing role of LAT-1 and its requirement in MS.

Growing evidence suggests that a Wnt signal from P2, E and C blastomeres polarizes adjacent cells (EMS, and AB4 cells) and results in asymmetric cell division (e.g. asymmetric *pop-1* levels in daughter cells). A Wnt signal from these cells i.e. P2 and C and to a less extent MS also aligns the mitotic spindle of responding cells (EMS and AB4 cells) along the anterior-posterior axis (which also in line with the site of cell contact between signaling and responding cells) (Park and Priess 2003).

Interestingly, mutant mosaic blastomere recombination experiments (graft and host cells were terms used in (Park and Priess 2003) to designate signaling and responding cell respectively) strongly argue that the nature of the polarizing signal from MS is different (or at least less prominent) than the signal emanating from P2, E or C blastomeres (Park and Priess 2003). Blastomere recombination experiments showed that just like P2, both C and E blastomeres can align EMS mitotic spindle along the axis perpendicular to the site of cell contact. In contrast, MS was not able to align EMS spindle nor was it able to induce endodermal fate in the proximal cell (Park and Priess 2003).

Similar recombination experiments showed that none of the AB4 cells has the ability to align AB4 cells. In wild type embryos; ABal makes cell contact only with MS and AB3 cells (AB3 cells lack the ability to align the spindle of AB4 cells). ABal normally aligns its spindle towards MS suggesting that alignment cues orienting ABal division originate from MS. The nature of the signal from MS to align ABal spindle is not well understood (in fact it is still unknown because, as discussion in the previous section, in *mom-2/mom-5* mutants, the division plane of ABal was correct, meaning that in the absence of Wnt signaling from the cell adjacent to ABal, the division plane is not disturbed). Several lines of evidence propose that the MS signal to ABal is independent (or at least parallel) to Wnt signaling: in Wnt mutants (e.g. *mom-2* and *mom-5* mutants) ABal division defect is not observed (Figure 3).

Furthermore, ability of MS to orient AB4 cells is not compromised in *mom-2/-5* mutants, or to induce POP-1 asymmetry in AB8 cells (Park and Priess 2003). Similarly, MS in *mom-2* mutants was able to orient ABar spindle in addition to inducing POP-1 asymmetry in ABar daughter cells relative to MS indicating that the signal from MS is not entirely Wnt-dependent strongly suggestion that the polarising signal emanating from MS is not dependent upon Wnt pathway.

Just like P2 descendents can induce high/low POP-1 asymmetry in AB2 cells; AB2 cells induce low/high POP-1 symmetry in P2 descendents. Likewise, just like MS induces high/low POP-1 asymmetry in AB4 daughters, AB4 induces low/high POP-1 in MS daughters (Park and Priess 2003). These results strongly suggest a mutual influence between signaling and responding cells.

In view of this "induction-response-induction" hypothesis, LAT-1 could be required in MS to align ABal (hence the ABal division defect observed in *lat-1* mutants) and for MS to respond to spindle alignment cues (hence the delayed division in MS). *lat-1* could be "that" proposed "alternative to Wnt signaling" in MS, and the delay in MS spindle alignment in *lat-1* mutants can be explained by the requirement of LAT-1 in MS to respond to Wnt spindle alignment cues (MS eventually aligns along the a-p axis as the signal from E is strong enough to rescue MS alignment (Langenhan, Promel et al. 2009)), furthermore, if the E blastomere can polarize EMS and induce endodermal fate in proximal cell, it should be capable of orienting the spindle of MS. To further dissect the relation between *lat-1* function and Wnt-spindle alignment pathway, more efforts should be invested to analyse the localisation of Wnt signaling components and of LAT-1 in the presence and the absence of a Wnt signal. Additionally, blastomere recombination assay between *lat-1* and Wnt signaling mutants would provide important insights regarding the signaling capacities of blastomeres lacking LAT-1.

Taken together, our analysis of blastomere division in *lat-1*, *mom* and *pop-1* mutants show that *lat-1* function is required for the a-p division plane in the anterior blastomere ABal and that *lat-1* is also required for MS function as indicated by the delay in its division. Moreover, the implication of *lat-1* in MS is more evident in the increased endoderm induction defect in *lat-1;mom-5* double mutants. The correct ABal division in *pop-1* mutants strongly suggest that the function of *lat-1* in orienting the mitotic spindle is independent of new gene transcription in responding cell. In summary, analysing the embryonic lineage in *lat-1* mutant embryos provide evidence implicating the adhesion GPCR *lat-1* in cell polarity emphasizing the evolutionary conserved function of adhesion GPCRs in cell polarity. Furthermore, the experiment was initially designed to scrutinize *lat-1* mutant embryos for defects prior to the morphogenesis stage that would be at the origins of the morphological defects observed in *lat-1* arresting L1/embryos. Indeed, after following the embryonic cell lineage, Tobias Langenhan, observed that the defective cell contact of ABala later results in further aberrant cell contacts in descendents of ABala and consequent mistakes in the cell fates of later blastomeres (Tobias Langenhan, personal communication).

The ABal division defect observed in *lat-1* mutant embryos was the result of the lack of maternal *lat-1* gene product as homozygous embryos from heterozygous mothers did not harbour this ABal division defect (Tobias Langenhan, personal communication). However, despite the presence of maternal *lat-1* gene product, homozygous embryos from heterozygous mothers also arrest as larvae indicating that early maternal expression in not enough to rescue lethality and that later zygotic LAT-1 is also required for viability (zygotic rescue of *lat-1* lethality is discussed in chapter III, section 1.2.3). Accordingly, the lethality of *lat-1* mutant embryos is the combination of the lack of early maternal expression as well as zygotic expression. To further investigate the function of LAT-1 in polarity, epistasis analysis between components of the Wnt signalling pathway (in addition to *mom-2* and

mom-5 mutants) would help reveal the functional relationship between LAT-1 and the Wnt signalling pathway and the polarizing capacity of blastomeres in double mutants. It would be interesting to analyse the subcellular localisation of Wnt molecules (such as the MOM-2 Wnt ligand) and Wnt receptors such as MOM-5 Frizzled receptor in *lat-1* mutant embryos to see whether lack of LAT-1 would affect the polarization/distribution of polarity signal components. Similarly, to differentiate between the requirement of LAT-1 in propagating a Wnt polarising signal and the function of LAT-1 in inducing polarity (as an independent signalling pathway parallel to the Wnt signalling pathway), one can determine the subcellular localization of LAT-1 in Wnt signalling mutants and perform blastomere recombination experiments between Wnt mutant blastomeres and *lat-1* mutant blastomeres. Additionally, the ABala blastomere in *lat-1* mutants fails to distant itself from the MS blastomere which could also be the consequence of ectopic adhesion formed between ABala and MS in the absence of LAT-1. However, visualisation of GFP labelled mitotic spindles in *lat-1* mutants revealed that the lost a-p division plane in *lat-1* mutant is actually caused by failure of the mitotic spindle to orient itself in an a-p axis (work done by Tobias Lanhenhan). Nevertheless, it would be interesting to perform electron microscopy to gain better insights into the nature of the cell contact formed between ABala and MS balstomeres (e.g. passive cell contact which would be the result of aberrant cell positioning as opposed to ectopic cellular adhesion formed between ABala and MS balstomeres). The ABal division defect identified in this study, was also independently observed by Tobias langenhan while in a collaboration work with Prof. Ralf Schnabel in Germany, the confirmation of the polarity defect by a leading expert further encouraged us to investigate the function of *lat-1* in cell polarity and the effect of this aberrant division axis on the development of *lat-1* mutant embryos. In view of this, T. Langenhan analysed the lineage of blastomeres in *lat-1* mutant embryos and was able to detect reproducible changes in the cell fate in later blastomeres (details can be fined in the supplement materials in (Langenhan, Promel et al. 2009)). Work

thus conducted by Tobias langenhan not only confirmed the ABal division defect observed in this study but also helped linking early blastomere division defect to morphological abnormalities/lethality that appear later during embryogenesis.

4. Materials and methods

4.1. *C. elegans* strains. Worm strains were cultured and maintained according to standard procedures described by S. Brenner (Brenner 1974). Animals were cultivated at 22'C unless otherwise noted. The animals described as wild type were the *C. elegans* variety Bristol strain N2. All strains listed herein were obtained from CGC unless stated otherwise. In this chapter the following strains were used:

LG I: *pop-1(zu189) dpy-5(e61)/hT1 I; him-5(e1490)/hT1 him-5(e1490) V* [JJ1057] (Lin, Thompson et al. 1995). *pop-1 (zu189)* embryos were obtained by ripping open DPY mothers. *unc-13(e1091) mom-5(zu193)/hT2 I; +/hT2[bli-4(e937) let-?(h661)] III; him-8(ec56) IV* [EU459] (Thorpe, Schlesinger et al. 1997).

LG II: *lat-1(ok1465)* [XA7542] (Langenhan, Promel et al. 2009), lat *lat-1(ok1465)/mIn1[mIs14 dpy-10(e128)] II ; him-8(e1489) IV* [XA7567]. *lat-1(ok1465) lat-2(tm463)* [XA7557]. *mIn1[mIs14(pes-10p::gfp myo-2p::gfp) dpy-10(e128)] II* (Edgley and Riddle 2001) is used as a balancer and it carries an integrated semi-dominant pharyngeal GFP element, such that one copy of *mIs14* can be distinguished from two copies by GFP signal brightness (Edgley, Baillie et al. 2006). *mln1* was used to balance the chromosomal region carrying the *lat-1* and *lat-2* loci.

LG V: *dpy-11(e1180) mom-2(or42) V/nT1[let-?(m435)] (IV;V)* [EU384] (Thorpe, Schlesinger et al. 1997). *mom-2(or42)* embryos that had no maternal inheritance were obtained by picking DPY hermaphrodites and ripping them open using a needle syringe as described in the following section **4.5**.

4.2. Identifying and scoring blastomere division planes. The division axis of blastomeres was determined as the axis parallel to the mitotic spindle alignment plane. The

mitotic spindle was distinguished from other parts of the embryos based on its image texture. The nucleus and mitotic spindles have a smother granule-free appearance which makes it relatively easy to identify. The mitotic spindle alignment plane was obtained by drawing a straight line between centrosomes at a time point when the cytokenesis cleavage furrow starts to form. The obtained line thus determined the division axis of each of the analysed blastomeres. Occasionally ectopic cytokenesis furrow formation was observed in *lat-1* mutant embryos (data not shown). The division axis was then compared to the embryo's orientation and correlated with the embryos axis: anterior-posterior axis (a-p) was parallel to the line connecting the nuclei centre of the P1 and AB blastomeres, dorsal-ventral (d-v) axis was parallel to the line connecting the nuclei centre of EMS and ABp blastomeres an axis that is also in parallel to the initial cleavage of the AB blastomere (initiated parallel to the future dorsal-ventral axis of the embryo), left-right axis (l-r) was parallel to the line connecting the nuclei centre of ABxl and ABxr blastomeres and is also parallel to the initial AB daughters division plane (Sulston, Schierenberg et al. 1983).

Analysis of ABal division axis in *lat-1* mutant embryos (n=52) showed rather random division planes. The wild type a-p ABal division places ABalp in contact with MS blastomere whereas ABala retires to the anterior most side of the embryos with no cell contacts with MS. Abnormal ABal division planes (i.e. Non- a-p) observed in *lat-1* mutants occasionally placed the ABal daughter cells in the correct configuration (i.e. only ABalp contacting MS). Accordingly, the scoring of ABal division defect was based on the final ABalp and ABala configuration: division planes that placed both ABalp and ABala in contact with MS were scored defective, whereas embryos that had only ABalp in contact with MS were scored as wild type.

4.3. Scoring endodermal induction. Generation of endodermal tissue was scored using polarised light microscopy as described in (Marston, Roh et al. 2008). Differentiated

endoderm is marked by Rhabditin granules which can be visualized by looking at the embryos under polarised light. Polarised light was obtained by setting the analyser and the polariser perpendicular to each other to give maximum extinction, and the birefringent Rhabditin granules will appear as bright spots. Endodermal induction was used to analyse the Wnt/B catenin pathway that is responsible to induce endodermal cell fate in the E blastomere. In order to make sure that the endoderm produced was originating from the E blastomere instead of ectopic endodermal induction we followed the lineage of both MS and E blastomeres. Wild type E blastomere exhibits delayed division pattern as compared to MS. Mutants in which the E blastomere has lost its endodermal fate show a presumed E lineage pattern that is accelerated (that is the E blastomere and its descendents divide at earlier time points). The E lineage was thus used in parallel to confirm the endodermal cell fate in E blastomere.

4.4. The Generation of double mutants. Crossing scheme for generating *lat-1;mom-2* double mutants. The *him-8* locus as well as the *nT1 (IV.V)* translocation are omitted from the crossing scheme for simplicity and because there were not followed during phenotypic selection of desired genotypes.

Step1. *lat-1* balanced males were mated to *mom-2* balanced hermaphrodites:

P0. $\frac{lat\text{-}1(ok1465)II}{mln1}$ ♂ × $\frac{dpy\text{-}11(e1180)\ mom\text{-}2(or42)\ V}{nT1}$ ♀

F1.

$\frac{lat\text{-}1(+)II}{mln1}$; $\frac{dpy\text{-}11(e1180)\ mom\text{-}2(or42)\ V}{dpy\text{-}11(+)\ mom\text{-}2(+)}$

$\frac{lat\text{-}1(+)II}{mln1}$; $\frac{dpy\text{-}11(+)\ mom\text{-}2(+)}{nT1}$ V

$\frac{lat\text{-}1(ok1465)II}{lat\text{-}1(+)}$; $\frac{dpy\text{-}11(e1180)\ mom\text{-}2(or42)}{dpy\text{-}11(+)\ mom\text{-}2(+)}$ V

$\frac{lat\text{-}1(ok1465)II}{lat\text{-}1(+)}$; $\frac{dpy\text{-}11(+)\ mom\text{-}2(+)}{nT1}$ V

Step2. F1 outcross progeny males that were GFP positive (i.e. carried *mln1* balancer) were mated with F1 non-GFP hermaphrodites:

P0. $\frac{lat\text{-}1(+)II}{mln1}$; $\frac{dpy\text{-}11(e1180)\ mom\text{-}2(or42)}{dpy\text{-}11(+)\ mom\text{-}2(+)}$ V ♂ × $\frac{lat\text{-}1(ok1465)II}{lat\text{-}1(+)}$; $\frac{dpy\text{-}11(e1180)\ mom\text{-}2(or42)}{dpy\text{-}11(+)\ mom\text{-}2(+)}$ V ♀

$\frac{lat\text{-}1(+)II}{mln1}$; $\frac{dpy\text{-}11(+)\ mom\text{-}2(+)}{nT1}$ V ♂ × $\frac{lat\text{-}1(ok1465)II}{lat\text{-}1(+)}$; $\frac{dpy\text{-}11(+)\ mom\text{-}2(+)}{nT1}$ V ♀

Step3. GFP positive outcross progeny was singled out and the desired genotype (*lat-1(ok1465)/mln1; dpy-11(e1180) mom-2(or42)/nT1*) was selected from the F1 outcross progeny based on percent embryonic lethality from the selfing of F2 DPYs as described herein. We preferred to follow the F2 DPY percent embryonic lethality due to the embryonic lethality caused by the *nT1*-caused aneuploidy and because we did not know what to expect from *lat-1;mom-2* double heterozygotes. Only *mnl1/ok1465* is depicted in the F1 outcross progeny for simplicity. *mln1/ok1465* genotype was verified by genotyping as described in later sections. The F2 DPYs were singled out and the embryonic lethality of each DPY selfing was counted. The original plate from which the DPYs were picked was only kept if none of the singled out DPY gave 100% viability.

F1. *lat-1(ok1465)II* / *mln1* ; *dpy-11(e1180) mom-2(or42)* *V* / *dpy-11(+) mom-2(+)* ⟹ selfing throws DPYs that would be:

1) *mln1/ok II; e1180 or-42 V* (selfing 100% emb.let)
2) *mln1II; e1180 or-42 V* (selfing 100% emb.let)
3) *mln1II; e1180 or42/wt V* (selfing 0% emb. let)

lat-1(ok1465)II / *mln1* ; *dpy-11(e1180) mom-2(or42)* / *nT1* *V* ⟹ selfing throws DPYs that would be:

1) *mln1/ok II; e1180 or42(I.V)* (selfing 100% emb.let)
2) *mln1 II; e1180 or-42 V* (selfing 100% emb.let)
3) *mln1II; e1180 or42/nT1(I.V)* (selfing> 60% emb. let)

lat-1(ok1465)II / *mln1* ; *dpy-11(e1180) mom-2(or42)* *V* ⟹ selfing produces 100% emb. let

lat-1(ok1465)II / *mln1* ; *dpy-11(+) mom-2(+)* *V* ⟹ selfing throws DPYs that are 100% viable (*mln1*)

lat-1(ok1465)II / *mln1* ; *dpy-11(+) mom-2(+)* *V* / *nT1* ⟹ selfing throws DPYs 75% (viable *mln1*)

lat-1(ok1465)II / *mln1* ; *nT1 (I.V)* or *nT1/wt* ⟹ these are not viable due to aneuploidy

4.5. Isolating *lat-1(ok1465);mom-2(or42)* embryos. First *lat-1(ok1465)II; dpy-11(e1180) mom-2(or42) V/nT1[let-?(m435)] (IV;V)* were isolated from the generated double heterozygous strain *lat-1(ok1465)II/mln1; dpy-11(e1180) mom-2(or42) V/nT1[let-?(m435)] (IV;V)* by picking non-GFP hermaphrodites under the dissecting microscope fitted with UV-illumination. Isolated non-GFP hermaphrodites were then incubated at 22'C and, self maintaining stable lines were selected. DPY hermaphrodites were picked (remember that *mom-2(or42)* is linked this DPY morphological mutation) and embryos were released from uterus by ripping these DPY hermaphrodites open using a syringe needle directly on the microscope slide.

4.5.1. Crossing scheme for generating *lat-1;mom-5* double mutants. The *him-8* locus as well as the *hT2 (I.III)* translocation are omitted from the crossing scheme for simplicity:

Step1. *lat-1* balanced males were mated to *mom-5* balanced hermaphrodites:

P0 $\frac{lat\text{-}1(ok1465)II}{mln1}\,\male \times \frac{unc\text{-}13(e1091)\ mom\text{-}5(zu193)\ I}{hT2}\,\female$

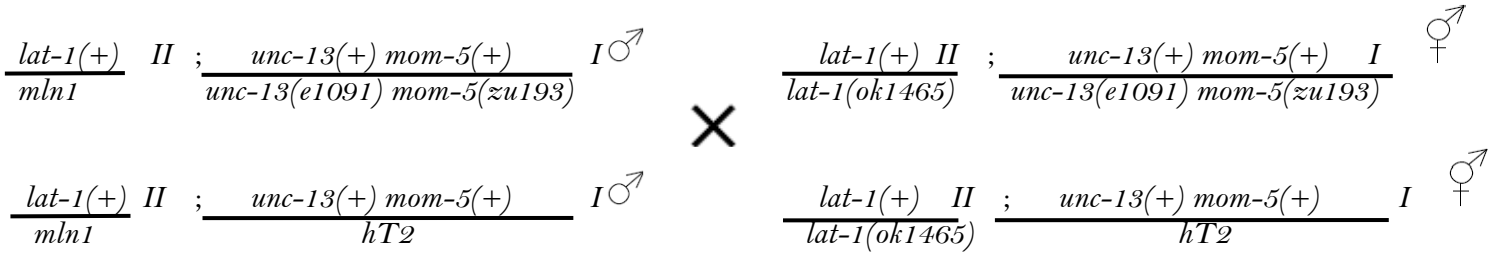
F1. $\frac{lat\text{-}1(+)}{mln1}\ II\ ;\ \frac{unc\text{-}13(+)\ mom\text{-}5(+)}{unc\text{-}13(e1091)\ mom\text{-}5(zu193)}\ I$

$\frac{lat\text{-}1(+)}{mln1}\ II\ ;\ \frac{unc\text{-}13(+)\ mom\text{-}5(+)}{hT2}\ I$

$\frac{lat\text{-}1(+)}{lat\text{-}1(ok1465)}\ II\ ;\ \frac{unc\text{-}13(+)\ mom\text{-}5(+)}{unc\text{-}13(e1091)\ mom\text{-}5(zu193)}\ I$

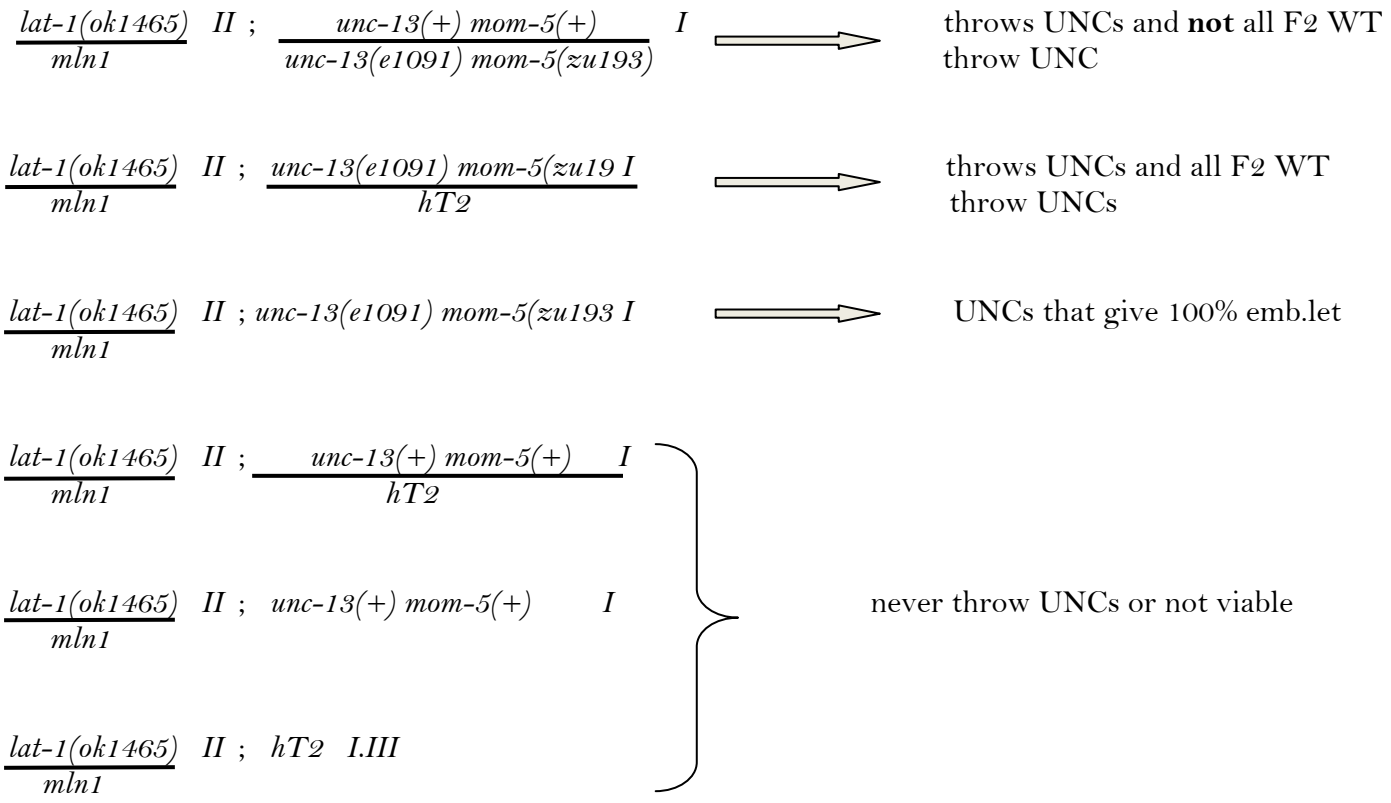
$\frac{lat\text{-}1(+)}{lat\text{-}1(ok1465)}\ II\ ;\ \frac{unc\text{-}13(+)\ mom\text{-}5(+)}{hT2}\ I$

Step2. F1 outcross progeny males that were GFP positive (i.e. carried *mln1* balancer) were mated with F1 non-GFP hermaphrodites:



Step3. GFP positive outcross progeny was singled out and the desired genotype (*lat-1(ok1465)/mln1; unc-13(e1091) mom-5(zu193)/hT2*) was selected from the F1 outcross progeny based on the segregation of UNC phenotype in the F2s as described herein. Only *mnl1/ok1465* is depicted in the F1 outcross progeny for simplicity. *mln1/ok1465* genotype was verified by genotyping as described in the section 4.1.9 of chapter III. The F2 WTs were singled out from plates that throw UNCs and their ability to throw UNC worms was followed throughout F3 generation. The original plate from which the WT were picked was only kept if all of the singled out WT throw UNC worms.

F1.


lat-1(ok1465) II / mln1 ; unc-13(+) mom-5(+) I / unc-13(e1091) mom-5(zu193)
throws UNCs and **not** all F2 WT throw UNC
lat-1(ok1465) II / mln1 ; unc-13(e1091) mom-5(zu19 I / hT2
throws UNCs and all F2 WT throw UNCs
lat-1(ok1465) II / mln1 ; unc-13(e1091) mom-5(zu193 I
UNCs that give 100% emb.let
lat-1(ok1465) II / mln1 ; unc-13(+) mom-5(+) I / hT2
lat-1(ok1465) II / mln1 ; unc-13(+) mom-5(+) I
lat-1(ok1465) II / mln1 ; hT2 I.III
never throw UNCs or not viable

4.6. **Isolating *lat-1(ok1465),mom-5(zu193)* mutant embryos**. First *lat-1(ok1465)* mutants were isolated from the generated strain *lat-1(ok1465)/mln1; unc-13(e1091) mom-5(zu193)/hT2 I; +/hT2[bli-4(e937) let-?(h661)]* by picking non-GFP under the dissecting microscope fitted with UV-illumination. Isolated non-GFP hermaphrodites were then incubated at 22'C and, self maintaining stable lines were selected. UNC hermaphrodites were picked (remember that *mom-5(zu193)* is linked this UNC morphological mutation) and embryos were released from uterus by ripping these UNC hermaphrodites upon using a syringe needle.

4.7. **Counting maternally rescued *mom-2* and *mom-5* mutants.** L4 hermaphrodites were singled out, incubated at 22'C and transferred daily to freshly seeded plates. The self progeny was incubated at 22'C and counted after 3 days or until adult stage. Selfing of *lat-1;mom-5* double heterozygotes [*lat-1(ok1465)/mln1; unc-13(e1091) mom-5(zu193)/hT2 I; +/hT2[bli-4(e937) let-?(h661)]* generates double heterozygotes that appear WT, maternally rescued mom-5 that are UNC and dead eggs. Self progeny of 32 *lat-1;mom-5* double heterozygote hermaphrodites were counted, and the ration UNC/WT represents the average of all UNC/WT (n=1256 F1 self progeny of 32 *lat-1;mom-5* double heterozygote mothers). Similarly, selfing of *lat-1;mom-2* double heterozygotes generates double heterozygotes that appear WT, maternally rescued *mom-2* homozygous (that appear DPY) and dead eggs. The DPY/WT ratio was the sum of all F1 self progeny counts (n=1943) from 43 hermaphrodites.

III. Chapter III: Phenotypic Characterization of *lat-1* mutants: *lat-1* mutants exhibit several morphological defects

1. Embryonic development defects in *lat-1* mutant.

1.1. Brief introduction.

The *C. elegans* embryo starts its development following fertilization with a series of cell division in a spatial-temporal reproducible division pattern (referred to as embryonic cell lineage). Embryonic cell division can be divided into two major groups: 1) **proliferation** and 2) **organogenesis/morphogenesis**. During proliferation, embryonic cells divide to produce approximately 550 cells. The first half of the proliferation phase governed by a complex network of inter and intracellular signaling pathways, leads to the generation of the founder cells (precursor cells that will give rise to specialised cells such as hypodermal, muscle, intestinal and germ line cells), whereas the second half is governed by bulk of cell division and highly controlled short range cell migration: **gastrulation** which marks the beginning of morphogenesis (Altun 2005).

Morphogenetic events transform the embryo from its spherical shape to a tube-like vermiform. Morphogenesis is achieved by extensive arrangement of epidermal cells throughout three morphological events: dorsal intercalation, ventral enclosure and elongation (Figure 7).

Like most morphogenesis events, the dynamic events of epithelial cell rearrangements are under transcriptional control. *die-1* encodes a C2H2 zinc finger transcription factor and is expressed in the nucleus of hypodermal, pharyngeal and muscle cells. *die-1* mutant embryos initiate but fail to complete the process of dorsal intercalation. In *die-1* homozygous embryos from mutant mothers, epidermal cells fail to migrate towards the dorsal mid line of the embryo despite the correct extension of basal protrusions. Interestingly, nuclear migration was reported to be functionally irrelevant to complete

dorsal intercalation, and that for dorsal intercalation to succeed, actin-rich filaments must be able to extend towards the midline and to make cell adhesions with the contralateral cells (Williams-Masson, Heid et al. 1998). Genetic evidence suggests that the early and late stages of dorsal intercalation (Figure 7) are genetically distinguishable. For instance, posterior dorsal cells in *die-1* mutant embryos fail to intercalate while the subsequent morphogenic rearrangement and dorsal cell fusion still take place (Williams-Masson, Heid et al. 1998). Several other studies proved the crucial role of cytoskeleton integrity in dorsal intercalation and ventral enclosure. For instance, mutations disturbing ventral enclosure and dorsal intercalation were associated with defects in the organisation of the actin filaments, and defects affecting actin-based motility such as mutations in *die-1*, SCAR/WAVE *wve-1*, *evl-20* and mutations affecting the Apr-2/Apr-3 complex (Heid, Raich et al. 2001) (Withee, Galligan et al. 2004) (Williams-Masson, Heid et al. 1998) (Antoshechkin and Han 2002).

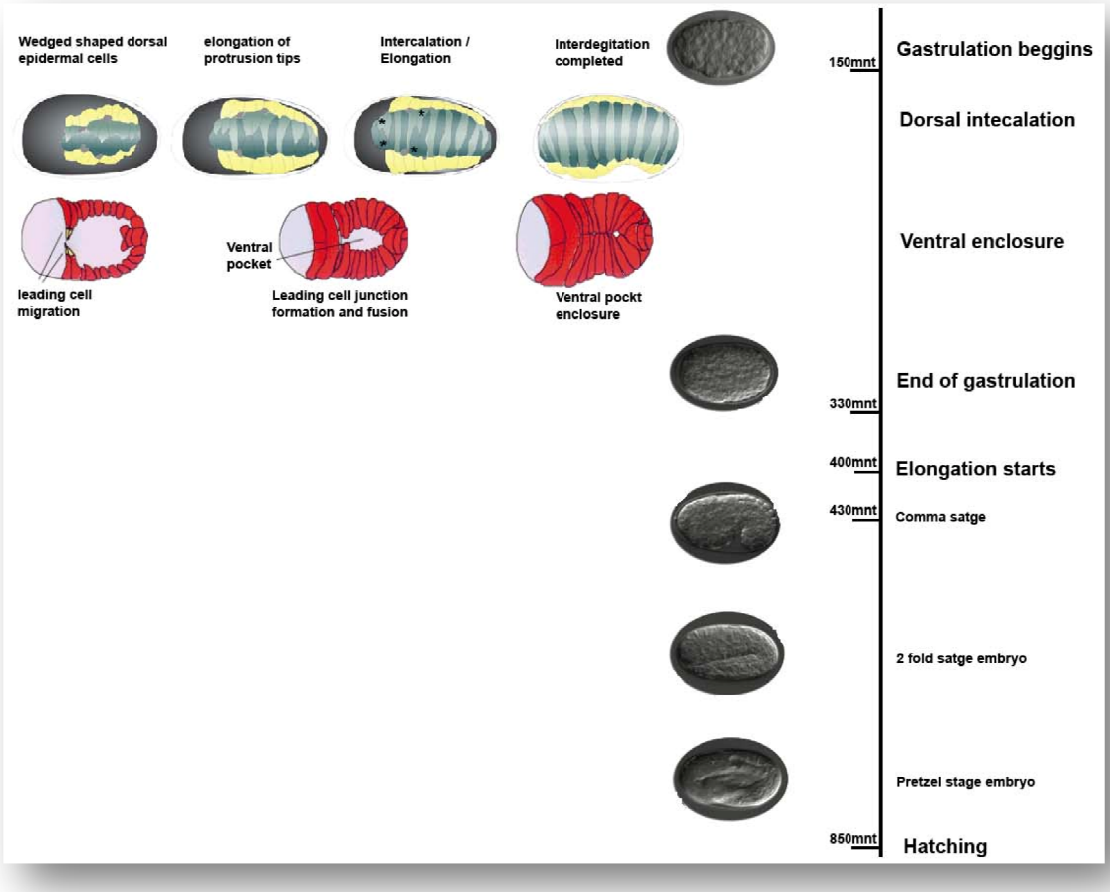


Figure 7. Schematic representation of embryonic developmental stages in *C. elegans*. DIC photograph of developing embryo are depicted against the time line of corresponding developmental stage at 22'C relative to first cleavage. Following fertilization, the embryo enters the proliferation stage during which founder cells are born. Gastrulation stage begins at approximately 150mnt following the first cleavage. Cells giving rise to internal tissues migrate to the midline of the embryo before they ingress into the inside of the embryo. Other short range cell migration and morphogenetic changes take place during dorsal intercalation and ventral enclosure. The initial dorsal intercalation event begins as dorsal epidermal cells (two rows of ten cells each) adopt a wedged shaped appearance before they start extending basal actin-rich processes towards the mid line. Dorsal intercalation completes when intercalating contralateral epidermal cells meet at the dorsal midline to form a single row of cells which later will fuse to form dorsal syncytium (not shown). The lateral most epidermal cells also migrate towards the ventral side of the embryo (ventral intercalation). Leading cells migrate first followed by pocket cells. These cells meet at the ventral side to contact and fuse with each other, thus sealing up the ventral cleft. Figure modified from (Simske and Hardin 2001; Altun 2005; Chisholm December 01, 2005).

In order for dorsal intercalation and ventral closure to proceed properly, the migrating cells must be correctly generated, able to extend basolateral filopodia filaments and able to migrate and form adhesions with contralateral cells. Mutations affecting the hypodermal cell fate specification cause failure in epidermal morphogenesis, such is the case in *elt-1* and *lin-26* mutants. These two genes control epidermal cell fate specification and maintenance of epidermal aspects respectively, and mutations cause dorsal intercalation and ventral closure failure (Page, Zhang et al. 1997; Gilleard and McGhee 2001). In addition, cell signaling between the underlying neuronal substrates and migrating epithelial cells, occupies a decisive function during epidermal cell migration as indicated by genetic analysis of the Efn/Eph receptor pathway which defines the migratory path of the epidermal cells during ventral enclosure. The embryonic phenotypes associated with mutation in the *vab-1* (variable abnormal) or efn (ephrin) ligands include enlarged and/or maintained ventral cleft, and failure of leading edge cells to migrate and/or failure of apical junction (AJ) formation (George, Simokat et al. 1998; Chin-Sang, George et al. 1999; Chin-Sang, Moseley et al. 2002). Rho-GTPase mediated cytoskeleton regulation is a downstream target of Eph signaling, again emphasising the importance of cytoskeleton integrity in epidermal morphogenesis (Schmucker and Zipursky 2001), for a detailed description of epidermal morphogenesis regulation, please refer to the epidermal morphogenesis chapter in wormbook (Chisholm December 01, 2005).

1.2. Results and discussion

1.2.1. *lat-1(ok1465)* causes embryonic lethality and larval arrest

lat-1 mutants exhibit a relatively high penetrant embryonic lethal phenotype. In an attempt to better understand the embryonic defect associated with *lat-1* mutation and the relationship between the ABal division defects and later morphological abnormalities, we designed an experiment to identify the stages during which *lat-1* mutant embryos arrest and

found that *lat-1* mutant embryos arrest at different stages of development and exhibit diverse embryonic morphological abnormalities. However the majority of arresting embryos are observed at different fold stages (2-fold, 3-fold and 4-fold stage, Figure 8).

In scoring the embryonic lethality of *lat-1* mutants, the XA7542 strain was used (*lat-1(ok1465)*). As stated in chapter I section 4, *lat-1* mutant strain was obtained by selecting viable homozygous *lat-1* mutants from the progeny of heterozygous *lat-1* hermaphrodites. Although chromosomal deficiencies that span the *lat-1* locus failed to complement *lat-1*, and although no *lat-1* transcript was detected in *lat-1* mutants, I used lat-1(RNAi) by feeding as an alternative method to the *lat-1* embryonic lethality (the RNAi feeding clone was a gift from Alison Wollard lab). *lat-1(RNAi)* experiments did cause embryonic lethality and larval arrest, but the results were sometimes irreproducible and variable from one replicate to another. The variability in RNAi results was observed each time the experiment was perfomed (*lat-1(RNAi)* experiment was also tried in the temperature sensitivity assay, and similarly, the results were variable).

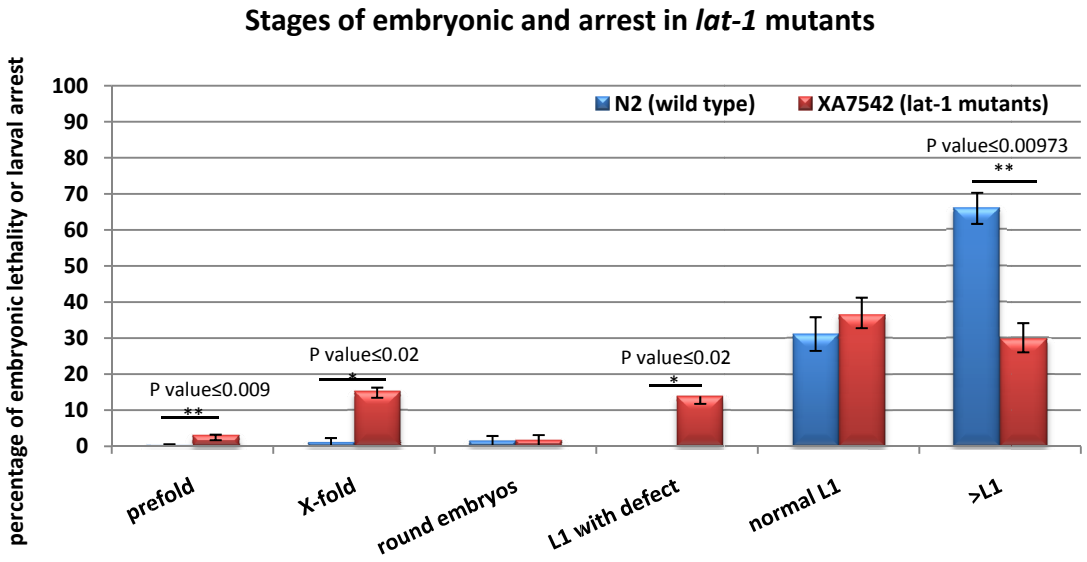


Figure 8. Developmental stages of embryonic arrest in *lat-1* mutants. Under our experimental conditions, 73% of arresting embryos are at 3/4-fold stages as judged by their appearance under the light microscope (pretzel shape). A small percentage of *lat-1* mutant embryos (strain XA7542) have a round rather than spherical shape. This round embryo phenotype is not very well understood, and although several round embryos do hatch, the majority of hatched larvae from round embryos have sever morphological defects or arrest development at the L1 stage (data not shown). The presence of >10% of L1 with morphological defects reflects that embryonic defects may not completely prohibit embryogenesis but are reflected later after hatching. The X-fold is used to refer to different fold-stages. Error bars= ±SEM. P value t-test is indicated on the top of each developmental stage, only statistically significant differences are shown.

1.2.2. Effect of temperature on *lat-1* mutants.

As *lat-1* encodes for a G protein coupled receptor (GPCR) we speculate that it could function in cell signaling-mediated coordination of embryogenesis. Additionally, the rather diverse (in severity) but reproducible morphological defects observed in *lat-1* mutants led us to hypothesize a role for LAT-1 in morphogenesis. Several cell signaling genes that function during embryogenesis and which mutations are associated with phenotypes similar to those observed in *lat-1* mutants (embryonic arrest, embryonic and larval morphological defects, larval arrest, reduced pharyngeal pumping, vulva defects and reduced brood size) show a temperature sensitive embryonic lethality (Walker, Gower et al. 2002; Thomas-Virnig, Sims

et al. 2004; Walker, Ly et al. 2004; Vazquez-Manrique, Nagy et al. 2008). Accordingly, we tested *lat-1* mutants for temperature sensitivity (Figure 9). Interestingly, *lat-1* mutant embryos were not sensitive to cold as is indicated by the absence of statistically significant difference between embryonic lethality at 15'C and 20'C (Figure 9). Although most arresting *lat-1* mutant embryos are observed at the 3/4-stage (Figures 8 and 9), at colder temperature, *lat-1* mutant embryos seem to be affected at an earlier embryonic stage earlier than either 2-fold, 3-fold or 4-fold stage: the pre-fold stage (Figure 9). Although the overall embryonic lethality was not affected by temperature change, the distribution of arresting/affected developmental stages show slight differences as indicated by the increase of pre-fold arresting embryos at 15'C as opposed to 20'C. More interesting is the nearly two-fold increase of L1 arresting larvae with defects at 15'C as compared to 20'C (13.9% and 7.8% L1 with defects at 15'C and 20'C respectively, Figure 9).

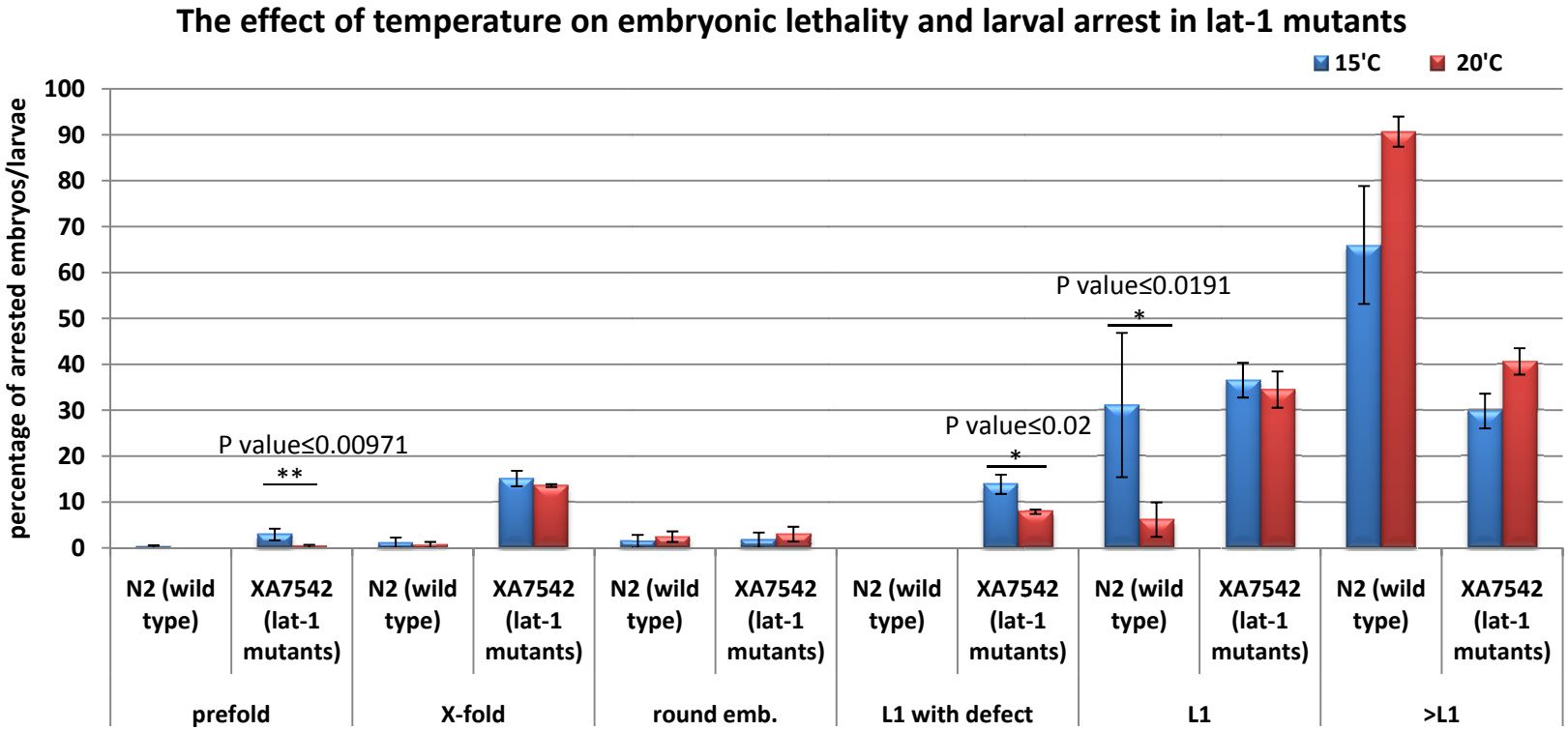


Figure 9: Effect of temperature on embryonic lethality and larval arrest in *lat-1* mutants. The following strains were used: wild type N2, *lat-1* mutants (XA7542). Temperature change did not affect the global percent embryonic lethality but did affect the distribution of the stage of developmental arrest. More pre-fold *lat-1* mutant embryos arrested at 15'C compared to 20'C and lower temperature caused more morphological defects in arresting L1 larvae. In *lat-1* mutants the percentage of L1 arrest without morphological defect was not affected by temperature proving that the mutants were allowed enough time to develop at lower temperature knowing that the latter slows down the rate of development. On the other hand, *lat-1 lat-2* double mutants do not appear to be affected by changes in temperature in the same way as *lat-1* single mutants do: L1 morphological defects were equally penetrant at 15 and 20'C and the overall distribution of stages of arrest were not affected neither. More L1 arrest was observed in *lat-1 lat-2* double mutants when compared to *lat-1* single mutants. Bars represent percent defects ± SEM. P-values t-test was used for the statistics; only P values of statistically significant differences are shown.

Bearing in mind the slow development phenotype of *lat-1* mutant embryos (section **1.2.5** of this chapter), the embryos were allowed to develop over a period of time that is enough to avoid over estimation of arrest caused by temperature-influenced growth rate. In view of this, the percent distribution of stage of developmental arrest is caused by the effect of temperature upon the mutants and not the affect of temperature on growth rate in general. In accordance with this statement, if the arrest observed in *lat-1* mutants was the cause of additive affect between low temperature-caused slow growth rate and *lat-1* mutants slow growth rate phenotype (in other words, low temperature slowing down the already slow development rate of *lat-1* mutants) then one would expect to see a difference in arresting L1 larvae at 15'C and 20'C. Conversely, temperature had no effect on the percentage of L1 arrest, confirming that the scored phenotypes are the consequence of lack of *lat-1* function at different temperature conditions.

The differences in pre-fold arrest and L1 with morphological defects at 15'C and 20'C led us to suggest two possible explanations. First is that at lower temperatures the growth rate slows down and consequently the embryos develop in an environment that is not challenged by time restrains and fast-happening developmental events, in other words, the mutant embryos are given more time to cope with their defects. However, this rather superficial solution later proves insufficient and the earlier "masked" embryonic defects appear later after hatching as indicated by the two-fold increase in L1 with defects at 15'C as opposed to 20'C. Second, the slight difference in the distribution of defects as affected by temperature suggests that LAT-1 might exert different functions at different stages: an early embryonic stage that is not affected by temperature and a later embryonic/larval stage during which LAT-1 function is impinged on by temperature. In veiw of the second explanation; *lat-1* mutants show a maternal effect lethality, and the mutation causes defects at different developmental stages: embryonic arrest, larval arrest with/without morphological defects, defects at later larval stages .e.g. vulva morphological defects at L4 stage, adult defects .e.g.

gonad migration and axon guidance defects, in addition to other functional abnormalities such as pharyngeal pumping and sensitivity to inhibitors of acetylcholinesterase.

The same temperature sensitivity assay was perfomed using RNAi as an alternative approach, but the results were variables from one replicate to another, and thus were not included in the results. The fact that the overall embryonic lethality was not affected by temperature invits to suggest that *lat-1* function is not affected by temperature, although in this experiment, only two conditions were used (15'C and 20'C), and in order to completly rule out the possibility that *lat-1* function is affected by temperatue, the same experiment should be repeated this time using an elevated temperature (25'C), as it might be that the mutants are sensitive to heat but not cold.

1.2.3. Zygotic and maternal *lat-1* expression is required for *C. elegans* development.

In order to test the second justification; that is LAT-1 is also required at later stages of embryogenesis and during larval development, we tried to rescue some of the *lat-1* lethality through zygotic expression of *lat-1*. Accordingly, the experiment was performed whereby zygotic *lat-1* expression was provided by wild type *lat-1* males. Interestingly, we found that zygotic expression of LAT-1 rescues to some extent the lethality caused by lack of maternal LAT-1 as indicated by the increase in the number of worms from *lat-1* mutant mothers reaching the adult stage (Figure 10).

This finding suggests that maternal LAT-1 is required for viability and that the early embryonic defects caused by lack of maternal LAT-1 can be "corrected" to some extent by zygotic expression of LAT-1. Furthermore, the results suggest that the lethality caused by *lat-1* mutation is due to the combination and accumulation of defects caused by lack of early maternal *lat-1* expression and also the lack of later embryonic/larval expression of wild type LAT-1. Additionally, this result allowed us to further refine the maternal effect of *lat-1* to

"partial maternal effect" as lethality can be partially rescued by paternal contribution (see material and methods for this chapter).

In combination with the ABal division defect discussed in chapter II, lack of maternal *lat-1* gene product causes defects in the division axis of the ABal blastomer, and the lack of zygotic *lat-1* gene product also seems to contribute to the lethality observed in *lat-1* mutants. Whether *lat-1* exerts similar functions in early and late-stage embryos in not known and this study was not sufficient to answer this question. It would be interesting the dissect the requirement of *lat-1* in early and late-stage embryos, or to further characterise the zygotic contribution to viability by comparing for example embryonic cell lineage of *lat-1* mutant embryos lacking maternal *lat-1*, embryos that have maternal *lat-1* gene product and embryos that show zygotic expression (embryos of paternally rescued outcross progeny).

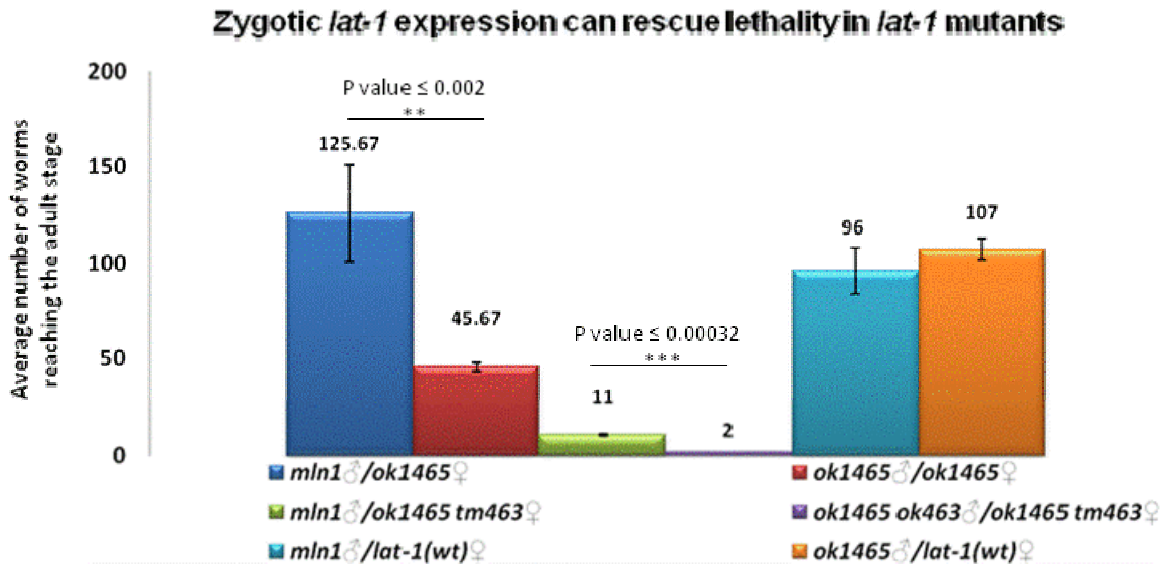


Figure 10: zygotic expression of *lat-1* can rescue lethality in *lat-1* mutants. Chart bars represent the averaged number of worms reaching the adult stage in each genotype. The average number of worms reaching the adult stage in *lat-1* mutants from mutant mothers (*ok1465♂/ok1465♀*, red bar) is 45 under these experimental conditions (see materials and methods). Paternal contribution of LAT-1 through mating (*mln1♂/ok1465♀*, dark blue bar) increased the number of adults from mutant mothers (from 45 to 126), indicating that zygotic expression rescued to some extent the partial maternal effect lethality. Early maternal expression is required for early embryogenesis and zygotic expression was not able to rescue the early embryonic ABal division defect (Tobias Langenhan, personal communication). However, combining late larval and adults' defects caused by *lat-1* mutation, with paternal zygotic rescue of *lat-1* lethality indicate that *lat-1* late embryonic/larval expression can correct early defects and rescue lethality. *mln1 [dpy-10(e128) mIs14]* is a chromosomal inversion rearrangement that effectively balances for the centre portion of LGII from *lin-31* through *rol-1* including *lat-1* and *lat-2*. It is also carries an integrated pharyngeal GFP element allowing for the identification of outcross progeny (see materials and methods). *ok1465* and *tm463* are loss of function alleles of both *lat-1* and *lat-2* respectively (Langenhan, Promel et al. 2009). Error bars = ±SEM. P value t-test was used for statistics; onyl statistically significant changes are indicated.

1.2.4. *lat-1* mutants show ventral enclosure defect.

Following the determination of the different stages of developmental arrest caused by lack of LAT-1, we analysed live recordings of developing embryos at high magnifications in the hope to determine the cause of elevated 3/4-fold arrested embryos over other stages of development (see Figure 8). Compared to N2 controls, *lat-1* mutant embryos from mutant mothers hatched in twice the time period (18-21hrs and 12-13hrs in *lat-1* mutants and wild type controls respectively, data not shown). Initial analysis of early blastomere divisions did not reveal any aberrations in *lat-1* mutant embryos. However, *lat-1* mutant embryos show low penetrance in ventral enclosure defect where the ventral cleft is wider and persistent (Figure 11). Moreover, the degree of severity in ventral cleft closure was different from one embryo to the other. In embryos where ventral cleft closure was severly affected, the embryos were unable to initiate ventral closure and elongation and were arrested without completing morphogenesis (pre-fold stage). In this category of embryos, it was possible to tell apart specialised tissues such as gut and hypodermal tissues (as judged by the morphology of cells). However, the differentiated tissue types were rather compartmented and internal organs were exposed including the pharynx and the gut (Figure 11, panels M and O) due to ventral rupture. Less sever ventral closure defects (affecting most embryos with ventral closure abnormalities) allowed the embryo to precede to the elongation step, whoever earlier minor defects in ventral closure can cause elongation arrest and/or morphological defects in early larvae, explaining thus the elevated number of 3/4-fold arrested embryos and larvae with morphological defects. Similarly, several studies indicated that early morphogenesis defects are reflected in abnormalities hitting later morphogenesis events. For instance, failure of pharyngeal precursor cells to ingress during gastrulation results in ventral cleft failure and elongation defects, presumably reflecting a defective substrate (Mango, Lambie et

al. 1994). Additionally, abnormal neuroblasts movement towards the ventral cleft prior to the initiation of epidermal ventral enclosure leads to elongation and severe morphological defects as shown in VAB-1 and *efn* mutants (Chin-Sang, Moseley et al. 2002) (George, Simokat et al. 1998) (Wang, Roy et al. 1999). These observations led us to propose that the elevated 3/4-fold stage embryonic arrest is probably the results of earlier embryonic defect such as defects in ventral enclosure.

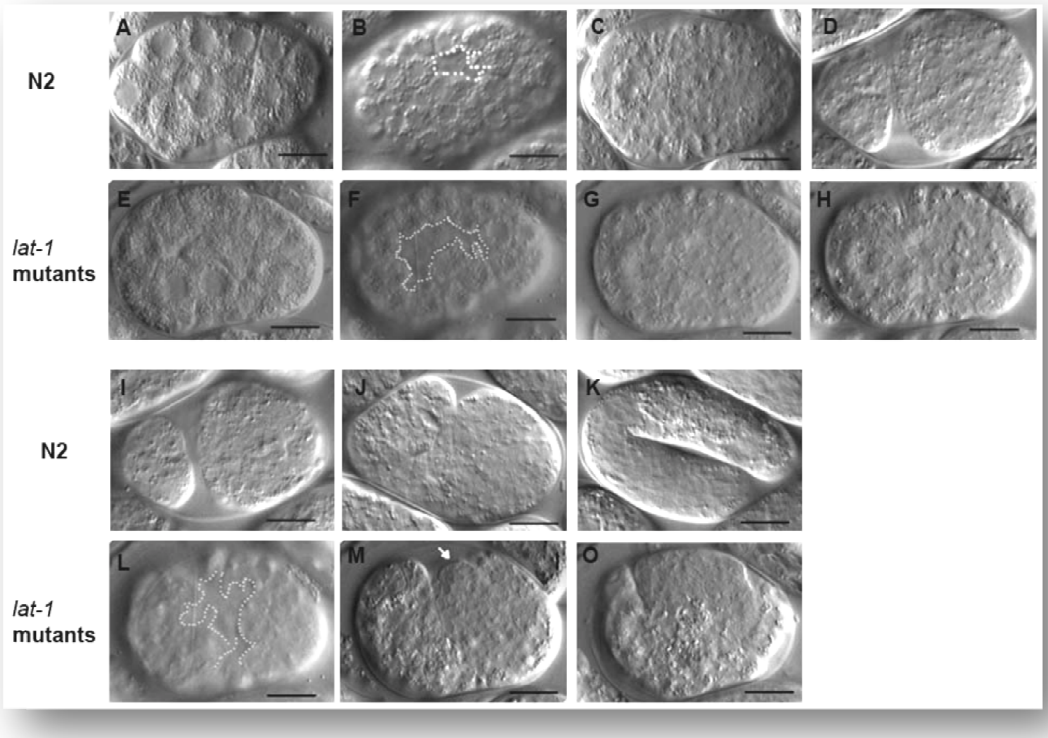


Figure 11: *lat-1* loss of function mutation causes embryonic lethality and affects several embryonic morphogenesis events. Differential interference micrographs of wild type and *lat-1* mutant embryos of equivalent development stage. (A, E) wild type and *lat-1* mutant embryos at the proliferation stage (beginning of gastrulation). Initial analysis did not reveal any defects during the proliferation stage of embryogenesis. B and F show the ventral cleft in wild type and *lat-1* mutant embryos respectively (periphery of ventral cleft is underlined by white dashed line). The ventral cleft is *lat-1* mutants is obviously wider and adopts an irregular shape. C, D, G and H depict the commencement of epidermal ventral enclosure as indicated by the restriction at either side of the anterior part of the embryo. In *lat-1* mutant embryos, the leading edge cells failed to reach the midline and the pocket cells do not appear (D and H). While wild type embryos complete ventral enclosure (I), whereas *lat-1* mutant embryos fail to complete ventral enclosure and ventral cleft is still persistent (L). As elongation starts, internal organs begin to burst (white arrow in M) and elongation fail to proceed (O). Bar scale=6uM

In order to further confirm that embryonic lethality observed in *lat-1* mutants is, at least partially, due to defects during embryonic morphogenesis, we analysed *lat-1* embryonic lethality in a genetic background sensitised for defects in epidermal cell movement during ventral enclosure. Ching-Sang and colleagues showed that the low penetrance epidermal cleft closure caused by delayed neuroblast migration in *efn-4*, *wsp-1* and *kal-1* mutants can be used as sensitizing genetic background for other pathways acting during epidermal morphogenesis (Chin-Sang, Moseley et al. 2002). Interestingly, *lat-1; efn-4* double mutants could not be isolated, and embryonic lethality was dramatically increased even in *lat-1* heterozygous mutants in an *efn-4* mutant background (Figure 12). Maternal contribution rescues embryonic lethality in *lat-1* mutant embryos from heterozygous mothers (figure 12)and the vast majority of homozygous worms arrest at the L1 stage. A hypomorphic *efn-4(e660)* allele proved enough to significantly increase lethality in *lat-1* heterozygote in an obviously more than just additive fashion, suggesting that lack of LAT-1 affects epidermal morphogenesis.

The ventral enclosure defect observed in *lat-1* mutant embryos could be the result of the earlier ABal division defect. Whether the lack of maternal *lat-1* gene product culminate in this ventral enclosure defect is not clear and this study was not sufficient to address this question. It would be interesting to analisze the ventral cleft enclosure in zygotically rescued embryos, as it would permit to see whether zygotic expression rescues this ventral enclosure defect. Additionally, to further understand the effect of the lack of early maternal *lat-1* experssion on ventral enclosure, we should analyse ventral enclosure defects in maternally rescued *lat-1* mutant embryos, as this will enable us to differenciate between ventral enclosure defect being the result of absent early expression and ventral enclosure defect being an independent additional abnormality, as work done by Tobias Langenhan, showed that maternal expression (homozygous from heterozygous mothers) rescues the ABal division defect, but whether it also rescues the ventral closure defect has not been

analysed. Additionally, it would be interesting to see what happens in the *lat-1* mutant embryos lacking maternal expression that do not show the early ABal division defect and whether they also exhibit the same ventral closure defect,

The conclusion derived from the increased embryonic lethality of heterozygote *lat-1* mutants in *efn-4* mutant backround is a hint that the morphegenesis stage in *lat-1* mutants is severly affected, however, the inability to isolate *lat-1;efn-4* double mutants limits the interpretation of this result, may be as an alternative approach, one could use RNAi directed againt *efn-4* in a *lat-1* mutant background and analyse the ventral closure in these embryos. in view of this, previous work with RNAi by feeding gave very variable results and was not thus routinely used in our lab.

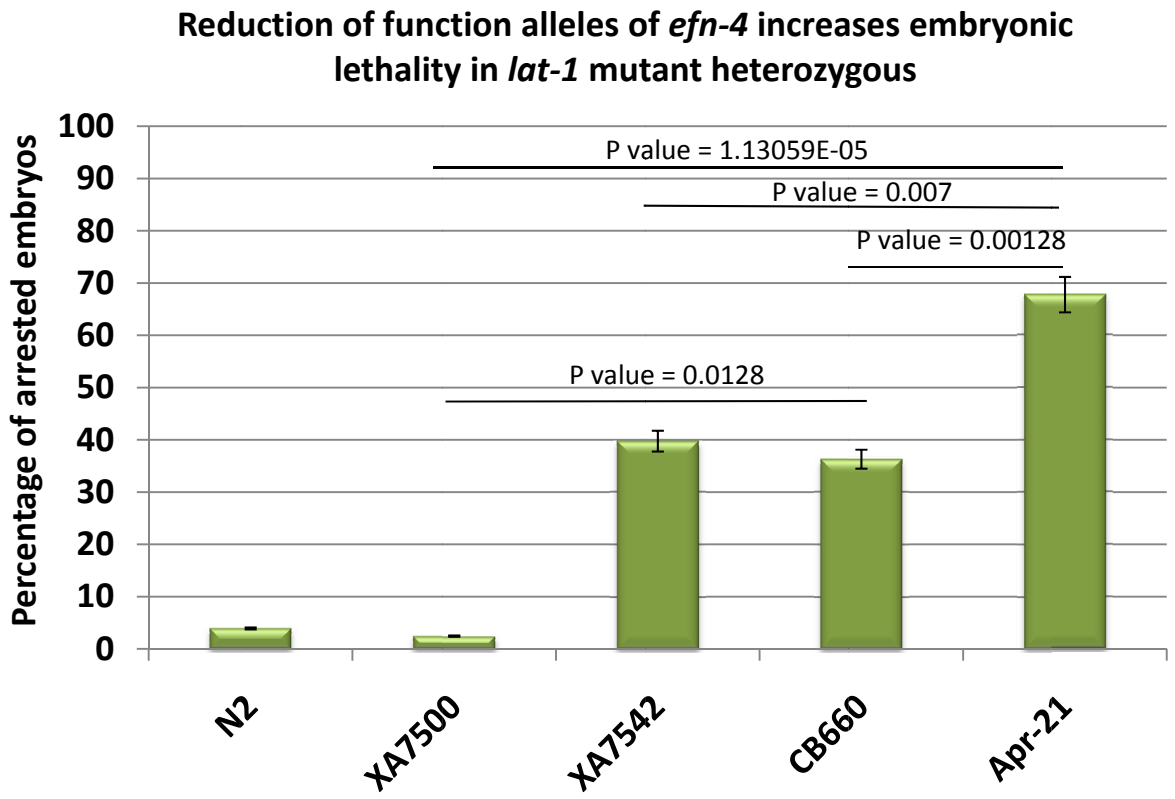


Figure 12: Embryonic lethality is dosage sensitive in a sensitised genetic background. *lat-1* heterozygous exhibit a small percentage of embryonic lethality (2.5%, strain XA7500 that is not statistically significant compared to N2) however, in an *efn-4* hypomorphic mutant background (APR21), *lat-1* heterozygous lethality increases to 68%, a percentage that is greater than either heterozygous and that is more than just additive**.** Strains used are the Bristol wild type strain N2, *lat-1* heterozygous [XA7500: *lat-1(ok1465)/mIn1[mIs14 dpy-10(e128)] II*], *lat-1* homozygous [XA7542: *lat-1(ok1465)* II]; *efn-4* hypomorphic mutants (CB660: *efn-4(e660)*IV], and *lat-1;efn-4* mutants [APR21: *lat-1(ok1465)/mIn1[mIs14 dpy-10(e128)] II*; efn-4(e660)IV]. Error bars=±SEM. P values t-test was used for statistics, and P-values are indicated.

1.2.5. *lat-1* mutants exhibit delays in blastomere divisions.

While analysing the development of *lat-1* mutant embryos we noticed that the mutants develop slowly. I would like to propose two possible explanations to this slow growth rate: first is that a particular developmental stage is heavily slowed down as a consequence of lack of LAT-1resulting in a barrier with a threshold that *lat-1* mutants cannot pass through, in other words, *lat-1* mutants struggle to meet the threshold criteria casing retarded development. Second, the slow growth rate is rather the accumulation of delayed developmental processes throughout the course of development (Figure 13). The second hypothesis was more credible for two reasons: although the majority of embryos arrested at the 3/4-fold stage, defects also impinge on different stages of embryogenesis. Moreover, analysis of early blastomere division revealed slight time differences in blastomere division: first mutant blastomeres divided few seconds late compared to wild type controls, and second, they completed division in a slightly prolonged period of time when compared to wild type embryos. Accordingly, the "accumulation" of late and prolonged cell division throughout the development could result is slow developing *lat-1* mutant embryos.

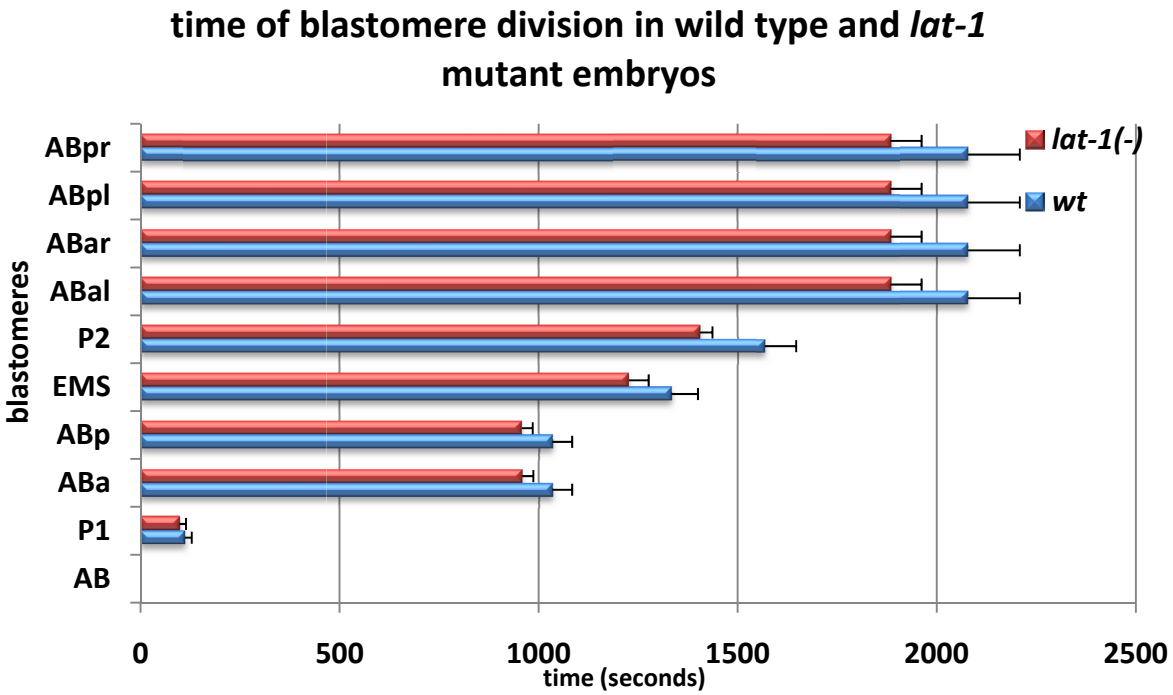


Figure 13: Chart representing the start of cell division of early balstomers. In *lat-1* mutants, early blastomeres showed delayed initiation of cell division and consequently delayed completion of cell division, as compared to wild type embryos. Our hypothesis concerning the delayed development observed in *lat-1* mutant embryos consists of accumulation of delayed cell division throughout embryogenesis. The time delay in initition blastomere division is not statistically significant in early blastomeres (this graph, and thus P values are not shown), but delays in initiating cell division accumulate as blastomeres enter their 5^{th} round of cell division Error bars= ±SEM.

2. *lat-1* mutants exhibit vulva morphological defects

2.1. Brief introduction

2.1.1. The development of vulva is a complex process

The *C. elegans* vulva is an excellent model for studying morphogenesis, and it has been used over several decades as the system of choice to shed light on the different pathways involved in cell pattern formation, cell fate induction, cell differentiation, invagination and morphogenesis (Kimble 1981) (Herman and Hedgecock 1990) (Sternberg 1988) (Ferguson, Sternberg et al. 1987). Laser ablation experiments showed that the integrity of somatic gonad is required for the proper morphogenesis and function of the vulva. Further refinement studies identified the Anchor cell to be the key somatic gonad cell for proper vulva eversion (Kimble 1981) (Seydoux, Savage et al. 1993).

The anchor cell is required at the L3 stage to induce vulva fate in the vulva precursor cells (VPCs), and is required at later stages (once vulva fate induction is complete) in vulva eversion and vulva-uterine connection (Seydoux, Savage et al. 1993).

The process leading to vulva opening is divided into four stages (Ferguson, Sternberg et al. 1987; Greenwald 1987), as outlined in Figures 14 and 15. First is the "generation stage" during which the specialised blast cells that will give rise to vulva cells are born along the dorsal side of the hermaphrodite larva. Second is the "fate specification stage" during which extracellular signals induce the vulva cell fate in target cells. Third is the stage throughout which the induced cells start dividing and differentiating and thus "executing" the fate that was induced in the previous induction stage. The fourth stage is the "morphogenesis stage" whereby the 22 vulva cells undergo extensive cell movement and morphogenesis to form the vulva opening and thus connecting the uterus to the outside environment (Eisenmann and Kim 2000).

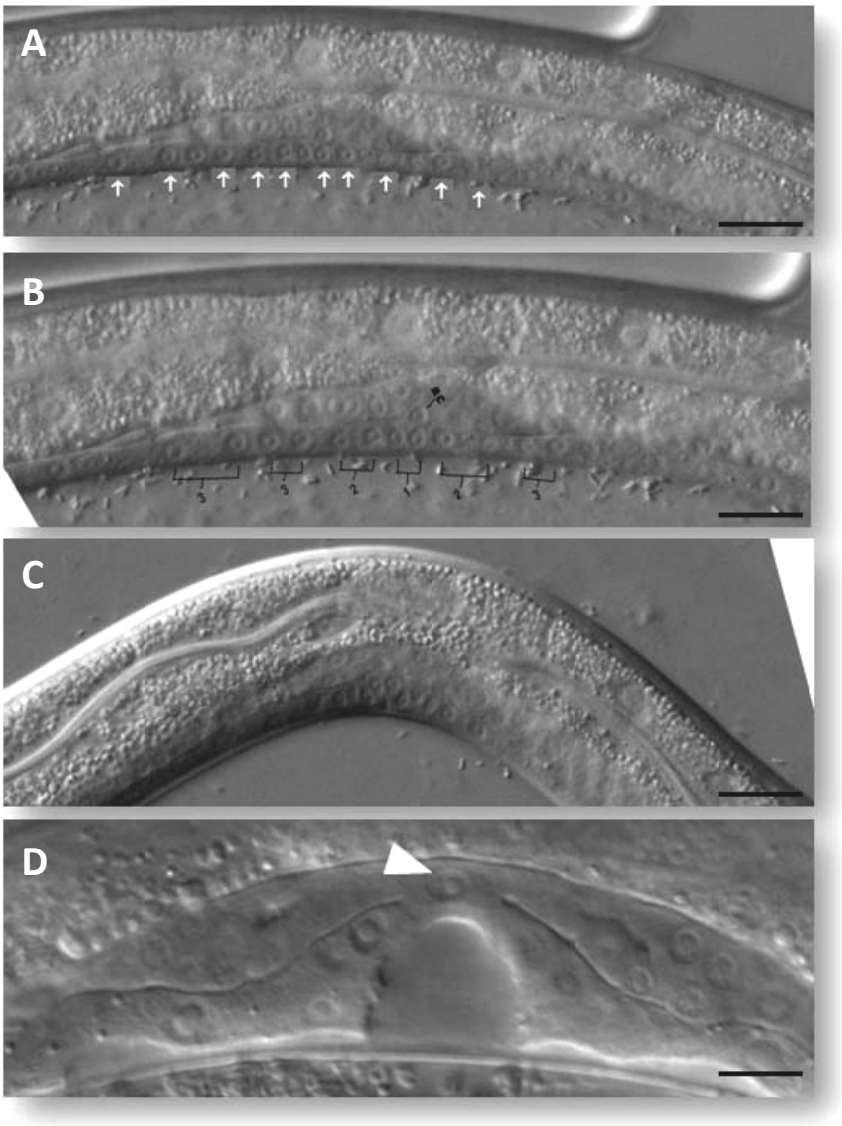


Figure 14. DIC pictures representing different steps during the process of vulva development in wild type worms. Anterior is left and dorsal is up in all figures unless indicated otherwise. The vulva development stats with the generation of the Pn.p hypodermal cells at the dorsal side of the larva (A, white arrows). (B) the anchor cell signals to the vulva precursor cells (VPCs) to adopt a vulva cell fate, and consequently, the VPCs execute the induction signal through a series of cell division in a defined spatial and temporal division pattern (C). By the L4 stage, the vulva acquires a Chrismass tree like shape that is symmetrically organised at either sides of the anchor cell (D, white arrow head).

The embryonic cells that will give rise to vulva tissue migrate from the ventrolateral position into the ventral midline. These 12P cells then divide in an anterior-posterior manner to give rise to anterior Pn.a neuroblast and posterior Pn.p hypodermal cells that later become vulva cells (Eisenmann and Kim 2000).

P3.p to P8.p represents the vulva equivalent group. These cells all express the Hox gene *lin-39*, and are all capable of adapting the vulva cell fate hence their name: vulva precursor cells (VPCs) (Sulston and Horvitz 1977; Sulston and White 1980; Sternberg and Horvitz 1986). *lin-39* expression is not sufficient to induce the vulva fate, as cells expressing the Hox gene *lin-39* and adopting the 3^0 fate (P3.p, P4.p and P8.p) do not make vulva tissue, but rather become part of the syncytium hypodermis (Ferguson and Horvitz 1985).

The vulva fate induction is achieved through three induction pathways. First is the induction signal from a somatic gonad cell called the Anchor Cell (AC), which induces the P6.p cell to adopt 1^0 fate (Kimble 1981). Second is the lateral inhibitory signal from the P6.p cell to both P5.p and P7.p (may be also in combination with reduced intensity of signal to these two cells from the anchor cell). P6.p inhibits both P5.p and P7.p to adopt 1^0 fate, and signal P5.p and P7.p to adopt 2^0 fate (Sternberg 1988). Third is another inhibitory signal from the surrounding syncytium hypodermis which sends an inhibitory signal antagonising that of the AC, ensuring that only cells that receive high amount of AC signal adopt a 1^0 cell fate (Herman and Hedgecock 1990). Accordingly, cells that are not induced adopt a 3^0 fate and divide once to fuse with the hypodermal syncytium (Eisenmann and Kim 2000).

2.1.2. Vulva defects: where things can go wrong.

In view of this, vulva defects can be the result of mutations affecting different steps of the vulva development process. Mutations affecting the generation of the Pn.p hypodermal cells, that will later specialise to become vulva cells, result in defective vulva, if a vulval tissue is made at all. Mutations in genes required for vulva development can be classified according to the stage during which they are necessitated. One such group includes genes

that function in the generation of the vulva precursor cells (the vulva equivalence group). Mutations in this group can affect different steps during the generation of the VPCs: (1) for example, *unc-83* and *unc-84* mutations affect the migration of the P cells into the ventral cord, thus preventing the generation of the vulva (the parents of P3-8.p cells, UNC-83 and UNC-84 co-localise to nuclear membrane during nuclear migration) (Sulston and Horvitz 1981; Greenwald, Sternberg et al. 1983; Ferguson and Horvitz 1985). Also, falling into this group of mutations, are genes that impinge on the generation of the Pn.p cells such as mutations in *lin-26* gene which cause the Pn.p cells to adopt the neural fate of their anterior sister cells, as judged based on nuclear morphology and division pattern (LIN-26 is required for the asymmetric cell fate determination in Pn.p cells to adopt vulva precursor cell fate (Ferguson, Sternberg et al. 1987)). Furthermore, mutations disabling vulva precursor cells to respond to the inductive signal also disturb the generation of vulva cell fates (Ferguson, Sternberg et al. 1987). Similarly, defects in the generation of signaling cells will affect the generation of the vulva tissue. In view of this, mutations in *lin-12* gene (which is involved in the generation of the signaling anchor cell) also affect the generation of vulva cells (Greenwald, Sternberg et al. 1983).

In addition to defects affecting the generation of the vulva precursor cells, vulva defects also raise from faults in the execution of the inductive signal. For instance, in their screen for protruding vulva mutants, Eissenmann and colleagues, identified genes that are required for the cells to respond to the Ras pathway and to adopt induced cell fate (Eisenmann and Kim 2000). The Ras pathway is activated in response to the inductive signal from the anchor cell and is required for the vulva cell fate instruction.

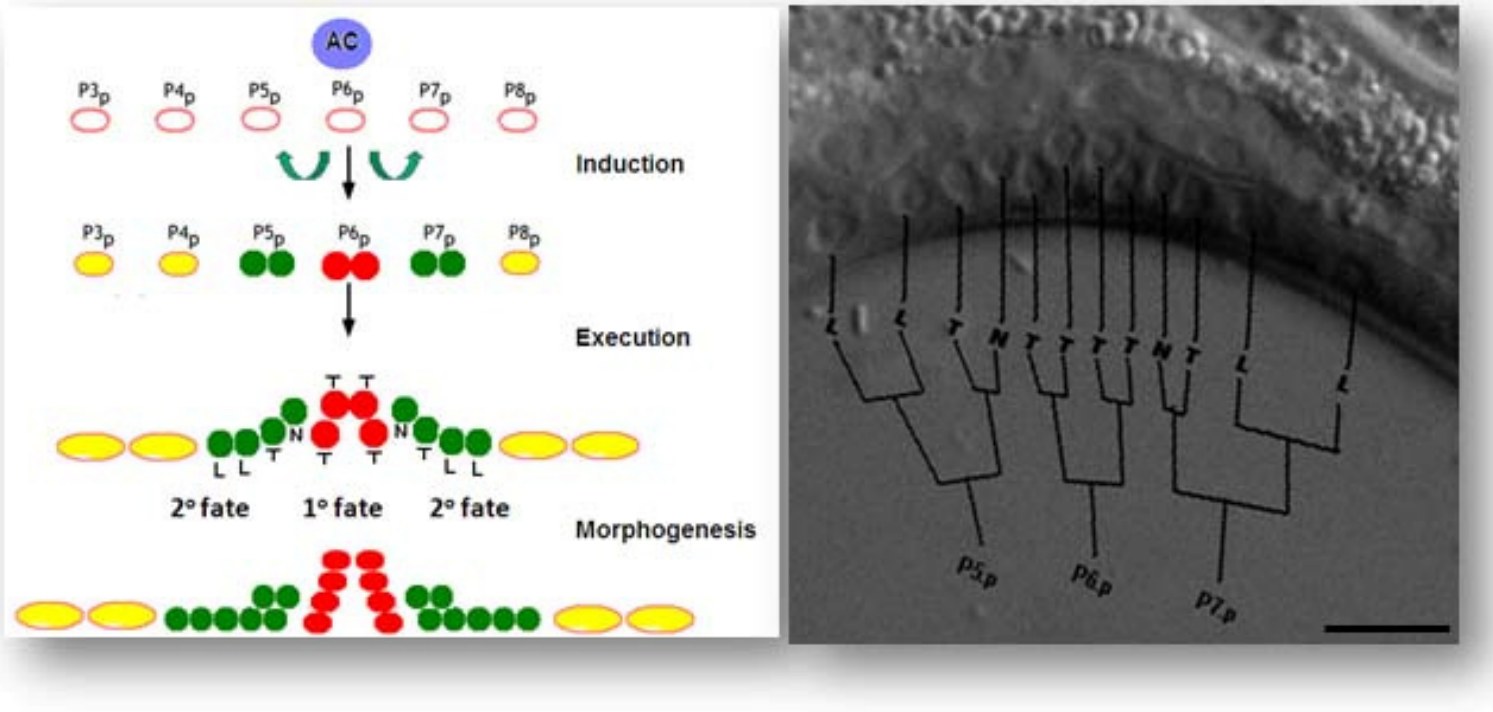


Figure 15. Stages of vulva development in wild type worms. Left is a diagram representing the steps of vulva development from the induction to the onset of morphogenesis. Right is a DIC micro-photograph of a late L3 larva depicting the first execution step of vulva precursor cells (VPCs) P5.p, P6.p and P7.p. Prior to the induction phase, the cells that will later give rise to the vulva tissue are born along the dorsal side of the larva at the L1 stage. During the L3 stage, the anchor cell forms the primary signaling centre which induces 1^0 fate in P6.p. The adjacent P5.p and P7.p are equally potent to adopt a 1^0 cell fate, however lateral signaling from the P6.p inhibit both cells from adopting 1^0 fate. May be with combination of low signal form the anchor cell (AC), both P5.p and P7.p adopt a 2^0 cell fate, whereas the remaining P3.p, P4.p and P8.p adopt the 3^0 fate to divide once each before they fuse with the hypodermal syncytium. The fate adopted by each VPC is defined by the pattern of its cell division. Cells adopting a 1^0 fate divide in a: TTTT pattern (T: Transverse (left/right), whereas 2^0 fate cells divide in a LLTN pattern (L: longitudinal anterior/posterior, T: transverse left/right, N: no division with characteristic compact morphology.

2.1.3. Benefitting from more sensitive screening approaches

Alterations to tactics of genetic screens improved from one experiment to another in order to allow more gene loci to be identified. For example, genetic screens for the vulva protrusion phenotype (instead of multivulva or vulvaless or suppressors of either phenotypes) identified new loci involved in vulva development such as *bar-1*, *mom-3/mig-14*, *egl-18* and *sem-4* (Eisenmann and Kim 2000). Protruding vulva mutants have subtle defects, some times affecting only one vulva cell, and which lead to either morphological defects or to vulva-uterus connection abnormalities leading to a protruding vulva and Egl phenotype with different levels of severity.

In *lat-1* mutants, while some hermaphrodites do not produce any progeny (Ste phenotype), other hermaphrodites look Egl, as judged by the late stage embryos inside the mother's uterus and the baggy appearance. In addition, occasionally, we observed mutants with aberrant vulva morphology. Such preliminary observations led us to further investigate the possible involvement of LAT-1 in vulva development/function. However the discrepancy in the vulva phenotypes and the changes in brood size from hermaphrodite to another, invites us to consider the potency of *lat-1* function in vulva development. One possible explanation is the redundancy and compensation between signaling pathways. Nevertheless, characterizing the vulva phenotypes in *lat-1* mutants would shed light on the engagement of *lat-1* in vulva development further to providing an additional system in which one could study the physiological role of LAT-1.

2.2. Results and discussion

2.2.1. *lat-1* mutants vulva defects begin at the L4 stage and not earlier

DIC microscopic analysis identified several vulva defects in *lat-1* mutants (Figure 16). In order to identify the stage(s) during which *lat-1* gene product is required for vulva development/function, we analysed the vulva morphology at different developmental stages

from L1 to adult stage. Interestingly, no defects were observed before the L4 stage. All 12 hypodermal cells were found at the L1 stage, and no lineage defect was observed as judged by the cell positions at the L4 stage. It is possible that subtle defects were not detected under our experimental conditions, however based on morphogenesis, number and position of vulva precursor cells, no defects were observed suggesting that *lat-1* does not function in the generation of vulva precursor cells or their migration to the dorsal side of the hermaphrodite. In future experiments, it would be helpful to use transcriptional constructs/antibodies to visualise the vulva precursor cells for a better identification of both cell numbers and cell position at the L1 stage.

2.2.2. L4 vulva is anteriorly tilted in *lat-1* mutants

Figure 16 depicts the different vulva morphological defects observed in *lat-1* mutants at the L4 stage. While the wild type vulva has a Christmas-tree like shape at the L4 stage arranged in mirror-image symmetry at either side of the anchor cell (the symmetry axis), some L4 *lat-1* mutant larvae have a vulva shape that is not completely symmetric, rather it is anteriorly tilted. Interestingly, all L4 stage that exhibit this rather abnormal asymmetric arrangement, the vulva tends to tilt anteriorly in 100% of the cases (Figure 16, panel B and C).

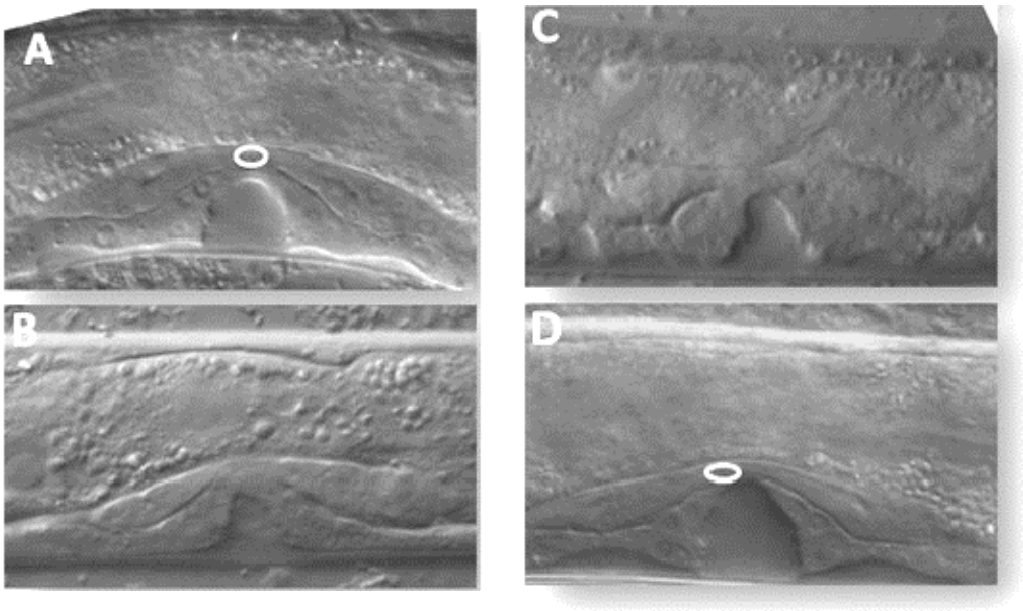


Figure 16. L4 *lat-1* mutants' vulva tends to tilt towards the anterior. (A) in wild animals, the vulva adopts a Christmas tree like shape organised symmetrically at either side of the symmetry axis (where the anchor cells is positioned, white circle in panel A). In some *lat-1* mutants (3.43% of observed cases (n=63)) the vulva is tilted towards the anterior side of the worm (B and C). Occasionally, the anchor cell location was shifted towards the anterior (white circle in panel D) but this did not lead to defects in vulva morphology.

2.2.3. LAT-1 might be required in establishing polarity of VPCs during vulva development.

Genetic evidence has identified genes that are required for the refinement of the cell polarity in order to assure this symmetric arrangement (Green, Inoue et al. 2008). This mirror image symmetry is achieved as VPCs P5.p and P7.p divide in opposite directions, and Wnt signaling has been directly involved in establishing the orientation of the cell division (Eisenmann, Maloof et al. 1998; Deshpande, Inoue et al. 2005) (Gleason, Szyleyko et al. 2006). Wnts are a class of secreted glycoproteins that are conserved amongst all metazoan, they bind to their Frizzled receptor to control different aspects of development in a canonical (involving beta-catenin) or a non-canonical (independent of beta-catenin) manner. In addition to Frizzled, Ror and receptor tyrosine kinases emerged as alternative Wnt-binding receptors (Green, Inoue et al. 2008). Three signaling pathways orchestrate the reproducible divisions of P5.p, P6.p and P7.p in the ventral epithelium, these are: Epidermal Growth Factor (EGF) signaling, Wnt signaling and Notch signaling pathways (for details on vulva development, refer to (Inoue, Wang et al. 2005)). LIN-44 and MOM-2 and their receptors frizzled LIN-17 and Ryk LIN-18 respectively, form parallel signaling pathways that orient the division of the P7.p progeny so that P7.p descendents are organised in a mirror-image to their P5.p counterparts, in order to assure the correct vulva invagination and morphology (Figure 17).

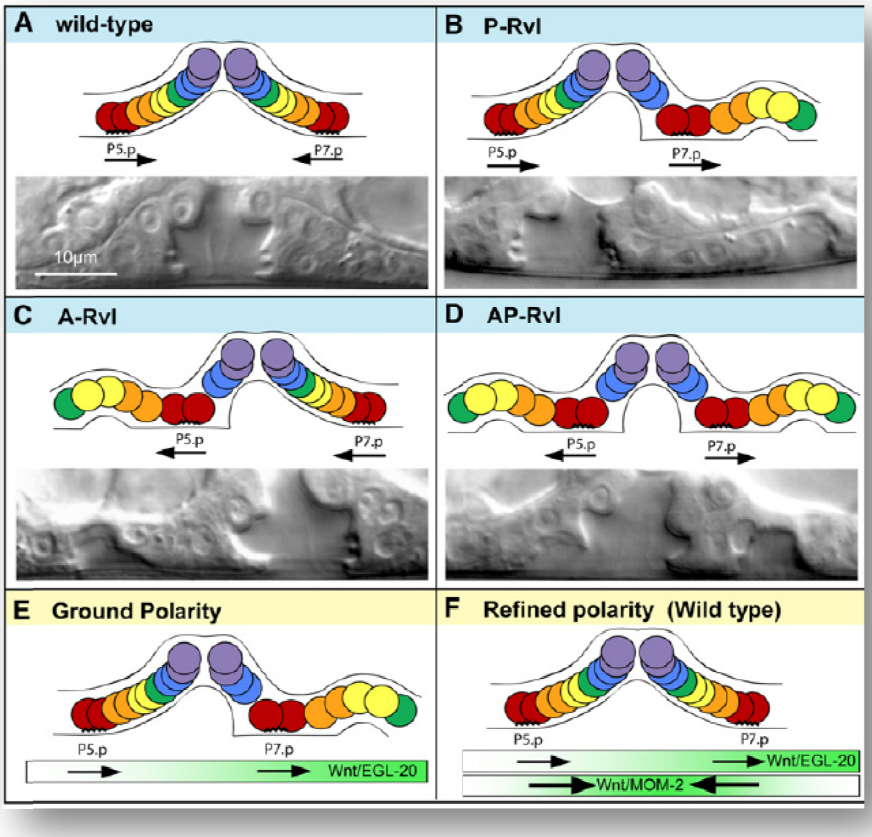


Figure 17. Default, ground and refined polarity in VPCs. In the absence of Wnt signaling, the P5.p and P7.p division is random leading to additional posterior and/or anterior invagination (C and D). A ground polarity is established by *egl-20* signaling in combination with the PCP component Vang gogh *vang-1* and Ryk Wnt receptor CAM-1. Ground polarity orients cell division towards the posterior (E), and MOM-2 and LIN-44 mediated signaling refines the polarity to orient cell division towards the centre (F). Picture taken from (Green, Inoue et al. 2008) for illustration.

The default polarity of P5.p and P7.p is random, and opposing Wnt pathways converge to control the division pattern of VPCs with Wnt *egl-20* in combination with PCP component van Gogh/*vang-1* and CAM-1 in establishing the ground polarity that orient both P5.p and P7.p towards the posterior. The orientation of P5.p and P7.p division is refined by action of MOM-2 and LIN-44 which orient the VPCs towards the centre (Figure 17) (Green, Inoue et al. 2008).

L4 *lat-1* mutant vulva defects were all abnormalities that appeared to affect the anterior side of VPCs, as the tilt observed in this defective vulva was towards the anterior in 100% of the

cases (Figure 16). Julie Gleason and colleagues reported that anterior VPPs were more strongly affected than posterior VPCs in Wnt mutants (Gleason, Szyleyko et al. 2006). More interestingly, green fluorescent expression patterns indicated that most and strongest Wnt expression are posteriorly located, suggesting that most Wnt ligands act over a distance to influence VPCs fate specification and P5.p lineage orientation. One possibility is that there exists a relay mechanism that propagates the Wnt signal from the posterior to the anterior side of the animal so that the anterior P5.p cells are exposed to the same intensity of Wnt signal as their P7.p counterparts. This proposition was previously suggested in embryogenesis, where a relay mechanism was proposed to transport the Wnt signal from the to the anterior side of the embryo (Bischoff and Schnabel 2006). The fact that only anteriorly tilted vulva was observed in *lat-1* mutants, in combination with the fact that no anterior-reversed vulval lineage was reported (at least prior to the ability to remove all Wnt signaling using CAM-1 over expression to sequester Wnt signaling (Green, Inoue et al. 2008)) reinforces the hypothesis that LAT-1 might function in a Wnt-relay mechanism during vulva development (the role of *lat-1* in polarity is discussed in chapter II). The vulva defects observed in *lat-1* mutants suggest that *lat-1* would be required so that the posteriorly originating Wnt signal reach to/affect more anterior vulva cells. However, the L4 anterior-tilted vulva phenotype observed in *lat-1* mutants was more severe in some mutants than others. This discrepancy in phenotypical severity is a phenomenon well described in pathways with redundant components such as the Wnt pathway. It is possible that lack of LAT-1 in our mutants cause the VPCs lineage to be random, meaning that VPCs can orient their cell division either anteriorly or posteriorly. However, based on DIC morphological analysis alone, it is not possible to discern the random posterior orientation of cell division because this latter looks like a wild type division pattern. Accordingly, it is possible that the penetrance of the defect in VPCs division pattern is underestimated in *lat-1* mutants.

One straightforward option to overcome this ambiguity is to search for vulva defects beyond the L4 stage: at the invagination stage of vulva development, and to observe the location of possible pseudo invagination (pseudo vulva). Invagination is the process by which cells fuse with the hypodermal lumen to form an opening connecting the uterus to the outside environment. The cells that contribute to the formation of the vulva opening are descendents of P6.p (10 cells), posterior descendants of P5.p and anterior descendants of P7.p. Defects in re-orienting P7.p division pattern leads to a P5.p like division pattern and formation of a posterior pseudo vulva, whereas defects in reinforcing the P5.p orientation of division lead to the formation of an anterior pseudo-invagination. Accordingly, defects causing random VPCs division pattern should lead to formation of both anterior and posterior pseudo-invaginations. This is indeed what we observed in *lat-1* mutants (Figure 18).

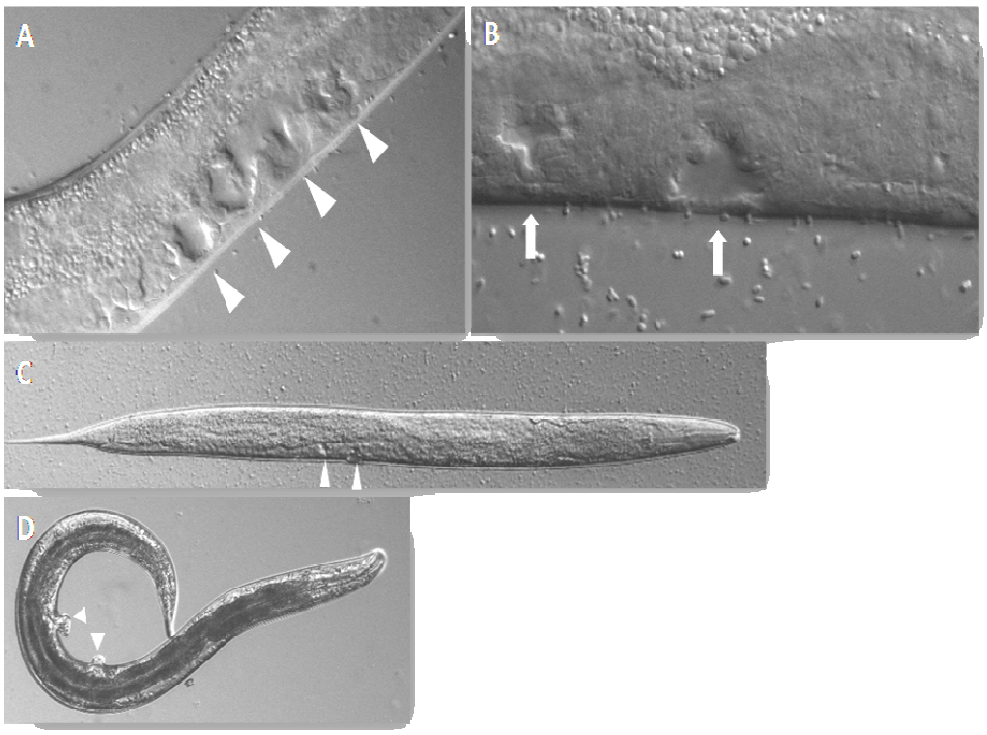


Figure 18. Anterior and posterior pseudo-invagination observed in *lat-1* mutants. DIC pictures of *lat-1* mutants at the late L4 and adult stages. Additional invaginations were observed in *lat-1* mutants at either side of the main vulva site. Pseudo-vulva were observed to form anterior to the main vulva site (white arrow in B and arrow heads in D) and to the posterior of the main vulva opening (C, white arrow heads) indicating that the pseudo-vulva formation happens in random orientations in *lat-1* mutants. A more severe phenotype is the presence of multiple vulva openings (A, white arrow heads). This phenotype was observed at very low frequency and was not further characterised in this study.

The formation of additional invagination sites to either the anterior or the posterior side of the main vulva opening indicates that the defects in VPCs division pattern are rather random. This is in accordance with the fact that in the absence of Wnt signaling, VPCs division patter is random as indicated by the presence of both anterior-reversed vulval lineage (A-Rvl) and posterior-reversed vulval lineage (P-Rvl) (Figure 17).

It is thus clear that *lat-1* is required during the development of the vulva. The results presented so far lead us to propose a role for LAT-1 in relaying Wnt signaling from the posterior to the anterior of the worm, as indicated by similar results between *lat-1* mutants and mutants that lack Wnt signaling, as far as vulva development is concerned. The additional observation that the AC cell is positioned at the wrong side in some of the *lat-1* mutants (Figure 16) led us to think that the role of LAT-1 is more complicated as opposed to simply propagating a polarising signal. In some cases, whether accompanied with vulva defects or not, the anchor cell position was not at the axis of symmetry, it was rather shifted towards the anterior (Figure 16). This is an invitation to put forward an alternative role, or even an additional role for *lat-1* in vulva development. The AC is reported to express the Wnt ligand MOM-2 (Green, Inoue et al. 2008). Moreover, the AC is born as a result of a series of a-p cell division of its precursor Z cell. The pre-AC cell is the source for a secreted signal LAG-2 which drives the AC/VU fate decision. Through a feedback mechanism, the mature AC becomes the highest source of LAG-2 secretion, inhibiting LAG-2 production in VU (refer to (Altun 2005). The fact that the dislocation of the anchor cell does not correlate with the presence of a vulva defect may be explained by redundancy in signaling mediators (also possibly owing to the low frequency of vulva defects observed in *lat-1* mutants, Figure 19).

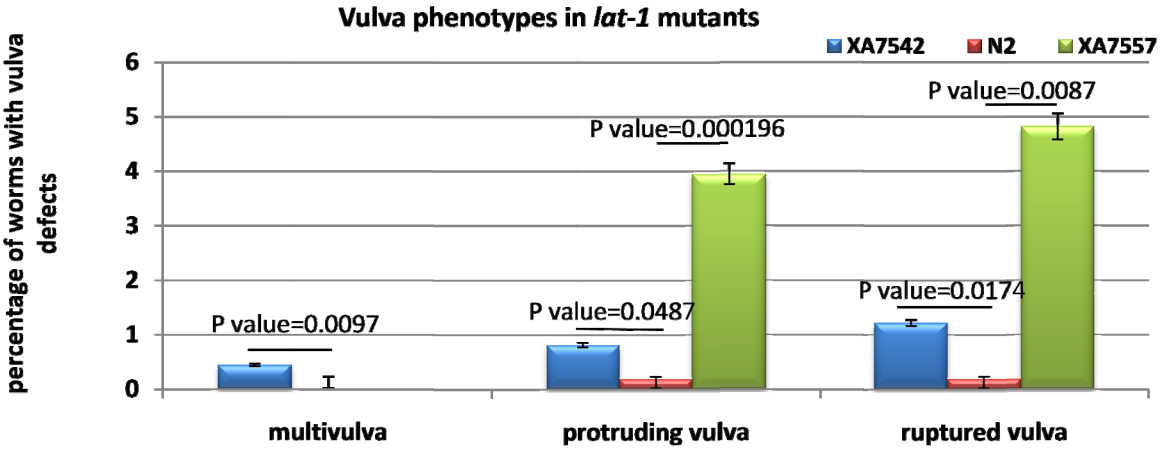


Figure 19. Percent penetrance of vulva defects observed in *lat-1* mutants. Three different vulva defects were observed: ruptured vulva (also referred to as burst vulva phenotype), protruding vulva and a less frequent multivulva phenotype. Strain used include the wild type Bristol strain N2, *lat-1* mutant strain XA7542 [*lat-1(ok1465) II*], and *lat-1 lat-2* double mutant strain XA7557 [*lat-1(ok1465) lat-1(tm465) II*]. Error bars are ±SEM. P value t-test was used for statistics, and t-test was performed between *lat*-1/2 mutants and the N2 control. P values are indicated.

In 2000, Eisenmann and colleagues showed that screening for mutations causing an alternative vulva phenotype such as protruding vulva (Pvl) led to the identification of novel loci that appear to play a role at different stages during vulva development (Eisenmann and Kim 2000). Protruding vulva phenotypes is the most highly penetrant defect in *lat-1* mutants compared to other vulva abnormalities (Figure 19), and previous Pvl phenotypes were associated with defects in VPCs fate induction, fate execution and VPCs generation (Eisenmann and Kim 2000). Despite the effect of lack of LAT-1 on vulva development, it is not possible to draw conclusion on the exact defect caused by *lat-1* mutation. Detailed expression pattern in combination with genetic synergy experiments (such as analysing the pseudo-vulva formation in *lat-1;mom-2* and *lat-1;egl-20* double mutants) would shed light on the extent of *lat-1* implication in vulva development and its potential role in defining the division orientation of P5.p and P7.p cells. Furthermore, the vulva morphological defects observed in adult *lat-1* mutants are most likely to be the result of an earlier defect because the percent penetrance of vulva defect is equally distributed between L4 and adult worms (table 2). Percent distribution of vulva defects between L4 and adult mutants was scored following incubation at 25'C alongside temperature sensitive *lin-12(n676n930)* hermaphrodites which show high penetrance of Pvl phenotype at restrictive temperatures. *lat-1* mutants on the other hand, were not affected by temperature as far as Pvl phenotype is concerned (data not shown). *lat-1(ok1465)* allele does not seem to be temperature sensitive neither in embryos nor in the vulval system (although the effect of elevated temperature, i.e. 25'C was not tested in embryos in thus study).

Table 2: Percent occurrence of vulva defect in L4 and adult *lat-1* mutants.

Genotype / stage	*lat-1(ok1465)*	N2	*unc-32(e189) lin-12(n676n930)III*
L4	3.43% (n=49)	0% (n=63)	N.D
adults	3.4% (n=68)	0.3%(n=81)	12.8%(n=8)

3. Complementation and deficiency mapping

3.1. Brief introduction

Early genetics screens aiming at identifying genes with controlling roles in embryogenesis led to the identification of several temperature-sensitive and maternal effect lethal mutations. More than 90% of embryogenesis genes identified in such screens are pure maternal effect lethal genes underlining the crucial role of maternal gene contribution in *C. elegans* embryogenesis (Kemphues, Kusch et al. 1988) (Wood, Hecht et al. 1980). Moreover, the fact that *lat-1* embryonic lethality is not temperature sensitive (i.e. non-conditional maternal effect lethality) reduces the magnitude of the probability that embryonic lethality in *lat-1* mutants is due to LAT-1 exerting an essential role in cell viability or proliferation rather than having controlling roles in embryogenesis (Wood, Hecht et al. 1980).

3.2. Results and discussion.

3.2.1. *lat-1* and selected loci are not allelic

Amongst the loci identified in such non-conditional genetics screens, two genes (*mel-8* and *mel-9*) were mapped close to *lat-1*, and share similar phenotypes to *lat-1* mutants (embryonic lethality, terminal embryonic morphological defects, larval arrest with morphological abnormalities as well as protruding vulva, a phenotype previously not associated with *mel-8* and *mel-9* mutations and observed during the course of this study). Additionally, similar to *lat-1* null allele, embryonic lethality in *mel-8* and *mel-9* mutants show a partial maternal

effect as it can be rescued by male mating. Accordingly, we performed complementation tests to find out whether the two loci are allelic. Our findings indicated first: that *lat-1* and *mel-9* are not allelic, and second: that *mel-8* is wrongly annotated as it showed no embryonic lethality (Figure 20, see material and methods).

To further refine the physical map position of *lat-1*, we performed complementation tests with several deficiencies previously described to cover the *lat-1* locus.

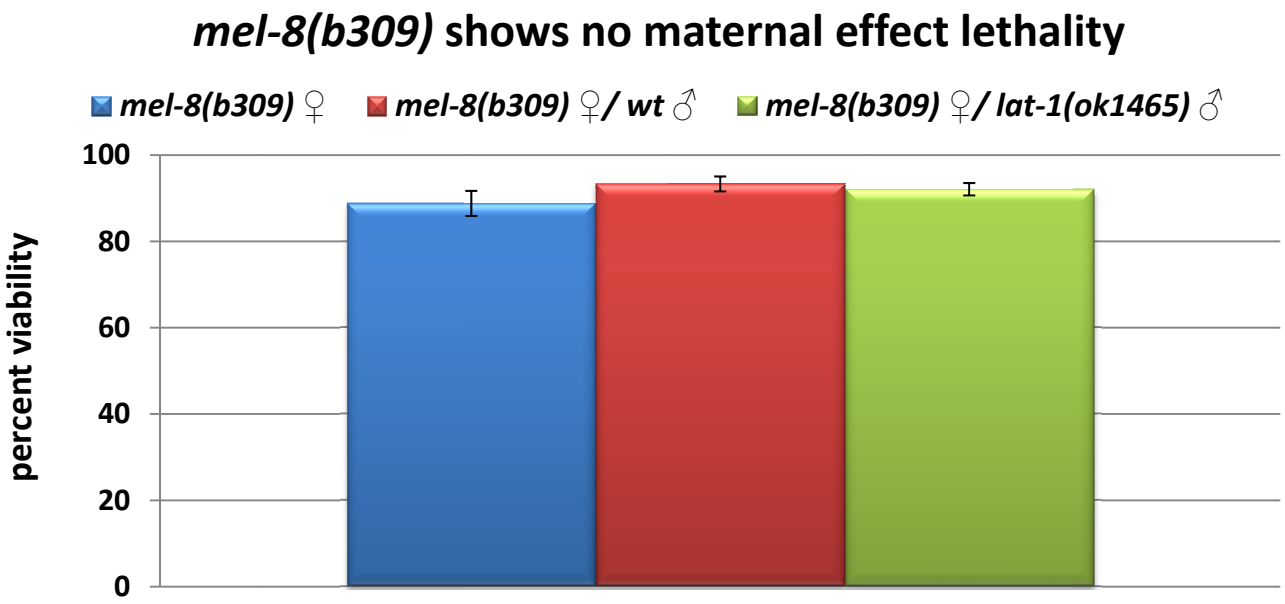
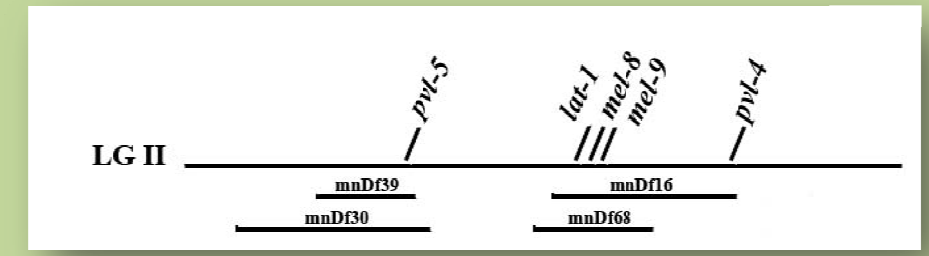


Figure 20: Percent viability in mated and unmated *mel-8(b309)* mutants. *mel-8* was previously identified in a non-conditional maternal effect lethal genetic screen (Kemphues, Kusch et al. 1988). The study presented *mel-8(b309)* allele as being a partial maternal effect lethal as embryonic lethality could be rescued by wild type male mating. Our results show that *mel-8(b309)* is neither maternal lethal nor is it temperature sensitive (data not shown): first, it was possible to maintain b309 allele in a homozygous state at restrictive temperatures, second male contribution of wild type copy of MEL-8 did not significantly improve viability. Mating *lat-1* mutant males with *b309* hermaphrodites produced out cross progeny similar in viability to both *b309/+* and *b309* homozygous. In order to verify the strain carrying b309, we sequenced across the *mel-8* loci and tested complementation between *mel-8* and deficiencies known to cover the *mel-8* region (mnDf68). *mel-8(b309)/mnDf68* cross progeny behaved like *mel-8(b309)* homozygous (date not shown). t-test was used for statistics and no significant different was observed and corresponding P values are thus not shown.

Further identified alleles exhibiting similar phenotypes with *lat-1* mutants were tested for complementation. These were mapped on linkage group II (LG II) near *lat-1* locus according to wormbase annotation. *pvl-5* and *pvl-4* exhibit vulva protrusion phenotypes (*pvl-5* mutants occasionally exhibit a multivulva phenotype at very low penetrance, data not shown) and the molecular lesions of alleles tested for complementation (*pvl-5(ga87)* and *pvl-4(ga96))* have not been characterised making it difficult identify outcross progeny, accordingly, we used deficiencies previously described to cover the *pvl-5* and *pvl-4* loci. Complementation test with deficiencies covering *pvl-5* and *pvl-4* loci revealed that *lat-1* and *pvl-5/4* are non-allelic (table 3).

Table 3: Complementation tests indicating that *lat-1* and the selected loci are not allelic. Deficiencies mapping and locus position are indicated as annotated on wormbase (www.wormbase.org). Last cell in the table depicts the distribution of selected loci alongside *lat-1* locus which is located between *mel-9* and *mel-8* loci.

Df boundaries / Deficiency / allele	Left arm	Right arm	Genes inside	Genes outside	Complement *lat-1* ?
mnDf16	0.867	1.84	*let-240, rol-6, mel-11, unc-4*	*mel-8, stu-16*	**No**
mnDf30	-0.745	0.297	let-178, let-179, let-263, **pvl-5**	mel-2, mel-5, mel-7	**Yes**
mnDf68	0.827	1.374	*mel-8, mel-9, mel-11*		**No**
mnDf39	-0.363	0.113	*Let-178,* ***pvl-5***		**Yes**
pvl-5(ga87) II.	0.11 ± 0.023				**yes**
pvl-4(ga96) II	1.80 ± 0.33				**yes**
mel-8(b309) II	0.98 ± 0.052				**yes**
mel-9(b293)	1.14				**Yes**



4. Materials and methods.

4.1. Embryonic development.

4.1.1. *C. elegans* strain. Worm strains were cultured and maintained according to standard procedures described by S. Brenner (Brenner 1974). Worms were cultivated at 22'C unless otherwise noted. The worms described as wild type were the *C. elegans* variety Bristol strain N2. The following genotypes were used:

LG II: *lat-1(ok1465)* [XA7500] (Langenhan, Promel et al. 2009), *lat-1(ok1465) lat-2(tm463)* [XA7557], *lat-1(ok1465)/mIn1[mIs14 dpy-10(e128)] II ; him-8(e1489) IV* [XA7567]. *mIn1[mIs14(pes-10p::gfp myo-2p::gfp) dpy-10(e128)] II* (Edgley and Riddle 2001) carries an integrated semi-dominant pharyngeal GFP element, such that one copy of mIs14 can be distinguished from two copies by GFP signal brightness (Edgley, Baillie et al. 2006). *mln1* was used to balance the chromosomal region carrying the *lat-1* and *lat-2* loci.

LG IV: *efn-4(e660)* [CB660] (gift from Jonathan Hodgkin lab). *e660* is a weak allele affecting a conserved proline residue and is proposed to affect the end of the beta-strand C in the tetramirization interface (Chin-Sang, Moseley et al. 2002).

4.1.2. Embryonic and larval arrest assay. 80-100 young adult hermaphrodites per strain were transferred to fresh NGM plates seeded with HB101 and allowed to lay eggs for 3 hours before they were removed. The embryos were washed with M9 buffer and 1% BSA (New England Biolabs) into a petri dish lid. Using a thinned Pasteur pipette connected to a mouth piece loader (Fisher Scientific), 5-3 embryos (with sufficient bacteria) were loaded in a 72-well flat-bottom Terasaki plates (Greiner Bio-One). Embryos were incubated at 22'C for 48 hours then were scored for embryonic or larval arrest using an inverted microscope.

4.1.3. Temperature sensitivity assay. F3 10x L4 hermaphrodites per strain were transferred to 10X fresh NGM plates seeded with HB101, incubated for three hours at 20'C or 15'C and left to lay egg during the incubation period before they were removed and the embryos washed with M9 buffer and 1% BSA and placed in a perti dish lid. The embryos were loaded into 72 microwell plate as described for the embryonic and larval assay. Embryos were incubated and left to develop at either 20'C or 15'C and each plate was scored after 48 hours by counting the number of progeny in their different developmental stages.

4.1.4. Zygotic expression rescuing assay. The assay aimed at analyzing the necessity extent of zygotic expression in order to better understand and differentiate between defects due to lack of early maternal expression and defects that occur later during development. A wild type copy of *lat-1* carried in the chromosomal rearrangement *mln1* was brought in a *lat-1* mutant background by mating balanced *lat-1* mutant males with *lat-1* mutant hermaphrodites. Balanced *lat-1* mutant males were used instead of wild type males because the wild type copy of *lat-1* can be easily distinguished as it is carried in the *mnl1* balancer which is GFP labelled. That is cross progeny F1 that are green carries a wild type copy of *lat-1* coming from the *lat-1/mln1* males. 20-25 XA7567 males [*lat-1(ok1465)/mIn1[mIs14 dpy-10(e128)] II ; him-8(e1489) IV*] were mated with 5-7 *lat-1* or *lat-1 lat-2* mutant hermaphrodites and the plates were incubated at 22'C. The harem was transferred daily to fresh NGM plates seeded with HB101. The outcross progeny was incubated at 22'C and allowed to reach adult stage before being scored. Scoring was performed under a microscope fitted with UV emission to allow the visualisation of GFP. GFP labelling integrated in the *mln1* chromosomal arrangement was used to select outcross progeny from selfing progeny. Average number of the outcross progeny expressing zygotic LAT-1 was counted as the average number of viable GFP adult worms. the number of *lat-1* mutants expressing zygotic LAT-1 only (i.e. GFP outcross progeny), was then compared to the brood size of *lat-1* mutants with neither maternal nor zygotic expression.

4.1.4.1. Crossing scheme for zygotic expression assay.

Zygotic rescue experiment in *lat-1* mutant background

P0. $\frac{\textit{lat-1(ok1465)}}{\textit{mln1}}$*; him-8(e1489)* ♂ × *lat-1(ok1465)* ♀

F1. *lat-1(ok1465);* $\frac{\textit{him-8(e1489)}}{\textit{him-8(+)}}$ non-GFP outcross F1 progeny, neither maternally nor zygotic expression.

$\frac{\textit{lat-1(ok1465)}}{\textit{mln1}}$; $\frac{\textit{him-8(e1489)}}{\textit{him-8(+)}}$ GFP F1 outcross progeny with zygotic expression (no maternal gene product).

4.1.4.2. Zygotic rescue experiment in *lat-1 lat-2* mutant background

P0. $\frac{\textit{lat-1(ok1465) lat-2(+);}}{\textit{mln1}}\ \frac{\textit{him-8(e1489)}}{\textit{him-8(+)}}$ ♂ × *lat-1(ok1465) lat-2(tm463)* ♀

F1. $\frac{\textit{lat-1(ok1465) lat-2(tm463)};}{\textit{lat-1(ok1465) lat-2(+)}}\ \frac{\textit{him-8(e1489)}}{\textit{him-8(+)}}$ non-GFP outcross F1 progeny, neither maternal nor zygotic *lat-1* expression.

$\frac{\textit{lat-1(ok1465) lat-2(tm463);}}{\textit{mln1}}\ ;\ \frac{\textit{him-8(e1489)}}{\textit{him-8(+)}}$ GFP F1 outcross progeny with zygotic *lat-1* expression and no maternal *lat-1*

4.1.5. Recording of live embryos. In order to understand the defects associated with embryonic arrest in *lat-1* mutants, gravid hermaphrodites were transferred from fresh NGM plates seeded with HB101 to a drop of M9 buffer on a microscope slide. Using a syringe needle, hermaphrodites were cut upon to release embryos from their uterus and early stage embryos were transferred onto a 1% agarose pad on a microscope slide using a thinned Pasteur pipette connected to a mouth piece loader (Fisher Scientific) and the slides were then sealed with molten Vaseline to keep moisture. Recording was performed using a Quimaging Regita Exi digital camera mounted on a Leica DM RXA2 microscope using Volocity v4 software (Improvision). Each recording was performed with 100X magnification and was adjusted to take a series of Z-tack snapshots with 1 micron depth difference between each frame of the same time point. Z-stack frames were taken every 30 seconds and each recording was performed until the embryos hatch. Recordings were then post-processed by analyzing slow motion replay of each recoding.

4.1.6. Generating *lat-1(ok1465)/mIn1[mIs14 dpy-10(e128)] II ; him-8(e1489) IV*]; *efn4(e660) IV.*

lat-1 balanced males were mated with L4 *efn-4(e660)* hermaphrodites. The harem was transferred daily to fresh NGM plates. Out cross progeny males were selected under UV-light fitted microscope and mated with L4 *lat-1* mutant hermaphrodites. GFP-positive outcross progeny were singled out infresh NGM plates seeded with OP50 and allowed to self at 22'C. *efn-4* mutations would be segregated in Mendelian ratio and thus ¼ of the selected GFP-positive outcross progeny would be heterozygous for *efn-4(e660)* mutation. F2 from outcross progeny were singled out into fresh NGM plates and their selfing progeny homozygous for *efn-4(e660)* were identified by sequencing across the efn-4 locus.

4.1.6.1. Crossing scheme, and sequencing-based selection of *e660* homozygous:

Step 1

P0. $\frac{lat\text{-}1(ok1465);\ him\text{-}8(e1489)}{mIn1}$ ♂ × *efn-4(e660)* ♀

F1. $\frac{lat\text{-}1(ok1465)}{lat\text{-}1(+)}$; $\frac{efn\text{-}4(e660)}{efn\text{-}4(+)}$; $\frac{him\text{-}8(e1489)}{him\text{-}8(+)}$ (50% non-GFP)

$\frac{lat\text{-}1(+)}{mIn1}$; $\frac{efn\text{-}4(e660);}{efn\text{-}4(+)}$ $\frac{him\text{-}8(e1489)}{him\text{-}8(+)}$ (50% GFP-positive)

Step 2 GFP positive males from outcross progeny form step one mated with *lat-1* mutant hermaphrodites:

P0. $\frac{lat\text{-}1(+)}{mln1}$; $\frac{efn\text{-}4(e660);}{efn\text{-}4(+)}$ $\frac{him\text{-}8(e1489)}{him\text{-}8(+)}$ ♂ × *lat-1(ok1465)* ♀

F1. $\frac{lat\text{-}1(ok1465)}{lat\text{-}1(+)}$; $\frac{efn\text{-}4(e660);}{efn\text{-}4(+)}$ $\frac{him\text{-}8(e1489)}{him\text{-}8(+)}$ (non-GFP)

$\frac{lat\text{-}1(ok1465)}{lat\text{-}1(+)}$; $\frac{efn\text{-}4(e660);}{efn\text{-}4(+)}$ *him-8(+)* (non-GFP)

$\frac{lat\text{-}1(ok1465)}{lat\text{-}1(+)}$; *efn-4(+)* ; $\frac{him\text{-}8(e1489)}{him\text{-}8(+)}$ (non-GFP)

$\frac{lat\text{-}1(ok1465)}{lat\text{-}1(+)}$; *efn-4(+)* ; $\frac{him\text{-}8(e1489)}{him\text{-}8(+)}$ (non-GFP)

$\frac{lat\text{-}1(ok1465)}{mln1}$; $\frac{efn\text{-}4(e660);}{efn\text{-}4(+)}$ $\frac{him\text{-}8(e1489)}{him\text{-}8(+)}$ (GFP-positive)

$\frac{lat\text{-}1(ok1465)}{mln1}$; $\frac{efn\text{-}4(e660);}{efn\text{-}4(+)}$ *him-8(+)* (GFP-positive)

$\frac{lat\text{-}1(ok1465)}{mln1\ )}$; *efn-4(+)* ; $\frac{him\text{-}8(e1489)}{him\text{-}8(+)}$ (GFP-positive)

$\frac{lat\text{-}1(ok1465)}{mln1}$; *efn-4(+)* ; $\frac{him\text{-}8(e1489)}{him\text{-}8(+)}$ (GFP-positive)

Step 3. GFP-positive outcross progeny hermaphrodites were singled out and allowed to self. Only selfing of *enf-4* heterozygous is shown here, *him-8* locus is omitted in the crossing scheme for simplicity:

$$\frac{lat\text{-}1(ok1465)}{mln1} \; ; \; \frac{efn\text{-}4(e660)}{efn\text{-}4(+)} \quad \circlearrowright$$

Selfing progeny:

$\frac{lat\text{-}1(ok1465)}{mln1} \; ; \; \frac{efn\text{-}4(e660)}{efn\text{-}4(+)}$	(1 in 4 would be double heterozygous)
$\frac{lat\text{-}1(ok1465)}{mln1} \; ; \; efn\text{-}4(e660)$	(1 in 8, appears WT and is GFP-positive)
$\frac{lat\text{-}1(ok1465)}{mln1} \; ; \; efn\text{-}4(+)$	(1 in 8, appears WT and is GFP-positive)
$lat\text{-}1(ok1465) \; ; \; \frac{efn\text{-}4(e660)}{efn\text{-}4(+)}$	(1 in 8, arrest and is GFP-negative)
$mln1 \; ; \; \frac{efn\text{-}4(e660)}{efn\text{-}4(+)}$	(1 in 8, DPY, and is bright GFP-positive)
$lat\text{-}1(ok1465) \; ; \; efn\text{-}4(e660)$	(1 in 4, arrest and is GFP-negative)
$mln1 \; ; \; efn\text{-}4(e660)$	(1 in 4, DPY and bright GFP-positive)

WT GFP-positive selfing progeny were singled out into fresh NMG plates seeded with OP50 and allowed to self at22'C. A swap of worms was taken using a platinum wire from each cloned population and whole worm DNA was extracted and sequenced across the *efn-4* loci as described in the following:

4.1.7. Worm lysis. Whole worm DNA was extracted by lysing worms in 5ul of the following lysis mix: worm lysis buffer (200ul) mixed with 5ul 1000x ProteinaseK

(Promega) (worm lysis buffer: 0.45% NP40, 50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM MgCl2, 0.01% w/v gelatine). The 5ul lysis mix (with transferred worms) was pipitted into PCR tubes covered with a drop of mineral oil to avoid evaporation, incubated at -80'C for 10 minutes (this will freeze-crack the worms and help release the DNA) then lysis was then performed in a thermal cycler using the following parameters: 65'C for 30 minutes then 95'C for 15 minutes. The lysis mix was then used as a DNA template to PCR-amplify across the *efn-4* locus as described in the next section.

4.1.8. PCR amplification across *efn-4* locus. Primers were designed using ApE plasmid editor (can be freely downloaded at: http://www.biology.utah.edu/jorgensen/wayned/ape/) and were manufactured by Eurofins MWG Operon (http://www.eurofinsdna.com/home.html).

Primers:

efn-4 1F: 5'- GGTCTCTAAGTGACAAAAGTTTTCTGTTT- 3'

efn-4 2R: 5'- TAATAATACGTCTGCCCTGGTTGATACTCT- 3'

PCR mix for a 30ul reaction:

21.75ul dH2O
0.25ul efn4-1F primer (20mM) for a final concentration of 165 nM
0.25ul efn4-2R primer (20mM) for a final concentration of 165 nM
0.5ul dNTPs (25mM each) for a final concentration of 416 nM
3ul 10xTaq buffer (containing $MgCl_2$)
0.25ul Taq polymerase (1.25 Units)
5ul DNA template (non-purified from whole worm lysate)

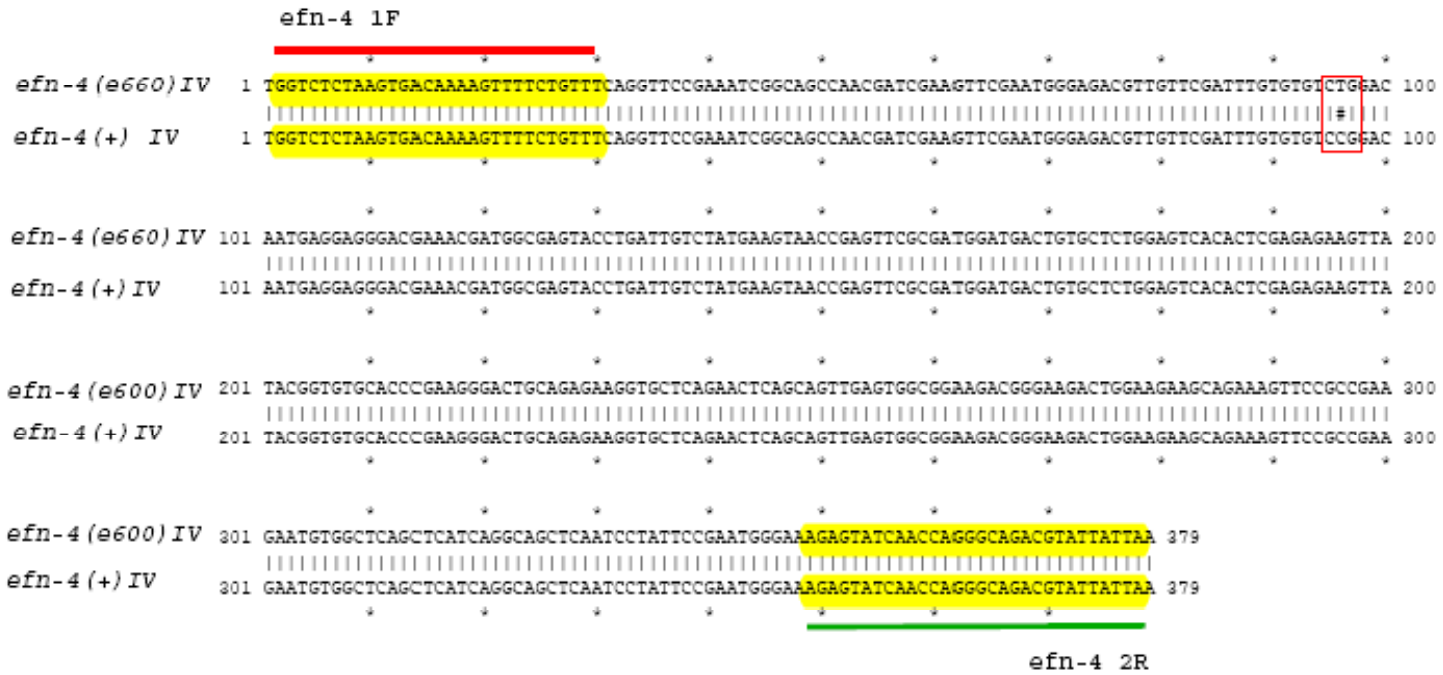
PCR amplification parameters:

Initial denaturation step: 94'C for 2 minutes

Denaturation step: 94'C for 30 seconds
Annealing step: 55'C for 30 seconds.
Elongation step: 68'C for minute.

} 30 cycles.

PCR reaction mix was then loaded on 1% agaose gel with 6X DNA loading dye, and run for electrophoresis at 60mA. The PCR-generated 379bp DNA fragment was cut off the gel and the DNA was purified using QUIAEX gel purification kit following manufacturers' advice. The purified DNA fragment was then sent for sequencing at the Geneservice sequencing centre in Oxford using efn-4 1F and efn-4 2R primers.



Sequence alignment of *efn-4* locus (as sequenced using efn-4 1F and efn-4 2R primers, highlighted sequences). Top is the sequence of *efn-4(e660)* mutants generated in a *lat-1* balanced background as described in the crossing scheme above. Bottom is the sequence of wild type efn-4 locus amplified from N2 worms and sequenced using the same efn-4 1F and efn-4 2R primers. *e660* mutation is a CCG to CTG mutation (Chin-Sang, Moseley et al. 2002). The e660 mutation is thus confirmed as is shownin the red box.

4.1.9. ***lat-1* genotyping**. The heterozygosity of *lat-1* locus was confirmed by genotyping as described in the following: worm lysis was performed as described in the previous section (section **4.1.7** of this chapter) and the PCR-based genotyping was performed using the following PCR parameters (for a detailed explanation on worm genotyping refer to www.wormbook.org)

PCR mix for 30 ul reaction:

21.5ul dH2O
0.25ul lat-1_17R primer (20mM) for a final concentration of 165 nM
0.25ul lat-1_15F primer (20mM) for a final concentration of 165 nM
0.25ul VC965/10R primer (20mM) for a final concentration of 165 nM
0.5ul dNTPs (25mM each) for a final concentration of 416 nM
3ul 10xTaq buffer (containing $MgCl_2$)
0.25ul Taq polymerase (1.25 Units)
5ul DNA template (non-purified from whole worm lysate)

PCR amplification parameters:

Initial denaturation step: 94'C for 2 minutes

Denaturation step: 94'C for 30 seconds
Annealing step: 55'C for 30 seconds.
Elongation step: 68'C for minute.

} 30 cycles.

lat-1 genotyping of wild type worms results in a single 0.7kb~ band whereas *lat-1* mutant heterozygous generate a 0.7kb~ and an additional 0.3kb~ band. *lat-1* mutants generate a single 0.3kb~ band.

4.1.10. Blastomere division time. Embryos were recovered from the uterus of hermaphrodites of the desired genotype and mounted on a slide for microscopy as described in section 4.1.5 of this chapter). The division time of each blastomere was obtained by analysing slow motion reply of the videos. The division time was noted as the time point at which the mitotic spindle elongates. Each recording was post-processed at least three times. The same procedure and parameters were applied for the scoring of each blastomere in each of the recorded embryos. The number of embryos assessed is: N2 (n=13), XA7542 [*lat-1(ok1465)*] (n=52).

4.2. Scoring the vulva phenotypes. *lat-1* mutant hermaphrodites of different developmental stages (L1, L4 and adults) were scrutinised for defects in vulva morphology following the procedures described below.

4.2.1. *C. elegans* strains. Worm strains were cultured and maintained according to standard procedures described by S. Brenner (Brenner 1974). Animals were cultivated at 22'C unless otherwise noted. The animals described as wild type were the *C. elegans* variety Bristol strain N2. The following genotypes were used:

LG II: *lat-1(ok1465)* [XA7500] (Langenhan, Promel et al. 2009), *lat-1(ok1465) lat-2(tm463)* [XA7557].

LG III: *unc-32(e189); lin-12(n676n930)/eT1 him-5.* eT1 is a reciprocal translocation that suppresses recombination on the right arm of chromosome III and the left arm of chromosome V, and that causes markers in these two regions to appear linked (Sundaram and Greenwald 1993) (Greenwald and Seydoux 1990). *lin-12* mutants were selected by picking UNC hermaphrodites.

For each phenotype (i.e. number of Pn.p cells, the position of the AC and the morphology of L4 vulva) more than 300 worms were scored for each genotype. For the developmental distribution of vulva defects, the number of worms scored was as follow: wild type (n=529), *lat-1* single mutants (n=894), *unc-32 lin-12* double mutants (n=456).

4.2.2. Microscopic visualisation of vulva morphology. Vulva defects were scored at different developmental stages. L1 larvae were selected from bleach-synchronised worm populations, immobilised with sodium azide, then mounted on the slide to be examined under Leica DM RXA2 microscope fitted with Quimaging Regita Exi digital camera at 100X magnification. The 11 Pn.p cells (VPCs) were identified and counted as described in (Sulston and Horvitz 1977). Mid to late L3 hermaphrodites were selected from synchronised worm population to score for the position of the Anchor Cell (AC) which occupies the midventral position during L3. L4 vulva defects, protruding and ruptured vulva defects were scored based on strict morphological abnormalities affecting the Christ-mass tree shape of the vulva at this developmental stage (Eisenmann and Kim 2000). Multivulva phenotype was observed at both L4 and adult stages, and a worm was scored positive for a multivulva phenotype if it has an additional invagination (pseudo vulva) at either sides of the main vulva invagination.

4.3. Complementation's and deficiency tests. Complementation tests with deficiencies were conducted as principally described in (Sigurdson, Spanier et al. 1984). All deficiencies used in this study are of the genotype *Df/mnC1[dpy-10(e128) unc-52(e444])II.* Deficiencies shown to cover *lat-1* were selected based on wormbase annotation of the current mapping data (www.wormbase.org). Strains used for complementation assay are as follow:

SP426 strain [*mnDf16/mnC1 dpy-10(e128) unc-52(e444)II*]

SP543 strain [*mnDf30 unc-4(e120)/mnC1 dpy-10(e128) unc-52(e444)II*]

SP637 strain [*unc-4(e120) mnDf68/mnC1 dpy-10(e128) unc-52(e444)II*]

SP552 strain [*mnDf39 unc-4(e120)/mnC1 dpy-10(e128) unc-52(e444)II*]

KK112 strain [*mel-8(b309) unc-4(e120) sqt-1(sc13)/mnC1 dpy-10(e128) unc-52(e444) I.*]

KK1557 strain [*mel-9(b293)II*]

EW10 strain [*pvl-5(ga87) II*]

Complementations with deficiencies were preformed by mating 10-15 *ok1465/mIn1[mIs14 dpy-10(e128)] II; him-8(e1489) IV* males with a single deficiency hermaphrodite (*Df/mnC1[dpy-10(e128) unc-52(e444])II*. The harem was transferred daily to new OP50 seeded NGM plates and the out cross progeny was incubated at 22'C and until it reached the adult stage. Each deficiency complementation test was performed in triplets. Using UV-illuminated dissecting microscope , the GFP label present in *mln1* and the DPY phenotype linked to *mnC1* balancer were used to identify the presumptive genotypes as follows: GFP positive outcross progeny: *mln1/mnC1* (these are GFP + and DPY), *mln1/Df* (these are GFP + and non-DPY). GFP negative outcross progeny: *ok1465/mnC1* (non-GFP non-DPY but selfing throws DPY) and *ok1465/Df* (non-GFP non-DPY). Non-complementation of *lat-1(ok1465)* for a deficiency was based on GFP+/GFP- ration. Deficiencies that complement *lat-1(ok1465)* segregate an outcross progeny with a 1:1 GFP+/GFP- ration. However, non-complementation of *lat-1(ok1465)* was indicated by the absence of *ok1465/Df* trans-heterozygotes due to the *lat-1*-caused L1 arrest and thus a GFP+/GFP- ratio of 2:1. Deficiency strains were all obtained from CGC.

Allelic complementation tests were conducted by analysing the phenotype of double heterozygote (i.e. outcross progeny) following the same principle described in (Yook, Proulx et al. 2001). Complementation tests with alleles of known molecular description (i.e. that can be identified either by genotyping or sequencing) were directly used in complementation crossing, however in cases where the molecular lesion of tested alleles was not previously described, we used deficiencies that were reported to cover the allele tested for complementation (based on the current mapping data, accessible at www.wormbase.org, *mnDf39* covers *pvl-5*, *mnDf168* covers *mel-8* and *mel-9*, and *mnDf16* covers *pvl-4*). Each allele was first confirmed by deficiency complementation using the herein indicated deficiencies. Allelic complementation assay was performed by crossing 10-15

ok1465/mIn1[mIs14 dpy-10(e128)] II; him-8(e1489) IV males with one mutant hermaphrodite (*pvl-5(ga87), pvl-4(ga96)*). The mating parents were transferred daily to new OP50 seeded plates and outcross progeny incubated at 22'C until it reached the adult stage. Similar to deficiency complementation, allelic complementation was indicated by the 1:1 GFP/non-GFP ration for example,non-complementation would be indicated by the absence of *ok(1465)/pvl-5(ga78)* or *ok(1465)/pvl-4(ga96)* adult outcross progeny.

Allelism between *lat-1* and *mel-8* and *mel-9* was tested using the paternal rescuing characteristic associated with the partial maternal lethality of *mel-8* and *mel-9* (Kemphues, Kusch et al. 1988). 10-15 *ok1465/mIn1[mIs14 dpy-10(e128)] II; him-8(e1489) IV* males were mated with single *mel-8* or *mel-9* mutant hermaphrodites (experiment was performed in 10 replicates), the harem transferred daily to new plates and the outcross progeny was incubated at 22'C (*mel-9b(293)* cross was performed at 15'C because *b293* is a temperature sensitive allele, (Kemphues, Kusch et al. 1988)). No-allelism was indicated by the ability of paternal *lat-1(ok1465)* to rescue the maternal effect of *mel-9* and *mel-8*.

IV. Chapter IV: Investigating the function of LAT-1 in neurotransmission: Results and Discussion

An uninterrupted communication between the external environment and the living organism is essential to coordinate biological responses with environmental stress and signals. Furthermore, cell-cell communication is essential during key developmental processes such as cell differentiation, migration, axon outgrowth and so forth. Amongst such cell communication methods is the secretion of neurotransmitters. Specific neurotransmitters become released from specialised neuron terminal structures (pre-synaptic vesicles, SV) into the synaptic cleft where the neurotransmitters are free to bind their receptors usually attached to the membranes of target cells (nerve or muscle) on the other half of the synaptic cleft. Exocytosis of neurotransmitters controls and modulates several behavioural aspects in *Caenorhabditis elegans* such as locomotion, feeding, egg laying, male mating and axon outgrowth (Raizen, Lee et al. 1995; Winnier, Meir et al. 1999; Hallam, Singer et al. 2000; Bany, Dong et al. 2003; Towers, Edwards et al. 2005).

The physiological state of neurotransmission in *C. elegans* can be addressed using known neuromodulators whose effect on neurotransmission are well characterised. In order to further investigate the involvement of LAT-1 function in neurotransmitter release (Frontali, Ceccarelli et al. 1976; Lelianova, Davletov et al. 1997), we sought at analysing the state of neurotransmission in *Caenorhabditis elegans lat-1* mutants by scrutinizing the impact of known neuromodulators on different behaviours that are known to be under the influence of neurotransmitter release and regulation.

1. Locomotion

1.1. Acetylcholine release at neuromuscular junction (NMJ) in *lat-1* mutants

1.1.1. Brief introduction

The *Caenorhabditis elegans* latrophilin homolog *lat-1* has been repeatedly implicated in neurotransmission. Toxicology experiments identified latrophilin as a binding partner of α-Latrotoxin (α-LTX) which is a component of the black widow spider venom. The binding of the toxin to its receptor causes massive release of neurotransmitter (acetylcholine) causing paralysis ensuing death (Krasnoperov, Bittner et al. 1997; Lelianova, Davletov et al. 1997; Ichtchenko, Khvotchev et al. 1998; Lang, Ushkaryov et al. 1998). It is noteworthy to state that the release of neurotransmitters from nerve terminals also happens via exocytosis: the neurotransmitter exocytosis mechanism requires the formation of synaptic vesicules that become filled with the neurotransmitter in question before they are docked to the active zone of the pre-synaptic membrane where they are ready to release their content upon changes in the action potential across the membrane. The process of neurotransmitter exocytosis is a very complex one, with a large number of proteins playing pivotal regulatory roles at different stages of neurotransmitter exocytosis. The *C. elegans* model organism has been largely used to identify genes required for neurotransmitter exocytosis and several players have been successfully identified. For example, the vesicular priming proteins *unc-13*/UNC-13 and *unc-64*/Syntaxin have been identified and shown to be required for the SV to become "competent" to fuse with the plasma membrane in response to Ca^{2+} signaling. *C.elegans* mutants in either *unc-13* or *unc-64* show decreased levels of acetylcholine release at neuromuscular junctions (NMJs). In view of this, when subjected to aldicarb treatment *unc-13*/UNC-13 and *unc-64*/Syntaxin mutants are more resistant to the paralytic effect of aldicarb due to their decreased levels of released acetylcholine. Aldicarb is an inhibitor of acetylcholinesterase and causes the accumulation of released acetylcholine at the synaptic

cleft resulting in increased stimulation of the post-synaptic cell, usually leading to paralysis (please refer to Figure 21).

In pursuit of characterizing the mode of action of both the toxin and its receptor, further research findings implicated *lat-1* in neurotransmission by showing that in response to the toxin α-LTX, latrophilin-dependent massive neurotransmission happened through a process that required the neurotransmitter vesicle priming molecule UNC-13, one of the protein necessitated for the docking of the synaptic vesicle prior to the release of neurotransmitters via exocytosis (Willson, Amliwala et al. 2004). The initial finding of the necessity of the vesicual priming protein UNC-13 proposed that the lethal release of neurotransmitter mediated by α-LTX occurs at least partly, through exocytosis. However, the data showing dependency on UNC-13 d has proved difficult to reproduce and studying the structure-function relationship of α-LTX revealed a more promising mode of action of the toxin independent of mobilizing vesicular exocytosis machinery (i.e. the effect of α-LTX on massive transmitter release also occurs through vesicular-independent manner). The lethal release of large amounts of neurotransmitters in the presence of the toxin is largely due to the capacity of the toxin to form high conductance unselective cation pores in the pre-synaptic cell membranes, which enables the influx of Ca^{+2} cations which subsequently triggers vesicle release. The pore formation ability of the toxin is dependent on the tetramerisation of the toxin. Tetramerisation happens in high concentration of the toxin, and Ca^{+2} cations (and other substitute bivalent cations such as Mg^{2+}, hence the unselective characteristic of the pores) help to stabilize toxin tetramers (Ashton, Rahman et al. 2000). Thus, the lethal neurotransmitter release seems to also be the result of the pores formed in the presence of the toxin. Whereas the mode of action of the toxin has been more successfully addressed, the role of latrophilin still remains to be elucidated in light of neurotransmission. In an attempt to shed light on the role of latrophilin in neurotransmission, Lelianova and colleagues addressed the question of whether latrophilin,

being a GPCR, may mediate its function on neurotransmission through G- proteins. Two experimental evidences invite to consider a potential role of Latrophilin receptor in neurotransmission: first, affinity chromatography experiments showed direct binding between *lat-1* and *goa-1* gene products (Lelianova, Davletov et al. 1997). *goa-1* encodes a G-protein $G_{\alpha o}$ which plays a sole role in mediating neurotransmission in several tissues of the *C.elegans* including the neuromuscular junctions (NMJs), pharynx and the egg laying system (Sieburth, Ch'ng et al. 2005; Minaba, Ichiyama et al. 2006; Dittman and Kaplan 2008; Tanis, Moresco et al. 2008). Second, in the vesicular pathway, in which the latrophilin receptor is believed to contribute to the neurotransmitter release in the presence of the toxin, was shown to be dependent on intact internal Ca^{2+} stores and functional phospholipase PLC both of which play pivotal roles in the exocystosis of neurotransmitters (Sugita, Ichtchenko et al. 1998; Ashton, Rahman et al. 2000). In accordance with the toxin-receptor induced neurotransmitter release, an α-LTX mutant LTX^{N4C} lacking the ionophore-like activity of wild type LTX, was shown to be still capable of neurotransmitter release upon binding to its receptor (neurotoxin and/or latrophilin). LTX^{N4C} action which necessitates extracellular calcium, requires mobilisation of internal Ca^{2+}. In view of this, blocking inositol(1,4,5)-triphosphate-induced Ca^{2+} release inhibits the action of LTX^{N4C}. Similarly, inhibiting the action of phospholipase C PLC (which produces IP3 and subsequent release of Ca^{2+} from IP3-sensitive stores) also attenuates the action of LTX^{N4C} further confirming the requirement of mobilizing internal calcium stores for the toxin-receptor induced neurotransmitter release (Capogna, Volynski et al. 2003). The use of pore-formation mutant LTX^{N4C} propose thus evidence for the toxin-receptor mediated release of neurotransmitters therefore implicating its receptor latrophilin in neurtranmitter release through a mechanism that requires the mobilisation of internal calcium stores. Correspondingly, Rahman and colleagues previously showed that latrotoxin stimulates a presynaptic receptor, latrophilin (LPH) which is linked to $G_{\alpha q}/11$ known to be involved in Ca^{2+} homeostasis (Rahman,

Ashton et al. 1999) further confirming the additional mode of action of the toxin involving its receptors including latrophilin (in addition to its ability to form high conductance pores).

To date, all studies referring to a role of latrophilin in neurotransmission involved the use of the toxin, and thus do not reflect the physiological function of latrophilin receptor where the toxin is not naturally present. For this purpose, we used the *C. elegans* latrophilin homolog *lat-1* knockout mutants to investigate the potential biological role of *lat-1* in neurotransmission. And to do so, we utilised the acetyl cholinesterase inhibitor aldicarb, and analysed its effect on *lat-1* mutants.

Aldicarb (2-methyl-2-(methylthio) propanal O-(N-methylcarbamoyl oxime) inhibits the action of acetylcholinesterase (Opperman and Chang 1991), and aldicarb treatment results in the accumulation of acetylcholine at the synapse and leads to paralysis due to prolonged muscle contraction (refer to Figure 22 for a description of the aldicarb effect). Worms with mutations that cause reduction in acetylcholine (Ach) release are thus resistant to aldicarb, whereas mutations increasing the release of Ach render the worms sensitive/hypersensitive to aldicarb and cause paralysis and subsequent death (Opperman and Chang 1991; Mahoney, Luo et al. 2006).

Accordingly, the use of aldicarb provides us with a simple method to assess the scope of Ach release at the neuromuscular junction (NMJs) in *lat-1* mutants.

Pharmacology experiments involving the use of α-Latrotoxin (α-LTX) showed that upon binding of the toxin to its latrophilin receptor, massive neurotransmission is triggered leading to paralysis and death. Based on these toxin-based experiments, three possible hypotheses can be proposed:

1) α-LTX binds latrophilin and inhibits its function, giving latrophilin a negative regulatory effect on neurotransmission.

2) α-LTX binds its receptor latrophilin and enhances its function in neurotransmission, causing massive release of neurotransmitters

3) The third hypothesis emerged from the structure-function studies which showed that fragmented or even truncated versions of LAT-1 (lacking the entire C-terminus and 7-TM domain) are capable of elucidating massive neurotransmitter release in presence of α-LTX despite their inability to interact with intracellular G-proteins (Sugita, Ichtchenko et al. 1998). The third hypothesis proposes thus, that the receptor latrophilin functions as a "tether" for the toxin to concentrate the latter at the presynaptic membrane, and the toxin then causes massive neurotransmission (Ichtchenko, Khvotchev et al. 1998). Finally, α-LTX was shown to form high conductance channels in membranes upon binding to its receptor providing an explanation for this secretagogous potential of the toxin-receptor complex (Ashton, Rahman et al. 2000; Hlubek, Stuenkel et al. 2000). *C.elegans* latrophilin homolog, like other α-LTX receptors (e.g. neurexins) would thus function as a tether to concentrate the toxin at the membranes. Still to be elucidated though, is whether latrophilin receptor plays a physiological role in neurotransmission and its regulation.

Cases of hypersensitivity to aldicarb include mutations in genes with negative regulatory effect on neurotransmission in general and Ach release in particular (Sieburth, Ch'ng et al. 2005; Mahoney, Luo et al. 2006). Hypersensitivity to aldicarb is described as the sensitive aptitude (manifested as paralysis) of worms in response to aldicarb at a lower concentration or a shorter exposure period compared to control animals.

1.2 Results and discussion

1.2.1 *lat-1* mutants are sensitive to inhibitors of acetylcholinesterase

In the hope to further understand the potential role latrophilin *lat-1* plays in neurotransmission we aimed at shedding light on the state of neurotransmission at the level of neuromuscular junctions (NMJs) by testing the response of *lat-1* mutants to aldicarb. Our

results showed that *lat-1* mutants are sensitive to aldicarb suggesting an increased acetylcholine release at the NMJs in our mutants (Figure 21).

As previously stated, sensitivity to inhibitors of acetyl cholinesterase is assessed by "rapid" onset of paralysis when compared to wild type controls. In viw of this, our results indicate that *lat-1* mutants become paralysed at an earlier time point as opposed to wild type controls. After one hour of treatment, nearly 50% of *lat-1* mutant worms were completely paralysed as opposed to 0% paralysis observed in wild type controls. Moreover, rescue lines previously shown to partially rescue *lat-1* lethality were not able to bring *lat-1* mutants' response to aldicarb to wild type levels. The inability to rescue the response to aldicarb could be due to over expression. Additionally, the rescuing line bearing the wild type copy of *lat-1* is not integrated in the genome, and mosaic and tissue-unspecific expression could interfere with control of gene expression especially under such challenging conditions.

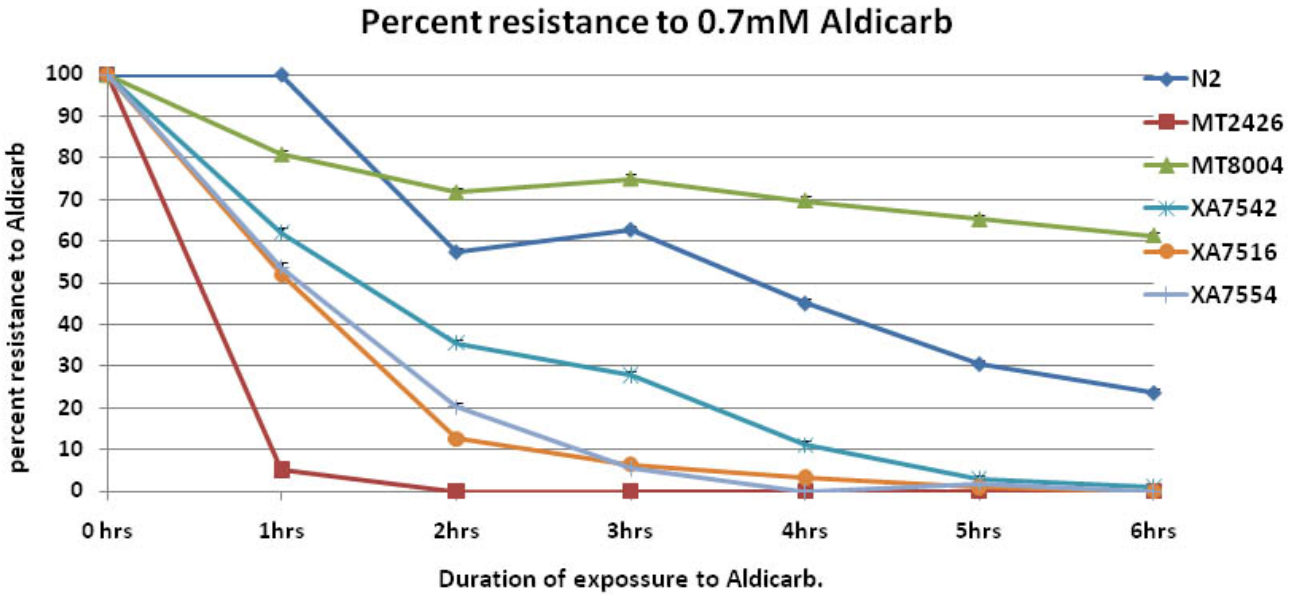


Figure 21 . *lat-1* mutants are sensitive to inhibitors of acetylcholinesterase. A graph showing the resistance pattern to 0.7mM Aldicarb. Strains used are as follows: wild type strain N2, hypersensitive strain MT2426[*goa-1(n1134) I*] harbouring a mutation is Gαo which is a negative regulator of acetylcholine release at neuromuscular junctions (NMJ), resistant strain MT8004[*unc-13(n2813) I*] harbouring a mutation in the vesicle priming protein UNC-13 a component of the SNAR complex necessary for acetylcholine release at NMJ, *lat-1* mutant strain XA7542[*lat-1(OK1465) II*], *lat-1* early pharyngeal rescuing line XA7554 [*lat-1(ok1465) II*; *qaEx7529*[*M05B5.2::LAT-1A unc-122::RFP*], and the full genomic rescue line XA7516 [*lat-1(ok1465) II*; *qaEx7513*[*pTL2(minicosmid) rol-6(su1006)*]. *lat-1* mutants (XA7542) become quickly paralysed when compared to wild type worms. After one hour of treatment with 0.7mM of aldicarb nearly 50% of *lat-1* mutants were paralysed compared to 0% paralysis in wild type worms. Following six hours of treatment, all *lat-1* mutant worms were paralysed while almost 25% of wild type worms were still crawling on the plate. On the other hand, the *goa-1* mutant worms which exhibit increased release of acetylcholine show same level of paralysis as *lat-1* mutants after six hours of treatment (although *goa-1* mutants become paralysed at an earlier time point, placing the sensitivity of *lat-1* mutants in between wild type and hypersensitive levels). The rescuing lines XA7516 and XA7554 that were previously shown to partially rescue lethality associated with *lat-1* mutation (Tobias Langenhan, personal communication) were not able to restore aldicarb sensitivity to wild type levels may be due to non-physiological levels of LAT-1 protein, over expression, or failure to coordinate *lat-1* expression under such stressful experimental conditions. Triplets of 60 worms were used for each genotype and the experiment was repeated three times, error bars indicate SEM.

Interestingly, G-protein $G_{\alpha o}$ was shown to be a negative regulator of Ach release at the neuromuscular junction and that reduction or loss of function mutations in *goa-1* render worms sensitive to aldicarb (Miller, Emerson et al. 1999). There are thus similar phenotypes between *lat-1* and *goa-1* mutants in their response to inhibitors of acetylcholinesterase in addition to resemblances in reduced pharyngeal pumping rate to be discussed in the next section. Therefore growing evidence brings *lat-1* and *goa-1* to a close phenotypic relationship. First Lelianova and colleagues showed physical interaction between *goa-1* and *lat-1* gene products (Lelianova, Davletov et al. 1997), second *lat-1* mutant lethality becomes more that just additive in *goa-1* mutant background (Lelianova, Davletov et al. 1997) and third, our experiments indicate a similar phenotype between *lat-1* and *goa-1* mutants in response to inhibitors of acetyl cholinesterase. This similar Hic phenotype (**H**ypersensitive to **I**nhibitors of **C**holinesterase) led us to ask the questions of whether *lat-1* and *goa-1* function in the same or parallel pathways in down regulating the release of acetylcholine at the neuromuscular junctions and whether *lat-1*'s function in neurotransmission is also observed in tissues and organs other than the neuromuscular junction.

For future experiments, epistasis genetics between *lat-1* and *goa-1* should be performed to see whether *lat-1* and *goa-1* function in the same or parallel pathways by testing the sensitivity of the double mutants to aldicarb. Additionally, rescueing experiments (such as the use of a rescueing *lat-1*or *goa-1* constructs) in the *lat-1;goa-1* double mutants would enable us to see whether *goa-1* functions upstream or downstream of *lat-1* in inhibiting the release of Ach at the NMJs.

1.2.2. *lat-1* mutants are unresponsive to serotonin-mediated tolerance to aldicarb

In order to further investigate the significance of the similar Hic phenotype observed in *lat-1* and *goa-1* mutants, we used the neuromodulator serotonin. Serotonin (5-

hydroxytryptamine, 5-HT) was previously shown to reduce sensitivity to aldicarb as far as locomotion is concerned: endogenous serotonin confers resistance to aldicarb as mutations in serotonin biosynthesis render worms hypersensitive to aldicarb, and exogenous serotonin (5-HT) treatment was shown to confer resistance to aldicarb through a pathway that involves *goa-1* and *dgk-1* (Figure 22) (Mendel, Korswagen et al. 1995; Segalat, Elkes et al. 1995; Nurrish, Segalat et al. 1999). The mechanism by which *goa-1*/$G_{\alpha o}$ confers resistance to aldicarb in the presence of serotonin was later revealed by Nurrish and colleagues. They showed that *goa-1*/$G_{\alpha o}$ deceases the abundance of UNC-13 at the synapse causing thus reduced acetylcholine release (Nurrish, Segalat et al. 1999). The exocytosis mechanism of neurotransmitter release from synaptic nerve terminals requires a harmonious coordination of several proteins that include the SNARE complex (soluble *N*-ethylmaleimide sensitive factor attachment receptor). Syntaxin is a member of the SNARE complex and it is required for the synaptic vesicle fusion with the plasma membrane. *unc-13*/UNC-13 is a diacylglycerol (DAG) binding protein with multiple C2 Ca^{2+}binding domains (Maruyama and Brenner 1991; Ahmed, Maruyama et al. 1992). UNC-13 proteins are required for exocytosis in *Caenorhabditis elegans*, mammals, and *Drosophila* (Brose, Hofmann et al. 1995; Betz, Ashery et al. 1998; Aravamudan, Fergestad et al. 1999; Tokumaru and Augustine 1999; Richmond 2005). UNC-13 and syntaxin physically interact (Betz, Ashery et al. 1998; Sassa, Harada et al. 1999; Tokumaru and Augustine 1999) and it is believed that UNC-13 is required to stabilise the "open" form of syntaxin which permits priming of recruited SV (synaptic vesicle) so that they become competent to fuse with the plasma membrane in response to Ca^{2+} signaling. (for a detailed description of the different steps governing the neurotranmitter release from nerve terminal in *C. elegans*, please refer to (Richmond 2005)). In their 1999 study, Nurrish and colleagues provided evidence for the mode of action of exogenous serotonin in down regulating acetylcholine release from nerve terminals: serotonin through its functional coupling with *goa-1*/$G_{\alpha o}$ would indirectly stimulate the *dgk-1* DAG kinase which upon phosphorylating its DAG targets reduce their availability to

UNC-13, thus reducing the amount of UNC-13 recruited at nerve terminals. The functional coupling of serotonin with goa-1/$G_{\alpha o}$ was evidence by genetic studies showing that *goa-1*/Gao which function upstream of *dgk-1* mutants are irresponsive to serotonin-mediated resistance to aldicarb (Mendel, Korswagen et al. 1995; Segalat, Elkes et al. 1995).

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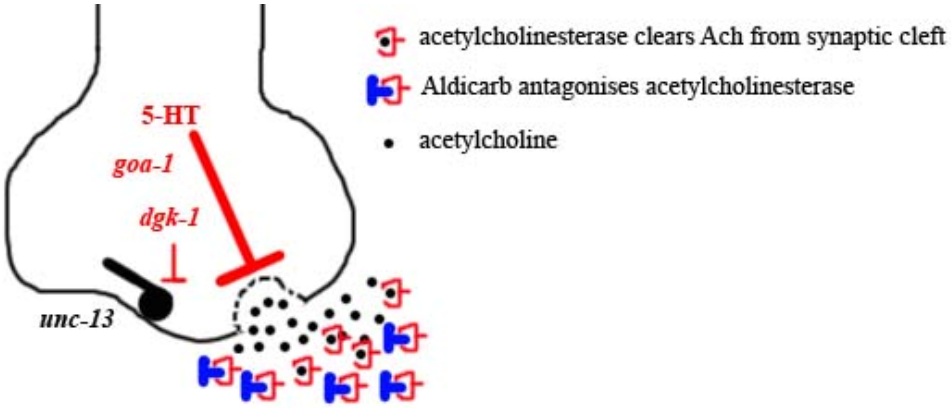
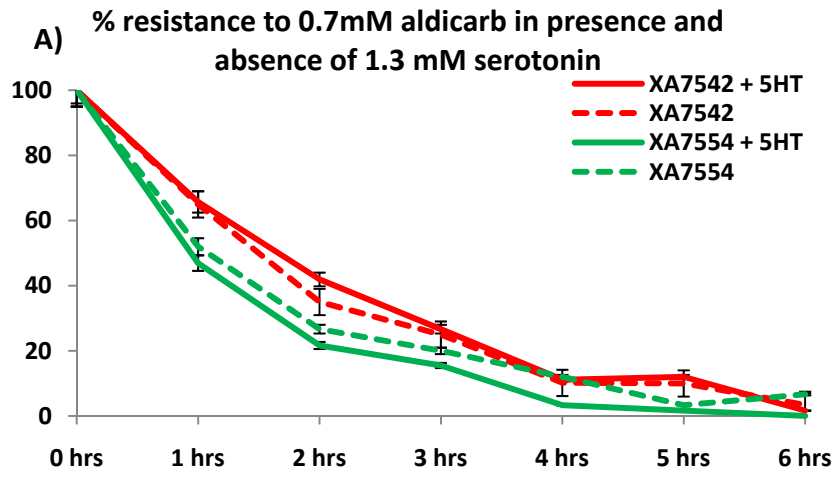
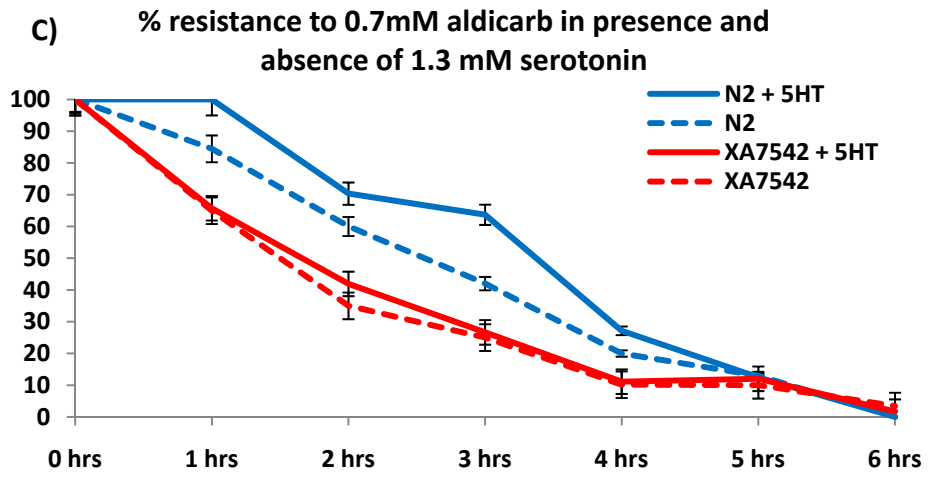


Figure 22: Schematic diagram explaining the mechanism by which serotonin confers resistance to the inhibitor of acetyl cholinesterase (aldicarb). Acetylcholine is released into the synaptic cleft and acts upon the postsynaptic target cells (not shown). The strength of the neurotransmitter signal is determined by the duration of the signal and the amount of the neurotransmitter available at the synaptic cleft. Amongst the several mechanisms regulating neurotransmission is the clearance of acetylcholine from the synaptic cleft by acetyl cholinesterase. Inhibiting the action of acetyl cholinesterase causes accumulation of acetylcholine at the synaptic cleft and over activation of post synaptic muscle cells leading to continuous muscle contraction and subsequent paralysis. Aldicarb inhibits the action of acetyl cholinesterase causing the build up of acetylcholine. Mutations that reduce the release of acetylcholine confer resistant to aldicarb due to the initial reduction of available neurotransmitter. Exogenous serotonin also confers resistance to aldicarb partly through a pathway that requires the negative regulator *goa-1* and *dgk-1* at the neuromuscular junctions (NMJs). Serotonin fails to reduce sensitivity to aldicarb in *goa-1* mutants (Nurrish, Segalat et al. 1999). The ability of serotonin to reduce sensitivity to aldicarb was widely used to identify genes required for the negative regulation of acetylcholine release at NMJs (Mahoney, Luo et al. 2006).

To further investigate the significance of the similarity in phenotypes between *lat-1* and *goa-1* mutants in the context of regulating neurotransmission we tested the ability of serotonin to confer resistance to aldicarb in *lat-1* mutants. Interestingly, serotonin was capable to reduce aldicarb-induced paralysis in wild type but not in *lat-1* mutant worms (Figure 23). This finding assigns a new phenotype to *lat-1* mutants in that they are irresponsive to serotonin as far as resistance to aldicarb is concerned. This observation raises the question of whether *lat-1* function is required in the same or in a parallel pathway to *goa-1* in down regulating Ach release in response to serotonin, or whether LAT-1 is necessitated in general in cells to respond to serotonin signals. The latter possibility can be directly tested by analysing the *lat-1* mutants' response to serotonin in tissues and organs other than the NMJs such as egg laying and pharyngeal pumping (described in the next sections). The fact that serotonin-mediated resistance to aldicarb happens through a process that seems to require LAT-1 strengthens the hypothesis that *lat-1* functions in down regulating neurotransmission. Additionally, it invites for the following additional propositions: first would be that the $G_{\alpha o}$-mediated reduction of acetylcholine release at neuromuscular junction is defective in *lat-1* mutants suggesting that *lat-1* functions in the same pathway as *goa-1*, second, *lat-1* negatively regulates Ach release in parallel to *goa-1* through a novel negative regulatory pathway that is also influenced by exogenous serotonin. Analysis of *lat-1;goa-1* double mutants' response to aldicarb and serotonin are required address such questions. It would be interesting to see if *lat-1;goa-1* double mutants are more resistant to aldicarb in the presence of exogenous serotonin than either single mutants as that would suggest theat the two genes function in parallel to dowregulate Ach release at the NMJs. Additionally, it would be interesting as well to use the hyperactive allele of *goa-1* in a *lat-1* mutant background and see if it rescues the aldicarb sensitivity, as this would further confirms that sensitivity to aldicarb is actually caused by increased Ach release in *lat-1* mutants, or whether a *goa-1* over expression rescues *lat-1* sensitivity to aldicarb.





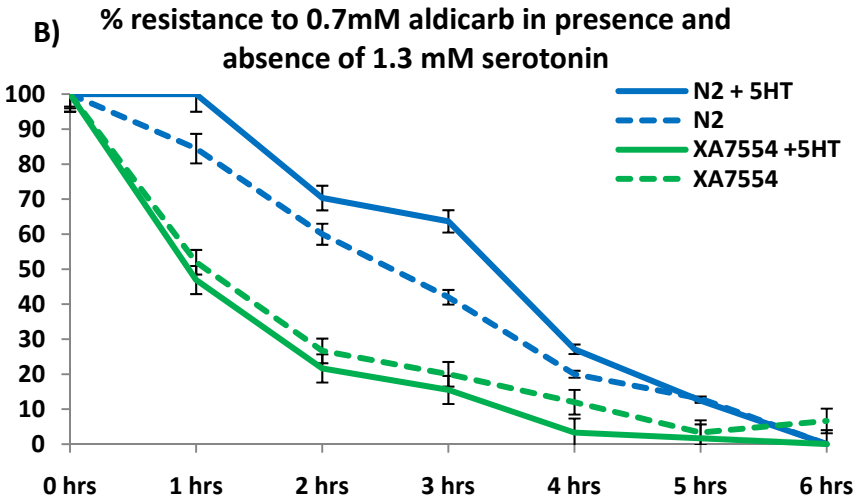


Figure 23. Percent resistance to 0.7mM aldicarb in presence and absence of 1.3mM serotonin (5-HT). Serotonin fails to reduce sensitivity to aldicarb in *lat-1* mutants. Strains used are as follows: wild type strain N2, *lat-1* mutant strain XA7542 *[lat-1(OK1465) II]* and *lat-1* early pharyngeal rescuing line XA7554 *[lat-1(ok1465) II; qaEx7529[M05B5.2::LAT-1A unc-122::RFP]*. Aldicarb causes worm paralysis in wild type and *lat-1* mutants. In wild type worms, serotonin was able to reduce sensitivity to aldicarb as indicated by the increased percentage of resistant worms treated with serotonin (panels B and C: solid Vs dashed blue lines). On the other hand, sensitivity to aldicarb was not ameliorated in *lat-1* mutants when treated with serotonin: there was no significant difference between *lat-1* mutants with or with out serotonin treatment (Panels A and C: solid Vs dashed red lines). Like in aldicarb experiment, the rescuing line XA7554 *[lat-1(ok1465) II; qaEx7529[M05B5.2::LAT-1A unc-122::RFP]* was not able to render *lat-1* mutants worms responsive to serotonin-mediated decrease of Ach release as indicated by the inability to reduce sensitivity to aldicarb). Quite the contrary, the early pharyngeal rescuing line made worms even more paralysed when treated with both serotonin and aldicarb (A and B). This could be due to over expression, and it also underlines the requirement of tight control over expression pattern. Error bars indicate SEM.

Our experiment did not aim to determine the response of *lat-1* mutants to serotonin as such, but to analyse the downstream effectors that respond to serotonin and lead to reduced Ach release. Our results propose that LAT-1 is required in a pathway through which serotonin reduces Ach release from the ventral cord motor neurons. While serotonin reduces aldicarb sensitivity through a pathway that engaged Gαo and UNC-13, the mechanism by which *lat-1* hypothetically reduces Ach release is still to be elucidated. In view of this, it would be interesting to analyse the abundance of UNC-13 for example at the presynaptic vesicles and see whether it is also reduced in *lat-1* mutants as is the case in *goa-1* mutants.

It is possible that *lat-1* functions in down regulating Ach release through a pathway that does not directly affect the synaptic vesicle cycle. For instance, mutations in *slo-1* which encodes a calcium-activated potassium channel also cause sensitivity to aldicarb. Experimental studies using emodepside which is a nematocide, showed that emodepside also reduces the sensitivity to aldicarb by activating *slo-1*. Potassium channels are well documented for their role in pre-synaptic regulation of neurotransmitter release with reports of both increased and decreased release (Robitaille, Adler et al. 1993; Robitaille, Garcia et al. 1993; Xu and Slaughter 2005) through a pathway independent of the synaptic vesicle cycle. However, it seems unlikely that *lat-1* responds to serotonin-mediated decrease of Ach through a pathway that engages potassium channels because emodepside reduces the locomotive paralysis caused by aldicarb in *lat-1* mutants (Guest, Bull et al. 2007). Furthermore, previous studies showed that in the presence of the toxin, latrophilin caused elevated cytosolic IP3 and cAMP in transfected COS cells (Lelianova, Davletov et al. 1997). IP3 function is required in the regulation of internal Ca^{+2} stores (Taylor, Taufiq Ur et al. 2009) keeping note that local rise in intracellular Ca^{2+} is required during synaptic vesicle fusion (Richmond 2005) suggesting an additional route through which *lat-1* might influence neurotransmitter release in that it plays a role in Ca^{2+} movement which indirectly affects synaptic vesicle fusion and subsequent release of neurotransmitters. Like many other

intracellular cascades, signaling pathways show a high degree of cross talk. In view of this, genetic analysis shows that activated Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is suppressed by perturbations of $G_{\alpha o}/G_{\alpha q}$ signaling network, which include loss of the GOA-1 $G_{\alpha o}$ (Robatzek, Niacaris et al. 2001). One approach to investigate the possibility of *lat-1* involvement in regulating Ca^{2+} release from internals stores and/or Ca^{2+}oscillation across synaptic membrane is to analyze Ca^{2+} currents in *lat-1* mutants. The fact that serotonin does not reduce sensitivity to aldicarb in *lat-1* mutants and the fact that *lat-1* is less likely to exert its function through potassium channels (based on emodepside experiments, (Guest, Bull et al. 2007)), suggest that serotonin influences several other pathways in addition to regulating the function of G-proteins. The routes through which serotonin (a widely used neuromodulator, which shows high potential for therapeutic applications) exerts its effects have not been characterised to completion, and our results suggest that thisis far from reaching a near-saturation point.

Furthermore, abnormalities in the response to exogenous stimuli (here serotonin) concentrates the defect on responding cells rather than serotonergic neurons defects, thus the resistance to serotonin-induced rescue of paralysis (caused by aldicarb) in *lat-1* mutants is unlikely to be due to serotonergic neuron defects. Additionally, serotonin downstream targets are differentially required in target tissues, for instance, *dgk-1* (encoding the ortholog of mammalian diacylglycerol kinase theta (DGKQ)) is required to respond to serotonin effect on locomotion but not on egg laying illustrating the complexity of serotonin modulation of behaviours (Nurrish, Segalat et al. 1999). In order to further analyse the requirement of *lat-1* in serotonin-mediated modulation of neurotransmission, we analysed other behaviours also known to be modulated by exogenous serotonin such as egg laying and pharyngeal pumping.

2. Pharyngeal pumping

2.1. Brief introduction

The free living nematodes *Caenorhabditis elegans* feed on bacteria through pharyngeal muscle contraction-relaxation cycles that transport bacteria posteriorly toward the terminal bulb from which it then enters the intestine through the pharyngeal-intestinal valve (Figure 24).

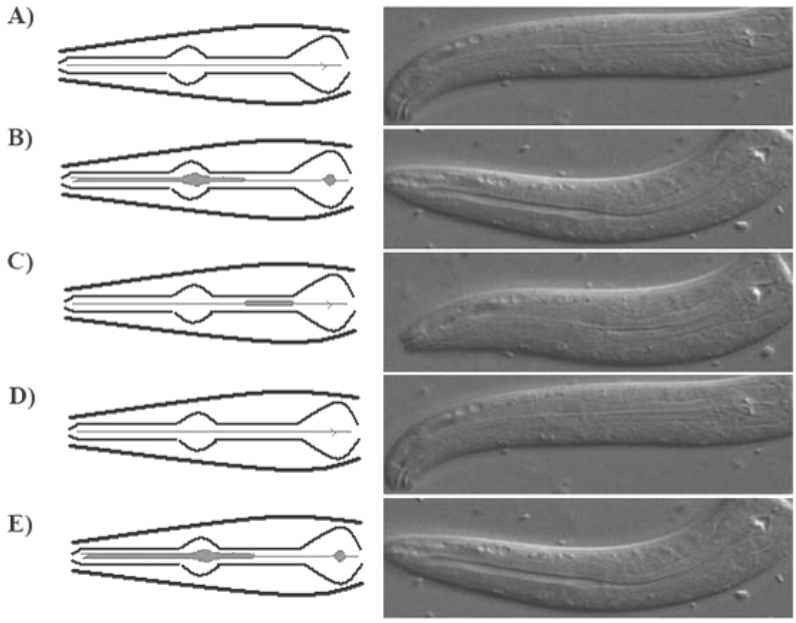


Figure 24: Schematic diagram of the mechanics of feeding in *C. elegans*. Two pharyngeal muscle movements govern the feeding process: pharyngeal pump and peristaltic movement. A pump is defined as the contraction-relaxation sequence in which take part all of the corpus, anterior isthmus and the terminal bulb in a near simultaneous contraction-relaxation cycles. A) during relaxation, the pharyngeal lumen is closed and the contraction of corpus, anterior isthmus and the terminal bulb opens the pharyngeal lumen (B) allowing the passage and trapping of bacteria which is then passed to the terminal bulb through the peristaltic movement of the posterior isthmus (C). The relaxation of corpus, anterior isthmus and the terminal bulb (D) takes place before a second pump starts again (E).

The rate of pharyngeal pumping varies according to the environment; it decreases in absence of food and increases upon availability of abundant food to reach approximately 250 pumps per minute (Avery and Horvitz 1990). Thus, regulating the rate of pharyngeal pumping not only does it represent an adaptation behaviour but also it is necessitated for proper coordination of muscle contraction and relaxation (defects disturbing coordinated movement of pharyngeal muscle perturb the feeding process and the growth rate (Avery 1993; Avery 1993)). Laser killing experiments showed that of the 20 pharyngeal neurons, MC and M3 motor neurons play sole roles in modulating the rate of the pharyngeal muscle contraction and relaxation respectively (Avery and Horvitz 1989).

MC is a sensory excitatory cholinergic motor neuron (Raizen, Lee et al. 1995) which upon firing triggers the release of Acetylcholine (Ach). Postsynaptically, Ach release causes depolarization across the pharyngeal muscle membrane and subsequent muscle contraction. M3 neuron on the other hand, is a glutamatergic inhibitory motor neuron (Dent, Davis et al. 1997; Li, Avery et al. 1997). Firing of M3 motor neuron occurs during the pharyngeal action potential and it causes the release of glutamate, which acts on the glutamate-gated chloride channel and results in inhibitory post synaptic potentials that can cease the muscle action potential thus causing muscle relaxation (Dent, Davis et al. 1997; Li, Avery et al. 1997).

2.2. Serotonin modulates pharyngeal pumping

Action of these two motor neurons (i.e. MC and M3 neurons) can be modulated by exogenous serotonin which increases the rate of pharyngeal pumping (Niacaris and Avery 2003). Electropharyngeogram (EPG) studies showed that serotonin-mediated increase of pharyngeal pumping rate is due to decreased duration of action potential and rapid repolarisation across postsynaptic membrane, thus allowing more pumps to happen in a shorter time. Serotonin-mediated shortening of action potential duration is partially due to the activation of M3 motor neuron to cause rapid repolarisation as indicated by EPGs

analyses in *eat-18; avr-15* double mutants where serotonin was not able to reduce duration of action potential due to defects in M3 signaling in *avr-15* mutants (AVR-15 is a subunit of the postsynaptic receptor for M3 and is required for muscles to respond to M3 motor neurons, (Dent, Davis et al. 1997), his study was performed in a genetic background that also uncouples the function of MC motor neuron through *eat-18* mutation). Additionally, serotonin proved able to reduce duration of action potential in a genetic background that functionally uncouples M3 neurons (*avr-15* single mutants) indicating that the action of serotonin on increasing pumping frequency is not solely dependent on M3 neuron pair. Simultaneously, in achieving increased pumping rate, serotonin acts on MC motor neurons to induce rapid depolarisation in a manner that require EAT-18 and EAT-2 (Figure 25) (Niacaris and Avery 2003). Interestingly, depletion of endogenous serotonin experiments suggested the presence of an additional mechanism to shorten the duration of action potential in a fashion independent of serotonin and M3 signaling(Niacaris and Avery 2003).

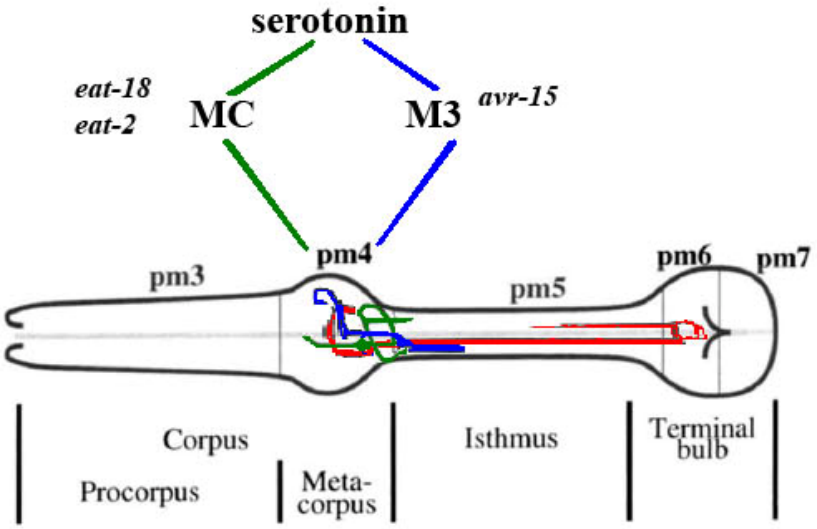


Figure 25: Schematic diagram illustrating the effect of exogenous serotonin on the excitability of pharyngeal muscles. Three neurons are required for effective feeding process: MC, M3 and M4 neuron pairs. MC are cholinergic excitatory neurons, located within the metacarpus and innervates pm4 to cause muscle contraction (MC neuron trajectory is depicted in green, only one lateral neuron is shown for simplicity). M3 are glutamatergic inhibitory neurons with outputs to the metacarpus and possibly the isthmus. M3 neurons fire upon corpus contraction to cause muscle relaxation (M3 neuron is shown in bleu, only M3R (right) neuron is shown for simplicity). M4 neuron function (shown in red) is required for the peristaltic movement in the posterior isthmus. M4 is a cholinergic motor neuron but it might also signal through another neurotransmitter because mutations in receptor of acetylcholine only partially block M4 function (Avery and Horvitz 1989). Serotonin increases the frequency of pharyngeal pumping by stimulating both MC and M3 neuron pairs. The function of *eat-2* and *eat-18* (encoding a nicotinic acetylcholine receptor (nAchR) subunit and a novel small protein required for the function of nAchR respectively) is required for the action of MC and it's response to serotonin. *avr-15* encodes for a chloride channel subunit and is required for the signaling by M3 and it's response to serotonin. The depicted neuron trajectories were taken from: worm atlas-alimentary system, pharyngeal neurons (Altun 2005).

The ability of exogenous serotonin to increase pumping rates bypasses the requirement of proper functioning of upstream effectors that are known to regulate the function of M3 and MC neurons. For instance, using laser killing experiments, Leon Avery elegantly showed that the function of M3 motor neuron is negatively regulated by interneuron I5. Accordingly, the capacity to respond to exogenous serotonin underestimates defects upstream of the I5-mediated negative regulatory effect on M3 pair of neurons. Under normal conditions, effective feeding demands not only pumping but also effective trapping of bacteria, a process that depends on tightly controlled timing of muscle relaxations (slippery phenotype described by L. Avery was linked to defective coordination of muscle relaxation (Avery 1993)). In view of this, a positive exogenous serotonin-mediated increase of pharyngeal pumping rate indicates the proper functioning of effectors that transmit the serotonin signal such as the responsiveness of M3 and MC motor neurons and their downstream targets to coordinate muscle contraction and relaxation during fast pumping caused by exogenous serotonin. Alternatively, it is eligible to think that serotonin can also modulate pharyngeal pumping frequency by directly acting on the postsynaptic targets of MC and M3 motor neurons to modulate their function. In accordance with this possibility, Niacaris and colleagues identified a serotonin receptor SER-1 expressed in pharyngeal muscles but not in MC or M3 neurons (Hamdan, Ungrin et al. 1999). Accordingly, if this theory is correct (i.e. serotonin modulates pumping by acting postsynaptically) then the ability to respond to exogenous serotonin indicates that postsynaptic target cells are functioning properly and thus any defects in response to exogenous serotonergic stimuli indicates a response defects rather than a signaling abnormality.

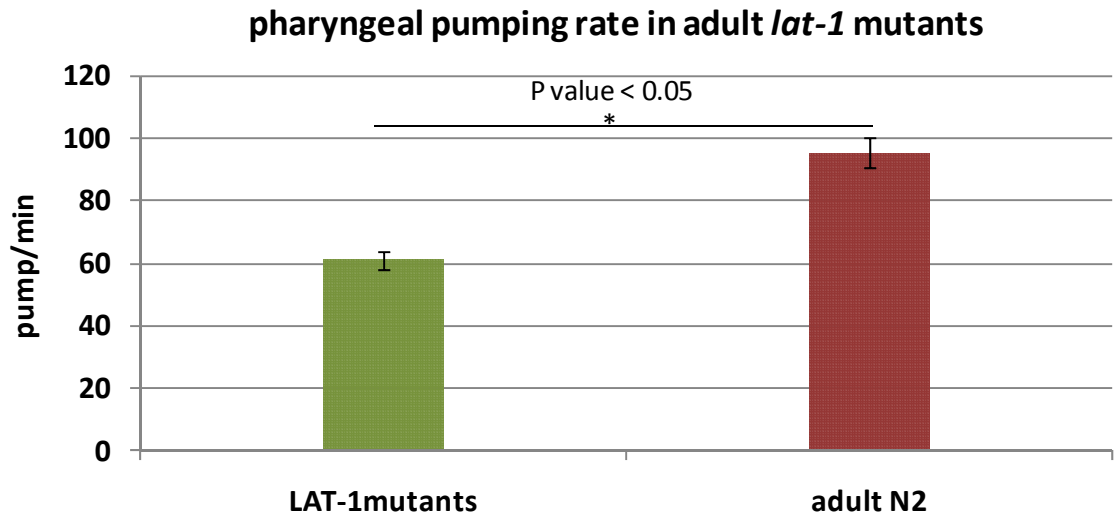
The complexity of pharyngeal pumping regulation is further demonstrated by the role small G proteins play in modulating pharyngeal pumping. Robatzek and colleagues showed that GPB-2 which is a G-protein beta -5 subunit, affects the function of M3 motor neurons (Robatzek, Niacaris et al. 2001). Additionally, $G_{\alpha s}$ subunit was also shown to regulate pharyngeal muscle contractions. Hobson *et al* identified a $G_{\alpha s}$-coupled serotonin like

receptor (SER-7b) that is exclusively expressed in the M4 motor neuron (M4 motor neuron synapses posteriorly with pm5 where peristalsis muscle movement occurs (Figures 25) (Hobson, Geng et al. 2003)) suggesting a mechanism of how serotonin stimulates pharyngeal pumping in that it stimulates neurotransmitter release from the M4 neuron (neurotransmitter release by M4 was also proposed by Avery and Horvitz (Avery and Horvitz 1989)). Moreover, mutations in regulators of G-protein signaling (RGS) such as *eat-16* and *sag-1* exhibited reduced pharyngeal pumping rates, a result that further illustrates the role that G proteins play in regulation pharyngeal pumping (Hajdu-Cronin, Chen et al. 1999). Later on in 2007, Tanis and colleagues beautifully revealed that G proteins regulate several aspects of C. *elegans* behaviours by modulating the biosynthesis of serotonin, at least in the egg laying system (Tanis, Moresco et al. 2008). However an informative profile of G-proteins function in regulating pharyngeal pumping is still to be completed. For example, it would be interesting to understand how G proteins that are known to regulate neurotransmission (such as *goa-1*/$G_{\alpha o}$) affect the rate of pharyngeal pumping and whether the effect of G-proteins on pharyngeal pumping is independent of neurotransmission. One possibility is that heterotrimeric G proteins would trigger calcium release and consequently this results in activating Ca^{2+} mediated neurotransmission. Further characterising the major players of neurotransmission in genetically uncoupled G protein mutant backgrounds would provide the missing link between G proteins-regulations of neurotransmission, calcium signaling and pharyngeal pumping.

2.3. Results and discussion

2.3.1. Pharyngeal pumping is impaired in *lat-1* mutants but does not cause lethality

The major behavioural response in the pharynx is the frequency of pharyngeal pumping. Amongst the several *lat-1* mutants defects is the L1 larval arrest (for further details, please refer to Chapter III). L1 arrest is a phenotype broadly observed in mutants that are unable to feed (Avery 1993). Accordingly, in addition to analysing the response of *lat-1* mutants to serotonin in the pharynx, we wanted to check whether this larval arrest is due to *lat-1* mutant's incapacity to feed. By analysing the pharyngeal pumping rate, *lat-1* mutants exhibited a pumping rate that is significantly reduced as opposed to wild type controls (that is fewer pumps per minute, Figure 26).



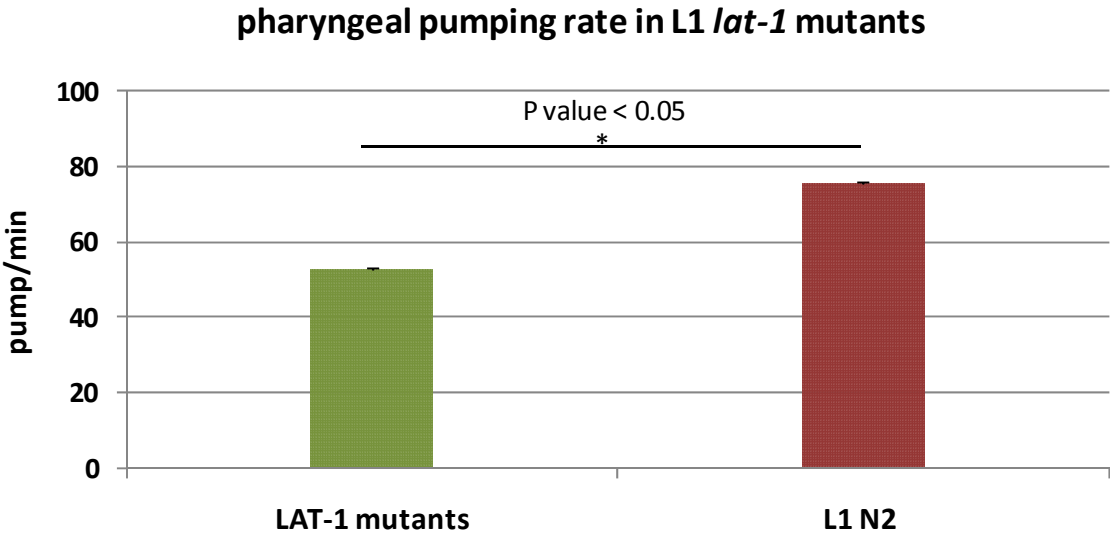


Figure 26. Pharyngeal pumping rate is reduced in *lat-1* mutant adults and arrested L1 larvae. Pharyngeal pumping rate is represented as the average of pumps per minute of recording ±SEM. Adult wild type worms have an average of almost 100 pumps per minutes whereas the pumping rate in *lat-1* adult mutants show almost a decrease of 40% to reach an average of 60 pumps per minute. Similarly, *lat-1* mutant arrested larvae have reduced pumping rate when compared to their wild type counterparts. No significant difference was observed in maternally rescued L1 when compared to L1 *lat-1* mutants (data not shown). 5 worms were recorded in each experiment, for a total recording length of 3 minutes for each worm. Error bars indicate SEM.

The decreased pharyngeal pumping rate observed in *lat-1* mutants led us to suggest two hypothesises:

1. Difficulties in initiating muscle contraction, thus reducing the number of pharyngeal pumps
2. Prolonged muscle contraction, thus allowing fewer pumps to happen.

In order to differentiate between the two possibilities, we looked at the frequencies of time difference between each pump in the terminal bulb and in the posterior isthmus (as initiation of muscle contraction in these regions requires inputs from different neurons). In wild type worms, and under our experimental conditions, the majority of pharyngeal pumps happen with an interval of 800ms, and in general, the time difference between pumps falls in the range of 800ms and 2s. However, in *lat-1* mutants the frequencies of time interval between each pump fall in several categories: 1Hz, 2Hz, 2.85Hz, 3.7Hz and 5Hz (Figure 27) indicating a rather irregular pharyngeal pumping.

In addition to reduced pumping rate, *lat-1* mutants show a great variability in their pumping capacity from one worm to another. Interestingly, *tph-1* mutants lacking serotonin also exhibit great variability in the rate of their pharyngeal pumping (Sze, Victor et al. 2000; Hobson, Geng et al. 2003; Donohoe, Jarvis et al. 2009) (refer to table 4). However, this reduced and irregular pumping frequency does not seem to account for the larval arrest phenotype in *lat-1* mutants, as other mutants exhibiting a far more severe pharyngeal pumping phenotype do not arrest as larvae such as *eat-8* mutants ((Avery 1993), Leon Avery, personal communication).

Table 4: Feeding defective mutants showing slow irregular pumping.

allele	gene	description	reference
ad465	*eat-2*	ligand-gated ion channel subunit, closely related to the non-α-subunits of nicotinic acetylcholine receptors (nAChR)	(Raizen, Lee et al. 1995)
ad426	*eat-3*	a mitochondrial dynamin-related protein, closely related to bacterial dynamin-like proteins	(Kanazawa, Zappaterra et al. 2008)
ad596	*ric-2*	orthologous to the synaptic vesicle protein synaptotagmin 1	(Nonet, Grundahl et al. 1993)
e2342	*unc-2*	a calcium channel alpha subunit	(Mathews, Garcia et al. 2003)
ad519	*unc-10*	a protein with zinc-finger motif, probably acts to regulate the priming step of presynaptic vesicle fusion by promoting conformational changes in syntaxin	(Gracheva, Hadwiger et al. 2008)
ad571	*unc-11*	encodes multiple isoforms of a clathrin-adaptor protein AP180, regulates neurotransmitter release and required for localization of SNB-1/synaptobrevin to synaptic vesicles.	(Nonet, Grundahl et al. 1993)
ad 486			
ad473	*unc-18*	UNC-18 function as a chaperone for UNC-64/syntaxin	(Hosono, Hekimi et al. 1992)
ad698	*unc-26*	unc-26 encodes synaptojanin, appears to function in multiple steps of synaptic vesicle recycling	(Harris, Hartwieg et al. 2000)
	unc-36	L-type voltage-dependent Ca2+ channel, alpha2/delta subunit	(Frokjaer-Jensen, Kindt et al. 2006)

The table lists a descriptive summary of mutations resulting in slow and irregular pharyngeal pumping. Mutations causing such phenotypes can be grouped into mutations that disturb the excitable cell function (*eat-2*, *unc-2*, *unc-36*) and mutations that disturb different steps of neurotransmitter release including vesicle priming, docking and fusion to presynaptic membrane (*ric-2*, *unc-10*, *unc-11*, *unc-18*). Defects distressing neuron transmission clearly affects pharyngeal pumping, illustrating the importance of regulating neurotransmission in the feeding behaviour.

It is not clear whether the exogenous serotonin-mediated modulation of pharyngeal pumping requires $G_{\alpha o}$ as is the case in locomotion. Interestingly, like in *lat-1* mutants, *goa-1* mutants have a reduced pharyngeal pumping rate (Segalat, Elkes et al. 1995). Despite its clear role in regulating pharyngeal pumping (Keane and Avery 2003) it remains ambiguous whether serotonin-mediated increase of pharyngeal pumping rate is a process that engages *goa-1*/$G_{\alpha o}$.

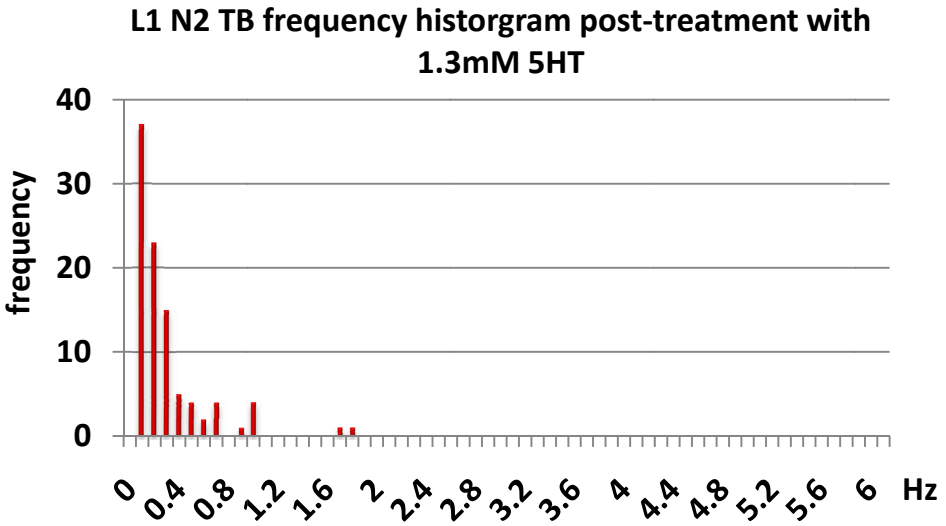
2.3.2. Pharyngeal pumping in response to serotonin in *lat-1* mutants

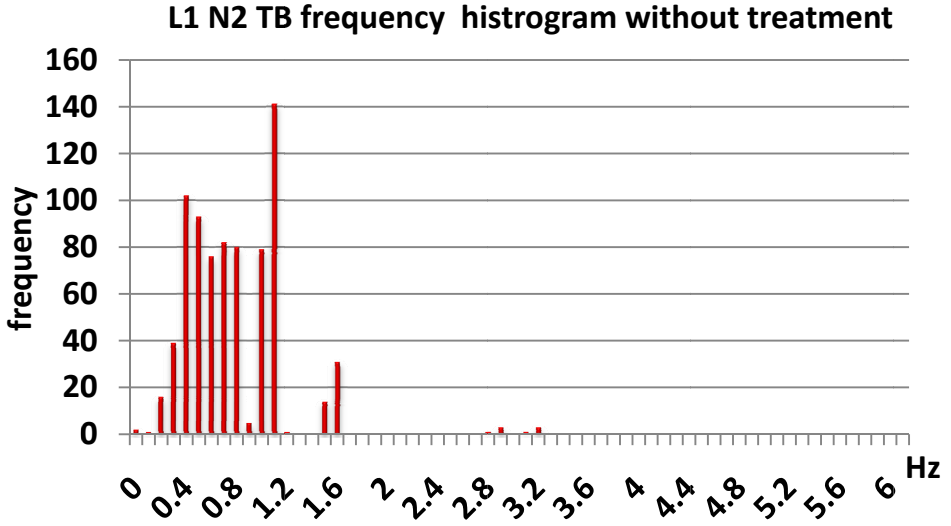
In presence of exogenous serotonin, *lat-1* mutants were sick and vacuoles quickly formed in the head region. In addition, the worms' pharyngeal lumen quickly became stuffed with bacteria and it was not possible to assess the frequency of pharyngeal pumping under such conditions. This bacteria-stuffed phenotype was also described in laser ablation experiments aimed at the M4 motor neuron. The M4 neurons stimulate the posterior isthmus to cause peristaltic contraction and thus opening of the pharyngeal lumen and the passage of accumulated bacteria to the terminal bulb (Ray, Schnabel et al. 2008). M4 is a cholinergic neuron, but it may also use other neurotransmitter as mutations in nicotinic acetylcholine receptor (nAchR) only caused partial block of M4 function (Raizen, Lee et al. 1995; Ray, Schnabel et al. 2008).

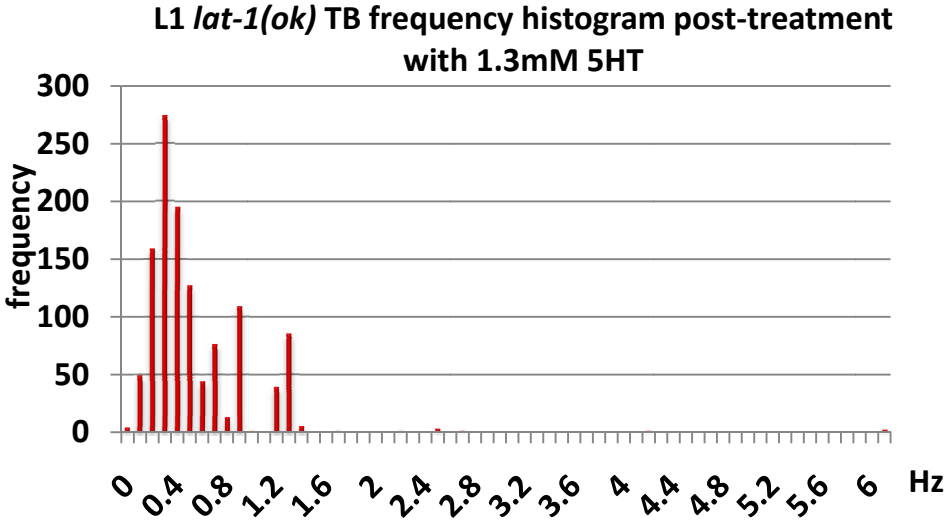
Experiments using antipsychotic drugs showed that removal of drugs that increase pharyngeal pumping causes a "withdrawal effect" represented by decreased pharyngeal pumping, and that this withdrawal effect is only observed in worms with intact serotonin signaling pathway as mutants in either serotonin biosynthesis of serotonin receptor did not show a withdrawal effect (Donohoe, Jarvis et al. 2009). Due to the sensitivity of *lat-1* worms to serotonin, we decided to test the effect of serotonin withdrawal instead.

Under our experimental conditions, in wild type untreated worms, a pump happened in average every 800ms, while in post-serotonin treatment; a pump happened every 5sec, as

also indicated by the reduced pumping rate owing to the serotonin withdrawal effect (Figure 27). The decrease in pumping rate in post-treatment with serotonin illustrates the withdrawal effect.


L1 N2 TB frequency historgram post-treatment with 1.3mM 5HT
frequency
0
10
20
30
40
0 0.4 0.8 1.2 1.6 2 2.4 2.8 3.2 3.6 4 4.4 4.8 5.2 5.6 6 Hz


L1 N2 TB frequency histrogram without treatment
frequency
0
20
40
60
80
100
120
140
160
0 0.4 0.8 1.2 1.6 2 2.4 2.8 3.2 3.6 4 4.4 4.8 5.2 5.6 6 Hz


L1 lat-1(ok) TB frequency histogram post-treatment with 1.3mM 5HT
frequency
0
50
100
150
200
250
300
0 0.4 0.8 1.2 1.6 2 2.4 2.8 3.2 3.6 4 4.4 4.8 5.2 5.6 6 Hz

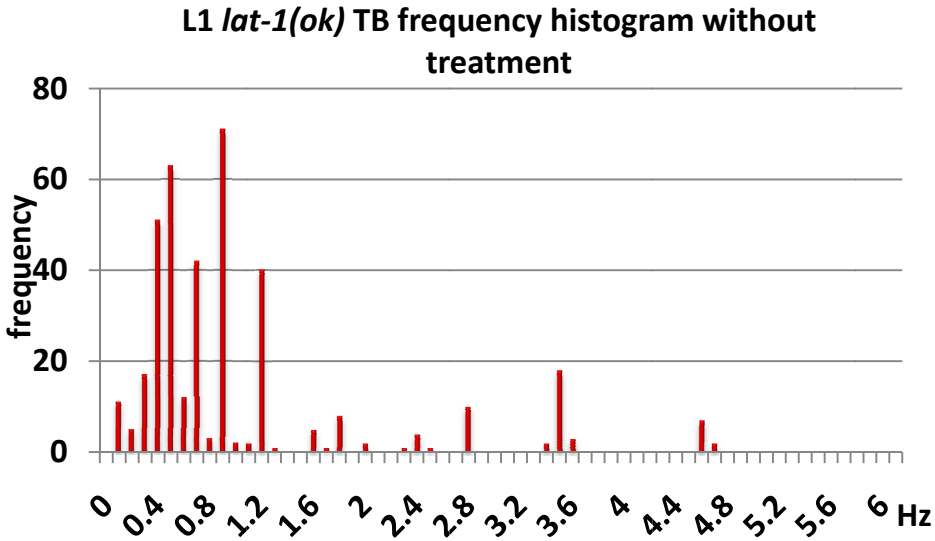

L1 lat-1(ok) TB frequency histogram without treatment
frequency
0
20
40
60
80
0 0.4 0.8 1.2 1.6 2 2.4 2.8 3.2 3.6 4 4.4 4.8 5.2 5.6 6 Hz

Figure 27. Frequency histograms of time intervals between each terminal bulb (TB) pump showing *lat-1* response to serotonin withdrawal. Under our experimental conditions, in wild type worms the majority of pharyngeal pumps happened every 800ms prior to serotonin treatment. Following removal of serotonin, the frequencies of time intervals shifted to accommodate the majority of pumps in the region of 0.2 Hz corresponding to 5 seconds interval between each pharyngeal pump. This decreased pumping rate is a consequence of the withdrawal of serotonin (withdrawal effect described in (Donohoe, Jarvis et al. 2009)). Similarly, *lat-1* mutants responded to serotonin withdrawal in a similar manner to wild type. Prior to treatment with 1.3mM 5-HT, the majority of pumps in *lat-1* mutants happened every 1.1 second. This time interval increased after removal of serotonin to accommodate a pump every 3 seconds. So, in both wild type and *lat-1* mutants, serotonin withdrawal caused reduced pumping rate indicating an initial serotonin-mediated increased pharyngeal pumping rate (Donohoe, Jarvis et al. 2009).

Likewise, *lat-1* mutants responded to serotonin withdrawal in a similar way: in untreated worms, the majority of pumps happened every 1.1s; while in post- treatment experiment a pump happened every 3sec (Figure 28). The ability to respond to serotonin withdrawal is an indication that serotonin was able to induced pharyngeal pumping in adult *lat-1* mutants. In accordance with this, EPGs analyses of action potential changes across *lat-1* mutants pharyngeal muscle membrane showed no significant difference in the cell membrane excitability when compared to wild type in response to serotonin (Tobias Langenhan, personal communication).

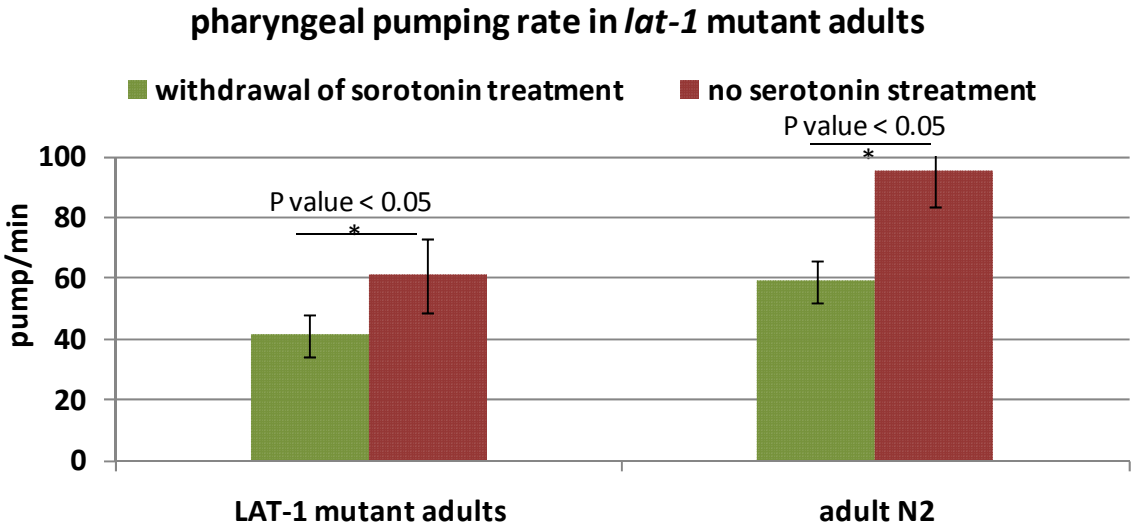
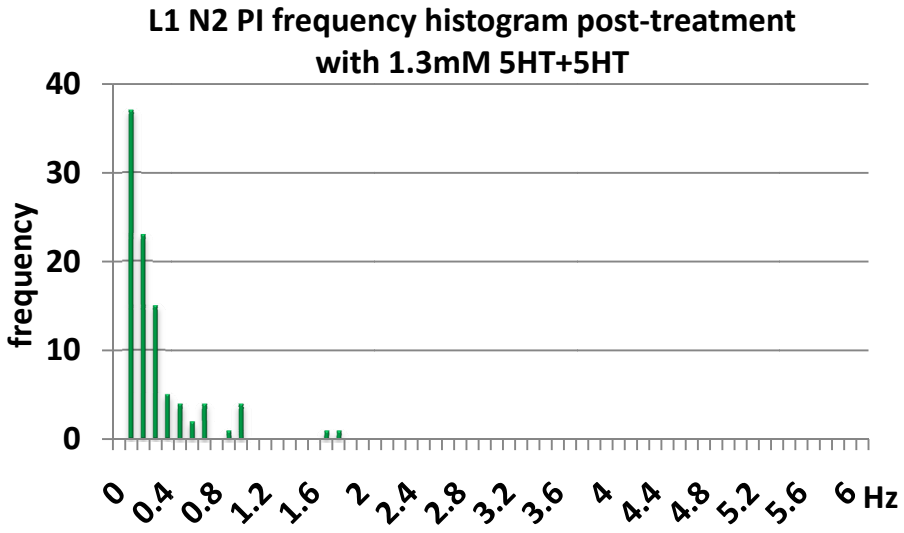
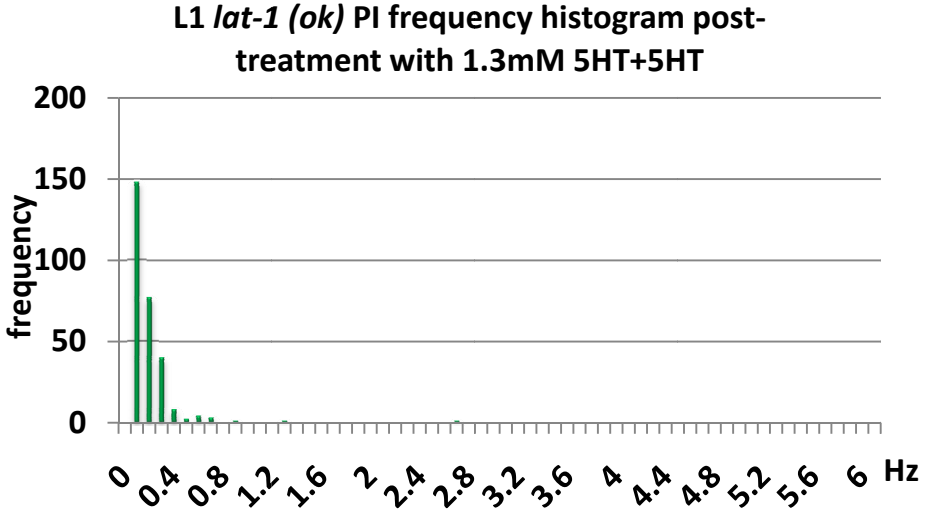
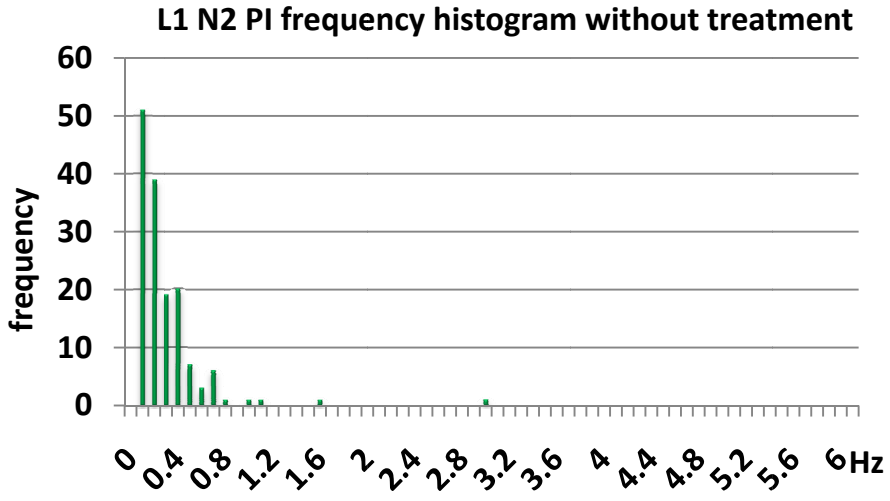


Figure 28. *lat-1* mutants pharyngeal pumping response to serotonin withdrawal. Pharyngeal pumping rate is represented as the average number of pumps per minute of recording ±SEM. Exogenous serotonin (1.3mM) increases pharyngeal pumping rate, and treatment withdrawal causes reduced pumping rate (Niacaris and Avery 2003) (Donohoe, Jarvis et al. 2009). In wild type worms, the average number of pumps per minute went down from 95 to 60 pumps post treatment with 1.3mM 5-HT. Similarly, *lat-1* mutants respond to serotonin withdrawal in a similar way in that the pumping rate was reduced following treatment with 1.3mM 5-HT from 60 to 40 pumps per minute. Error bars indicate SEM.

There is no evidence of a direct serotonin-mediated increase in peristalsis frequency. Pharyngeal posterior isthmus peristalsis frequencies did not change following treatment with 1.3mM serotonin, suggesting that serotonin does not directly influence muscle contractions at the posterior isthmus, at least under our experimental conditions (Figure 29).


L1 N2 PI frequency histogram post-treatment with 1.3mM 5HT+5HT
frequency
40
30
20
10
0
0 0.4 0.8 1.2 1.6 2 2.4 2.8 3.2 3.6 4 4.4 4.8 5.2 5.6 6 Hz


L1 lat-1 (ok) PI frequency histogram post-treatment with 1.3mM 5HT+5HT
frequency
200
150
100
50
0
0 0.4 0.8 1.2 1.6 2 2.4 2.8 3.2 3.6 4 4.4 4.8 5.2 5.6 6 Hz


L1 N2 PI frequency histogram without treatment
frequency
60
50
40
30
20
10
0
0 0.4 0.8 1.2 1.6 2 2.4 2.8 3.2 3.6 4 4.4 4.8 5.2 5.6 6 Hz

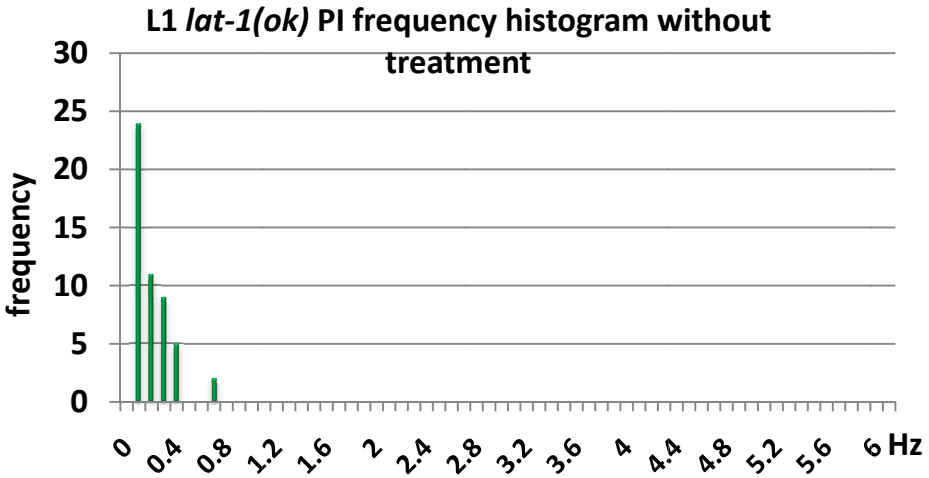

L1 lat-1(ok) PI frequency histogram without treatment
frequency
30
25
20
15
10
5
0
0 0.4 0.8 1.2 1.6 2 2.4 2.8 3.2 3.6 4 4.4 4.8 5.2 5.6 6 Hz

Figure 29. Frequency histograms of time intervals between each posterior isthmus (PI) peristaltic movement, showing *lat-1* response to serotonin withdrawal. In both wild type and *lat-1* mutant worms, we were not able to observe serotonin withdrawal effect on posterior isthmus peristalsis as it was the case in the terminal bulb. According to Javis *et al*, withdrawal effect depends on previous response to serotonin (Donohoe, Jarvis et al. 2009), accordingly, under our experimental conditions, it could be that serotonin did not have an effect on the posterior isthmus peristalsis. An alternative explanation is that the terminal bulb and the posterior isthmus have different adaptation capacities.

Defects affecting the feeding process can disturb either pumping or peristaltic muscle movements in posterior isthmus or both. Mutants affecting pumping show reduced, elevated and/or irregular pumping rate and/or "slippery" phenotype (Avery 1993; Avery 1993) whereas defects reducing or eliminating the peristaltic movement of posterior isthmus greatly limit or arrest growth as the trapped bacteria cannot be passed from the anterior of the pharynx to the terminal bulb. Mutations affecting M4 neurons exhibit a "stuffed" phenotype as the pharynx becomes clogged with bacteria due to the incapability to transport bacteria to the terminal bulb (Raizen, Lee et al. 1995; Ray, Schnabel et al. 2008). Interestingly, longer exposure to serotonin caused the "stuffed" phenotype in *lat-1* mutants but not wild type worms (Figure 30). This stuffed pharynx phenotype observed in *lat-1* mutants, could be due to the inability of the posterior isthmus to cope with the serotonin-caused increased amount of trapped bacteria suggesting an impaired coordination between pumping muscle contractions and peristalsis of the posterior isthmus.

Collectively, our pharyngeal pumping experiments showed that *lat-1* mutants are able to respond to exogenous serotonin and that their reduced pharyngeal pumping rate is unlikely to account for their larval arrest.

The failure of serotonin to reduce aldicarb sensitivity is thus unlikely to be the result of irresponsiveness, thus ruling out the requirement of LAT-1 to response to serotonin at least in the pharyngeal system.



Figure 30: DIC photographs showing abnormalities observed in the pharynx of *lat-1* mutants in the presence of serotonin. A panel shows the head region of a wild type larva in the presence of serotonin, which in the *lat-1* mutants; results in the formation of large vacuoles in the head region (B) and the larvae pharynx becomes quickly stuffed with bacteria (C).

3. Egg laying

3.1 The neurophysiology of egg laying in *lat-1* mutants

3.1.1 Brief introduction

Egg laying is activated by $G_{\alpha q}$ and inhibited by $G_{\alpha o}$ in a process that require HSN neurons (Hermaphrodites Specific Neurons). Ventral type C neurons are the only known neurons with synapses directly projecting at the HSN neurons and the egg laying muscle cells. Conversely to its positive effect on locomotion, synaptic Ach has been shown to negatively regulate egg laying (Brundage, Avery et al. 1996; Bany, Dong et al. 2003).

goa-1/$G_{\alpha o}$ negatively regulate Ach release at neuromuscular junction in the vulva, as mutation in *goa-1* causes hyperactive egg laying: Ach release is increased in *goa-1* mutants, indicating both the negative regulation of *goa-1* on Ach release, and the inhibitory effect of Ach on egg laying (Bany, Dong et al. 2003).

Interestingly, several mutations that increase egg laying also render animals sensitive to inhibitors of acetylcholinesterase (aldicarb) an observation that further emphasizes the negative effect of acetylcholine release on the egg laying behaviour. For instance, mutations in *goa-1*, *eat-16* and *dgk-1* increase egg laying and also cause worms to be sensitive to aldicarb (Hajdu-Cronin, Chen et al. 1999; Bany, Dong et al. 2003).

Response to endogenous serotonin requires intact signaling cells and intact serotonin target tissues/cells. Exposure to exogenous serotonin however requires intact serotonin target cells in addition to intact downstream effectors that mediate serotonin action. Furthermore, mutations associated with defects in neuronal structure, including axonal guidance, fasciculation and synaptic projections showed hyperactive egg laying (e.g. mutations in *unc-115*, *unc-34* and *unc-5*) (Colavita and Culotti 1998; Lundquist, Herman et al. 1998; Gitai, Yu

et al. 2003). For instance, defects in the VC neuron processes cause hyperactive egg laying due to the inability of proper signaling (Bany, Dong et al. 2003).

VC type neurons signal to the HSN neurons by releasing Ach which then binds its receptor *gar-2* expressed at the synaptic terminals of HSN. A proposed model is that Ach released by VC neurons, blocks the serotonin signaling pathway in the HSN neurons. Another model implies that HSN induces egg laying by causing egg laying muscle contractions while VC neurons acts directly on the vulva muscle cells to cause relaxation and thus inhibit egg laying (Bany, Dong et al. 2003). However, there is no evidence of the requirement of intact structure of this type of neurons for the animals to respond to exogenous serotonin. Therefore, whether VC neurons mediate the action of exogenous serotonin is not clear yet.

At the neuromuscular junctions, it was shown that serotonin-mediated inhibition of Ach release is dependent on *goa-1* (Mendel, Korswagen et al. 1995; Segalat, Elkes et al. 1995). Whether *goa-1* is required for the serotonin-mediated stimulation of egg laying is also unclear. An evident question to ask is whether the action of exogenous serotonin requires *goa-1* in target muscle cells or in HSN neurons to induce muscle contraction and subsequent egg release. In view of this, Shyn and colleagues showed that serotonin directly stimulates the activity of the vulva muscle cells in a process that require *egl-30* $G_{\alpha q}$ but not $G_{\alpha o}$ (Shyn, Kerr et al. 2003). Additionally, the same group proposed that *goa-1* negatively regulate muscle activity, at least in part, through intrinsic action within the egg laying muscle cells. Further, it appears that *goa-1* functions independently and antagonistically to serotonin to inhibit the Ca^{2+} transients in vulva muscle cells, and that the effect of *goa-1* on serotonin take place within the serotonergic HSN motor neurons where *goa-1* provides a negative feedback loop to decrease the release of serotonin by HSN neurons (Shyn, Kerr et al. 2003) (Figure 31). This finding is interesting in that it shows that G-proteins affect Ca^{2+}

transients in the egg laying system, and calls for validating the same effect of Ca^{2+} transients at the level of pharyngeal pumping for example.

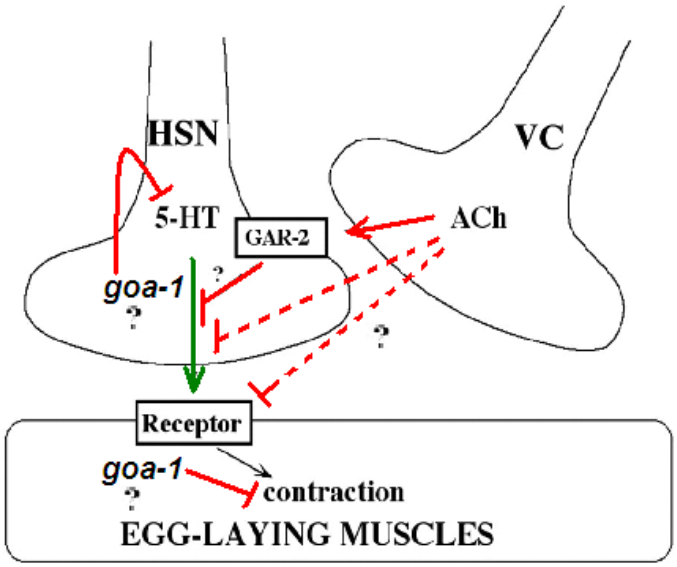


Figure 31. A model of VC-released acetylcholine in inhibition of egg laying. The cholinergic VC neurons release acetylcholine which inhibits the HSN-mediated stimulation of vulva muscle contraction by acting on it muscarinic receptor GAR-2 expressed in the HSN neurons (solid red arrow). Acetylcholine may also be inhibiting egg laying through a mechanism that is independent of HNS and serotonin (5-HT) (dotted red lines) (Bany, Dong et al. 2003). *goa-1* $G_{\alpha o}$ was proposed to act intrinsically within the vulva muscle cells to inhibit their contractions, and in the HSN neurons where it provides a negative regulatory loop that decreases the release of serotonin (5-HT) from the serotonergic HSN neurons (curved red line, (Shyn, Kerr et al. 2003)). Diagram taken from (Bany, Dong et al. 2003) and adapted according to (Bany, Dong et al. 2003; Shyn, Kerr et al. 2003).

Interestingly, it is also proposed that acetylcholine might signal through another receptor/mechanism as *gar-2* mutations only partially increase egg laying as opposed to acetylcholine *cha-1* mutant (Bany, Dong et al. 2003). Acetylcholine has thus an additional route through which it influences egg laying behaviour.

3.2 Results and discussion

3.2.1 Serotonin induces egg laying in *lat-1* mutants

goa-1 Gαo is expressed in most neurons and in egg laying muscles and controls several aspects of behaviours in C. *elegans* including egg laying. Reduction of function mutations cause hyperactive locomotion and premature egg laying amongst other phenotypes (Mendel, Korswagen et al. 1995). Further, mutation in the *goa-1* gene mimics the phenotypes observed in mutants lacking the neurotransmitter serotonin (5-HT), and *goa-1* mutants, as far as locomotion is concerned, are partially resistant to exogenous serotonin, making $G_{\alpha o}$ an important but not unique mediator of several behavioral effect of serotonin (Segalat, Elkes et al. 1995). In the egg laying system, *gar-2* is the only muscarinic acetylcholine receptor shown to b expressed in any cell of the egg laying system (Lee, Park et al. 2000). Amy Bany and colleagues proposed a model of how acetylcholine inhibits egg laying (Figure 31). In brief, they suggested that the six VC neurons release acetylcholine, which upon binding to its muscarinic receptor GAR-2 in the HSN neurons inhibit the serotonin-mediated stimulation of egg laying (Bany, Dong et al. 2003). How this inhibitory effect is mediated remains to be elucidated.

We wanted then to test the effect of serotonin on egg laying in *lat-1* mutants and found that *lat-1* mutants were perfectly capable to respond to exogenous serotonin (Figure 32), further affirming that the failure of serotonin to confer resistance to aldicarb in *lat-1* mutants was not due to *lat-1* mutants being unable to respond to serotonin but to the requirement of *lat-1* in down regulating Ach release at the NMJs in the presence of serotonin.

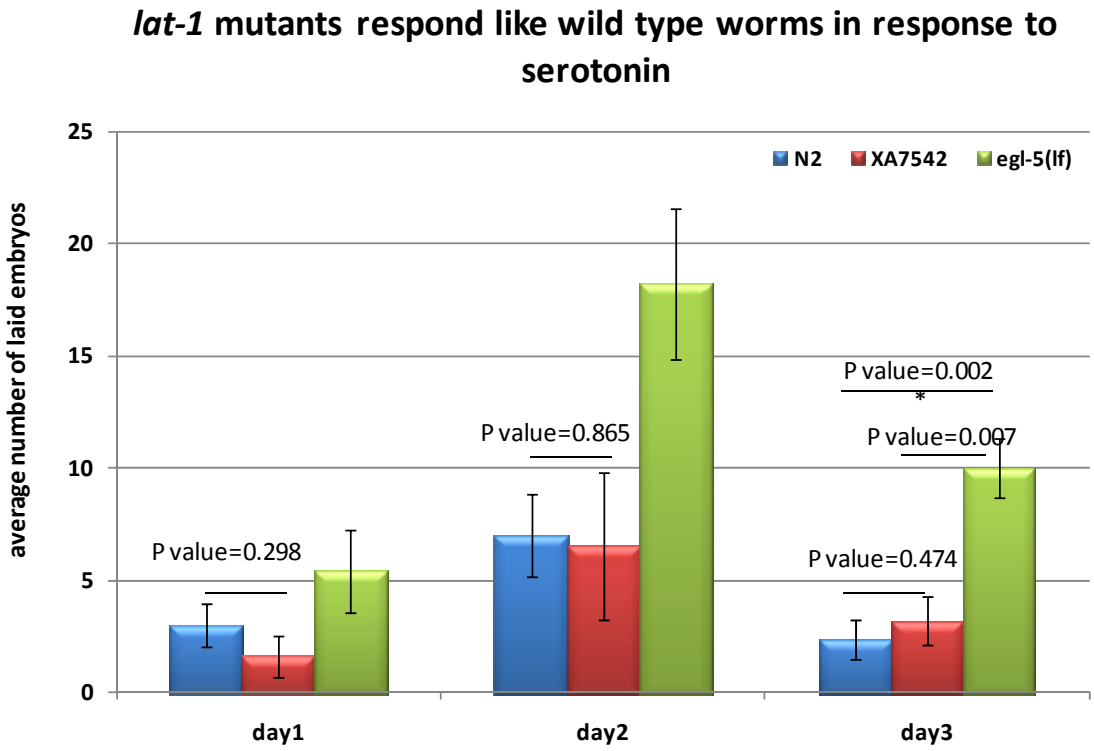


Figure 32. Serotonin induces egg laying in *lat-1* mutants. Day 1, 2 and 3 hermaphrodites were selected from synchronized population and treated with 1.3mM serotonin. The average number of eggs laid is shown in the graph with error bars indicating SEM. In all of days 1,2 and 3 there is no significant difference between number of laid eggs between wild type (N2) and *lat-1* mutants (XA7542[*lat-1(ok1465) II*]) (P-values indicated). *egl-5* loss of function mutation had significantly increased number of laid eggs at day 3 because the mutation causes the worms to retain more eggs by day three (P-value indicated).

In 1-day old adult hermaphrodites, the number of eggs released by *lat-1* mutant hermaphrodites was lower compared to wild type. This could be the result of the delayed growth observed in *lat-1* mutants which could slow down the reproductive system. The ability of *lat-1* mutants to respond to exogenous serotonin indicates that the downstream effectors of serotonin are not impaired in these mutants and that the vulva muscle cells are

perfectly excitable by exogenous serotonin. The *egl-5* mutant controls have a defective HSN signaling (Desai, Garriga et al. 1988; Desai and Horvitz 1989) and the fact that control *egl-5* mutants were similarly stimulated by exogenous serotonin indicate that exogenous serotonin acts directly on postsynaptic vulva muscle cells to induce contraction and expulsion of the egg off the uterus. This further affirms that the downstream serotonin targets are not defective and that *lat-1* mutants are capable to respond to exogenous serotonin.

3.2.1.1 *lat-1* mutants retain fewer eggs

Mutations in *goa-1* and other gene shown to negatively regulated egg laying exhibit a hyperactive egg laying phenotype known as Egl-C (constitutive) due to the continuous laying of eggs. Accordingly, such mutants retain fewer eggs in their uterus (Bany, Dong et al. 2003). Because the *lat-1* mutants' egg laying system is able to respond to serotonin and because exogenous serotonin does not challenge the state of acetylcholine release in the egg laying system, we wanted to know more about the state of acetylcholine release and see whether *lat-1* mutants exhibits such Egl-C phenotype. Increased acetylcholine release inhibits egg laying causing more eggs to be retained in the uterus, and lowered acetylcholine release leads to fewer eggs being retained in the uterus. Surprisingly, *lat-1* mutant day 3 hermaphrodites retained fewer eggs than wild type hermaphrodites of the same age (Figure 33).

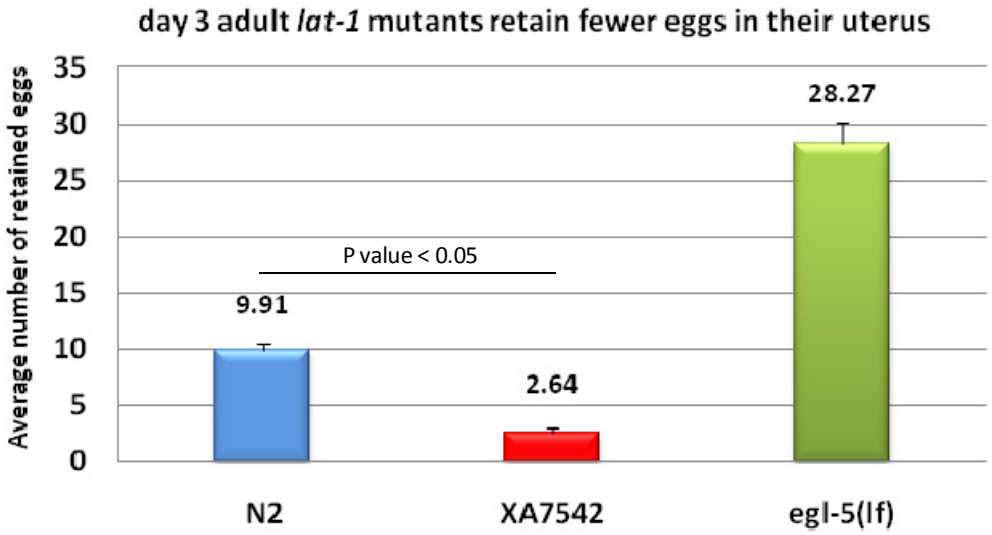


Figure 33. *lat-1* day 3 adult mutants retain fewer eggs in their uterus. The number of retained eggs in the hermaphrodite's uterus was used by many researchers to assess egg laying behaviour. *lat-1* mutants (XA7542[*lat-1(ok1465) II*]) retain fewer eggs in their uterus when compared to wild type worms (N2). Reduced acetylcholine signaling was evidenced to cause hyperactive egg laying and cause fewer eggs to be retained in the uterus (Bany, Dong et al. 2003).

This result was rather in contradiction with our expectations deduced from the hypersensitivity to aldicarb observed in *lat-1* mutants and which postulates a rather increased Ach release. Fewer retained eggs propose a decreased Ach release. This has left us to think of an alternative hypothesis whereby LAT-1 plays a role in the proposed additional route through which acetylcholine inhibits vulva muscle contractions (Figure 31). LAT-1 is expressed in vulva muscle cells (Tobias Langenhan, personal communication). An alternative and more plausible explanation is that LAT-1 would be required in vulva muscle cells (and possibly vulva neurons) to respond to the direct effect acetylcholine on vulva muscle cells, where it activates $G_{\alpha o}$ to inhibit egg laying. Accordingly, in *lat-1* mutants, this $G_{\alpha o}$ mediated negative inhibitory effect is reduced leading to increased rate of egg laying and

consequently, fewer eggs retained in the uterus (Figure 33). As evidenced by Shy and colleagues, $G_{\alpha o}$ is not required to respond to exogenous serotonin and so is *lat-1* (Figure 31 and 33). Detailed protein localization analysis would allow us to Figure out the site of action of LAT-1 and to find out whether *lat-1* gene product is required in the egg laying system in signaling cells or in the responding target cells or both. Possibly, the coupling of *lat-1* function to distinct heterotrimic G proteins could explain the discrepancy observed in the state of Ach release at the NMJ and the egg laying system. The proposition that *lat-1* could function via $G_{\alpha o}$ is in accordance with the aldicarb hypersensitivity phenotype in *lat-1* mutants. The seemingly elevated Ach release in *lat-1* mutants could be the result of the absence of a negative regulatory pathway. The coupling of *lat-1* to $G_{\alpha o}$ also fits the reduced number of retained eggs. *goa*-1 mutants have reduced number of uterus-retained eggs due to the absence of egg laying inhibition. Accordingly, it is plausible to suggest that both *lat-1* and *goa-1* function in inhibiting egg laying. It is not possible to say whether *lat-1* activates $G_{\alpha o}$ inhibitory pathway or whether they function on parallel/different pathways. Analysing the uterus-retained eggs in *lat-1;goa-1* double mutants would help us shed light on any synergy between the two genes. Alternatively, LAT-1 might simply execute different functions in the egg laying system and body neuromuscular junctions. Aschapter III, *lat-1* mutants exhibit several morphological defects including defects in the development of vulva tissue. It is then possible that this apparently reduced Ach release associated with low number of retained eggs is due to signaling defects that are caused by anomalies in the development of the vulva tissue and even vulva tissue connectivity, especially knowing that amongst the many *lat-1* mutants' phenotypes is some axonal guidance defects (Tobias Langenhan, personal communication). It is true that axonal migration defects were not observed in the vulva tissue, however detailed analysis were not performed to date and reconstruction electron microscopy could reveal into more details the scope of tissue connectivity in the vulva and whether the two main neuron types (HSN and VC neurons)

make correct branching points to properly enervate target vulva muscle cells in *lat-1* mutants. Additionally, we do not know the potency LAT-1 exerts in the egg laying system. For example, the low number of retained eggs phenotype was more apparent in some mutant worms than in others. To further complicate the interpretation of this phenotype; as mutant hermaphrodites got older (more than 4 days old) some of them even appear Egl retaining more eggs in their uterus acquiring thus a baggy phenotype whereas others were completely sterile. The reduced number of retained eggs in *lat-1* mutants led us to think that *lat-1* might function in the regulation of Ach at the egg laying system. It would be interesting to use *lat-1* and *egl-30*/$G_{\alpha q}$ double mutants to see if this reduced number of retained eggs can be rescued in the double mutants as mutations is the $G_{\alpha q}$ mutants retain more eggs. Further more, epistasis genetic analyses of *lat-1* mutants and Ach receptor mutants in the egg laying system (such as *gar-2*/nAchR hypomorphic mutants) would help make firm conclusions about the egg laying defects observed in *lat-1* mutants and the effect of the lack of LAT-1 on Ach release in the egg laying system. Additionally, we could characterise *lat-1* over expressors and see whether they show the opposite phenotype, i.e. whether they retain more eggs in their uterus, and whether *lat-1* over expression is capable of rescueing/reducing the Egl-C phenotype of *goa-1*/$G_{\alpha o}$ mutans.

Neuronal activity can be inhibited by neurotransmitters either by opening chloride channels or by activating G-proteins coupled receptors that signal through the inhibitory $G_{\alpha o}$. Both forms of inhibitory neurotransmissions are adopted by the HSN to regulate egg laying. Acetylcholine signaling, serotonin signaling, tyramine-activated chloride channels and GABA signaling are together integrated to control egg laying. Furthermore, a particular player down regulating egg laying might function by integrating more than one inhibitory pathway. For example, the orphan GPCR encoded by *egl-47* regulate egg laying through $G_{\alpha o}$ and also by activating chloride channels (Moresco and Koelle 2004; Tanis, Bellemer et

al. 2009). Furthermore, the inconsistency of the egg laying phenotype from one *lat-1* hermaphrodite to another can be the result of functional redundancy as $G_{\alpha o}$ (which is a key G-protein regulating egg laying) integrates signaling inputs from several GPCRs simultaneously (Segalat, Elkes et al. 1995; Shyn, Kerr et al. 2003; Moresco and Koelle 2004; Tanis, Bellemer et al. 2009).

Considering the results obtained from the aldicarb assay, it seems that *lat-1* might exert its function through coupling to heterotrimic G proteins, more precisely on $G_{\alpha o}$ based on phenotypes' similarities, instead of solely regulating the release of acetylcholine. For example, at the neuromuscular level, *lat-1* could function through $G_{\alpha o}$ which then decrease the release of neurotransmitter (Ach) and loss of function mutation in *lat-1* causes defects in this $G_{\alpha o}$ negative regulatory pathway resulting in increased release of acetylcholine as illustrated by the Hic phenotype. More importantly, the Hic phenotype in *lat-1* mutants was not improved in the presence of exogenous serotonin, a phenomenon observed when the *goa-1*/$G_{\alpha o}$ negative regulatory pathway is compromised. Similarly, in the egg laying system, *lat-1* could function through $G_{\alpha o}$ and loss of function mutation in *lat-1* results in reduced $G_{\alpha o}$-inhibitory effect on egg laying leading to more eggs being laid out as illustrated by the low number of retained egg in *lat-1* day 3 adult mutants. Assessing the stage during which the eggs are laid in *lat-1* mutants would further detail the Egl-C phenotype (a characteristic of the Egl-C phenotype is that mutants lay early stage embryos as is the case in goa-1/$G_{\alpha q}$ mutants).

4 Materials and methods

4.1. Aldicarb assay. Experimental procedures were modified from (Nurrish, Segalat et al. 1999; Mahoney, Luo et al. 2006) as described below:

4.1.1. *C. elegans* strains. Worm strains were cultured and maintained according to standard procedures described by S. Brenner (Brenner 1974). Animals were cultivated at 22'C unless otherwise noted. The animals described as wild type were the *C. elegans* variety Bristol strain N2. The following genotypes were used:

Linkage Group LG I: *goa-1(n1134)* [MT2426] (Nurrish, Segalat et al. 1999), *unc-13(n2813)* [MT8004] (Yook, Proulx et al. 2001; Langenhan, Promel et al. 2009)

LG II: *lat-1(ok1465)* [XA7500] (Langenhan, Promel et al. 2009).

The transgenic lines were used: *lat-1(ok1465) II; qaEx7513[pTL2(minicosmid rol-6(su1006)]*[XA7516], *lat-1(ok1465) II; qaEx7529(M05B5.2::LAT-1A unc-22::RFP)* [XA7554].

4.1.2. Aldicarb and Serotonin treatments. Nematode Growth Medium (NGM) was prepared following standard method described in (Stiernagle 2006). NGM plates were treated with aldicarb as follows: aldicarb (sigma- PS734) was first dissolved in 70% ethanol to reach a final concentration of 100mM then it was added to cooled NGM plates to a final concentration of 0.7mM aldicarb. The plates were incubated at room temperature and allowed to dry overnight. Few hours prior to the start of the experiments, plates were seeded with a small drop of HB101 bacteria strain (gift from Leon Avery) in LB medium, and the plates' lids were removed to allow the bacteria to dry before the worms were gently transferred to the plates with a platinum wire mounted on glass Pasteur pipette. Young adult worms < 2days were used in the experiments. Similarly, prior to seeding the plate with bacteria, serotonin was added to cool NGM to a final concentration of 1.3mM. 65mM

Serotonin stock was first prepared by dissolving 0.26g of serotonin in 10ml of 0.1mM HCl (following manufacturers' advise). NGM plates containing serotonin were incubated at room temperature in the dark to avoid degradation.

4.1.3. Worm population synchronization. Worm populations were synchronized by bleaching gravid adult hermaphrodites as described in (Stiernagle 2006). Briefly, a 10ml bleaching solution was prepared by mixing 7.5ml dH_2O, 2ml of 100% hydrocholrite and 0.5ml of 10N NaOH. The solution was mixed using a vortex, and 2ml of the bleaching solution was used to wash gravid mothers off NGM plates. Worms washed in the bleaching solution were then transferred to 2ml eppendorf tubes and the tubes were inverted couple of times, before they were spun down in a Mini Spin Plus Eppendorf at low speed (3500 rpm) to collect embryos down the tube. The supernatant were discarded and the embryos were washed twice with 2ml of M9, and the embryos were then transferred to fresh seeded NGM plates either by pipetting or by taping the eppendorf tubes. Embryos recovered from the bleaching procedures were allowed to hatch then transferred to fresh plates when they reached the L4 stage (worms were considered to be at the L4 stage as judged by the clear-coloured patch around the vulva and the absence of embryos in their uterus). Day 1 adult animals were then transferred to the previously prepared NGM plates. Triplets of 20 worms were used per genotype per experimental condition. Each experiment was repeated three times with fresh materials preparation each time.

4.1.4. Behavioural assay. The paralytic effect of aldicarb was tested by probing the worms individually with an eye lash. A worm was scored resistant to the paralytic effect of aldicarb if it was able to move when gently poked three times with an eye lash. Scoring was performed every 1 hour for each genotype.

4.2. Pharyngeal pumping assay.

4.2.1. *C. elegans* strains. Worm strains were cultured and maintained according to standard procedures described by S. Brenner (Brenner 1974). Animals were cultivated at 22'C unless otherwise noted. The animals described as wild type were the *C. elegans* variety Bristol strain N2.

LG II: *lat-1(ok1465)* [XA7500] (Langenhan, Promel et al. 2009).

4.2.2. Videomicroscopy of pharyngeal pumping behaviour. L1 larvae were synchronized by bleaching mixed worm populations as described above (section 4.1.3. of this chapter). L1 larvae were placed on a drop of M9 on 1% agarose pad mounted on a microscope slide, covered with a coverslip and sealed with molten Vaseline to lock moisture (Vaseline allows gas exchange but prevents desiccation). Recording was performed using a Quimaging Regita Exi digital camera mounted on a Leica DM RXA2 microscope using Volocity v4 software (Improvision). Each worm was recorded with a frame speed of 200 frames per second at 100X magnification for total recording duration of 5 minutes. Each recording was then post-processed by analyzing slow motion replay of each recoding, and time points corresponding to the happening of a terminal bulb pump/peristalsis movement were manually logged on a spreadsheet. Pumping rate (pumps/minute) was evaluated as the average number of pumps per minute and was obtained by dividing count of pharyngeal pumping/peristalsis movement by the length of recording in minutes. Frequency histograms of pharyngeal pumping intervals (instantaneous pumping frequency) were obtained by binning all instantaneous frequency values (bin size used=0.1Hz) divided by the total number of minutes. Similarly, Videomicroscopy recording of adult worms was obtained using the same procedure. Under all experimental conditions, worms were mounted on the slides in presence of bacteria. 5 worms were used for each of the assessed genotypes and each experiment was repeated three times.

4.2.3. Serotonin modulation of pharyngeal pumping behavioural assay. To test the feeding behaviour response of *lat-1* mutants to serotonin, *lat-1* mutants were first treated with serotonin by placing the worms on NGM plates treated with serotonin (to a final concentration of 1.3mM, prepared as described above, section 4.1.2. of this chapter). The worms were incubated at 22'C for one hour, before they were mounted on the slides for recording.

4.3. Egg laying behavioural assay.

4.3.1. *C. elegans* strains. Worm strains were cultured and maintained according to standard procedures described by S. Brenner (Brenner 1974). Animals were cultivated at 22'C unless otherwise noted. The animals described as wild type were the *C. elegans* variety Bristol strain N2.

LG II: *lat-1(ok1465)* [XA7500] (Langenhan, Promel et al. 2009).

LG III: *egl-5(n1439) III; him-5(e1490) V* (strain generated in Jonathan Hodgkin lab, and kindly gifted by Allison Wollard lab).

4.3.2. Serotonin effect on egg laying behaviour. NGM plates were prepared as described in (Stiernagle 2006) and treated with serotonin to a final concentration of 1.3mM as described in the previous section (section 4.1.2. of this chapter). Day1, day2 and day 3 adult worms were selected from synchronized worm populations (obtained by bleaching mixed population worms plates as described in section 4.1.3. of this chapter). Worms were individually transferred to serotonin-treated NGM plates, left for two hours, then the number of egg laid were counted under the microscope. Triplets of 20 hermaphrodites were used in each experiment and each experiment was repeated three times.

4.3.3. Retained eggs assay. Young adult hermaphrodites were picked and transferred to a 1-2ul drop of M9 containing 25mM sodium azide (NaN3) on 1% agarose pad

mounted on a microscope slide, covered with a coverslip and sealed with molten Vaseline to lock moisture. NaN_3 anesthetizes the worms so that they will not move allowing accurate counting of embryos inside the uterus. Worms were then scrutinized under a Leica DM RXA2 microscope fitted with Quimaging Regita Exi digital camera. Total number of hermaphrodites used is as follows: *lat-1* mutants (n=52), wild type worms (n=35) and *egl-5* mutant hermaphrodites (n=32).

V. Conclusion

The results presented in this work illustrate the complexity of GPCRs function. Despite the fact that the *C. elegans* genome carries two homologs of latrophilin; *lat-1* and *lat-2*, knocking down the gene function of a single copy results in a large range of morphological defects observed throughout the development of the worm and very likely performing different functions. We have identified a polarity defect affecting the anterior-posterior division axis in a blastomere that was not identified in previous screens. How the lack of LAT-1exactly results in a lost anterio-posterior division axis in not known. In future experiments, it would be interesting to test the integrity of the cytoskeleton and the subcellular localisation of microtubules and components of the mitotic spindle in the ABal blastomere while it divides, in addition to performing epistasis analysis with mutants on other components of the Wnt signalling pathway. Polarity defects were previously reported to be caused by mutatons in other adhesion GPCRs such as the *Drosophila* Flamingo and its *C. elegans* ortholog *cdh-6* (*cdh-6* mutants exhibits axon outgrowth defects). Whether latrophilin controls the polarity in other cells/tissues (such us the patterning of the vulva tissue) remains to be further investigated. The role of adhesion GPCRs in cell guidance has been reported in prevous works in *Drosophila*, and the *C. elegans* latrophilin also functions in a pathway that is required for cell arrangement: *lat-1* mutatnts has axon guidance defects (Langenhan, personal communication) as well as a highly penetrant a-p polarity defect in the embryos. However, whether *lat-1* has an adhesive function is not known, and future experiments should be directed towards investigating the mode of action of *lat-1* and its possible role in adhesion, such as performing electron microscopy analysis to dissect the nature of cell adhesion between ABala/p and MS blastomeres. So far, we were able to link another adhesion GPCR to function in regulating cell polarity, however whether it signals through small G proteins in not know and requires further investigation. In view of this, we found very similar phenotypes between *lat-1* and *goa-1* muatnts in that they are both

sensitive to inhibitors of acetylcholinesterase suggesting an increased Ach release at the NMJs in addition to their irresponsiveness to serotonin when it comes to rescueing this sensitivity to aldicarb. In order to see whether *lat-1* signals through *goa-1*/$G_{\alpha o}$ we would need to analyse the scope of Ach release in the double mutants and perform *lat-1* and *goa-1* rescue experiments in the double mutants. Furthermore, in order to put in context our study which aims at characterizing the function of an adhesion GPCRs (due to the high therapeutic potential as a future drug target), we would need to try and rescue *lat-1* lethality with human latrophilin gene, especially that latrophilin has now been directly implicated in the ethiology of ADHD (Attention Defecit Hyperactivity Disorder). Further characterizing the function and mode of action of lat-1 would bring us one step forward to understand the ethiology of ADHD disorder in hope to find future therapies.

VI. References

Adler, P. N. (2002). "Planar signaling and morphogenesis in Drosophila." Dev Cell **2**(5): 525-535.

Ahmed, S., I. N. Maruyama, et al. (1992). "The Caenorhabditis elegans unc-13 gene product is a phospholipid-dependent high-affinity phorbol ester receptor." Biochem J **287 (Pt 3)**: 995-999.

Altun, Z. F. a. H., D. H. (2005). Handbook of C. elegans Anatomy. In WormAtlas.

Antoshechkin, I. and M. Han (2002). "The C. elegans evl-20 gene is a homolog of the small GTPase ARL2 and regulates cytoskeleton dynamics during cytokinesis and morphogenesis." Dev Cell **2**(5): 579-591.

Aravamudan, B., T. Fergestad, et al. (1999). "Drosophila UNC-13 is essential for synaptic transmission." Nat Neurosci **2**(11): 965-971.

Arcos-Burgos, M., M. Jain, et al. (2010). "A common variant of the latrophilin 3 gene, LPHN3, confers susceptibility to ADHD and predicts effectiveness of stimulant medication." Mol Psychiatry **15**(11): 1053-1066.

Ashton, A. C., M. A. Rahman, et al. (2000). "Tetramerisation of alpha-latrotoxin by divalent cations is responsible for toxin-induced non-vesicular release and contributes to the Ca(2+)-dependent vesicular exocytosis from synaptosomes." Biochimie **82**(5): 453-468.

Avery, L. (1993). "The genetics of feeding in Caenorhabditis elegans." Genetics **133**(4): 897-917.

Avery, L. (1993). "Motor neuron M3 controls pharyngeal muscle relaxation timing in Caenorhabditis elegans." J Exp Biol **175**: 283-297.

Avery, L. and H. R. Horvitz (1989). "Pharyngeal pumping continues after laser killing of the pharyngeal nervous system of C. elegans." Neuron **3**(4): 473-485.

Avery, L. and H. R. Horvitz (1990). "Effects of starvation and neuroactive drugs on feeding in Caenorhabditis elegans." J Exp Zool **253**(3): 263-270.

Bany, I. A., M. Q. Dong, et al. (2003). "Genetic and cellular basis for acetylcholine inhibition of Caenorhabditis elegans egg-laying behavior." J Neurosci **23**(22): 8060-8069.

Bei, Y., J. Hogan, et al. (2002). "SRC-1 and Wnt signaling act together to specify endoderm and to control cleavage orientation in early C. elegans embryos." Dev Cell **3**(1): 113-125.

Betz, A., U. Ashery, et al. (1998). "Munc13-1 is a presynaptic phorbol ester receptor that enhances neurotransmitter release." Neuron **21**(1): 123-136.

Bischoff, M. and R. Schnabel (2006). "A posterior centre establishes and maintains polarity of the Caenorhabditis elegans embryo by a Wnt-dependent relay mechanism." PLoS Biol **4**(12): e396.

Bittner, M. A., V. G. Krasnoperov, et al. (1998). "A Ca2+-independent receptor for alpha-latrotoxin, CIRL, mediates effects on secretion via multiple mechanisms." J Neurosci **18**(8): 2914-2922.

Bjarnadottir, T. K., R. Fredriksson, et al. (2005). "The gene repertoire and the common evolutionary history of glutamate, pheromone (V2R), taste(1) and other related G protein-coupled receptors." Gene **362**: 70-84.

Bjarnadottir, T. K., R. Fredriksson, et al. (2007). "The adhesion GPCRs: a unique family of G protein-coupled receptors with important roles in both central and peripheral tissues." Cell Mol Life Sci **64**(16): 2104-2119.

Bockaert, J. and J. P. Pin (1999). "Molecular tinkering of G protein-coupled receptors: an evolutionary success." Embo J **18**(7): 1723-1729.

Bourne, H. R. and E. C. Meng (2000). "Structure. Rhodopsin sees the light." Science **289**(5480): 733-734.

Brauner-Osborne, H., A. A. Jensen, et al. (2001). "Cloning and characterization of a human orphan family C G-protein coupled receptor GPRC5D." Biochim Biophys Acta **1518**(3): 237-248.

Brauner-Osborne, H. and P. Krogsgaard-Larsen (2000). "Sequence and expression pattern of a novel human orphan G-protein-coupled receptor, GPRC5B, a family C receptor with a short amino-terminal domain." Genomics **65**(2): 121-128.

Brenner, S. (1974). "The genetics of Caenorhabditis elegans." Genetics **77**(1): 71-94.
Brose, N., K. Hofmann, et al. (1995). "Mammalian homologues of Caenorhabditis elegans unc-13 gene define novel family of C2-domain proteins." J Biol Chem **270**(42): 25273-25280.
Brundage, L., L. Avery, et al. (1996). "Mutations in a C. elegans Gqalpha gene disrupt movement, egg laying, and viability." Neuron **16**(5): 999-1009.
Capogna, M., K. E. Volynski, et al. (2003). "The alpha-latrotoxin mutant LTXN4C enhances spontaneous and evoked transmitter release in CA3 pyramidal neurons." J Neurosci **23**(10): 4044-4053.
Chin-Sang, I. D., S. E. George, et al. (1999). "The ephrin VAB-2/EFN-1 functions in neuronal signaling to regulate epidermal morphogenesis in C. elegans." Cell **99**(7): 781-790.
Chin-Sang, I. D., S. L. Moseley, et al. (2002). "The divergent C. elegans ephrin EFN-4 functions inembryonic morphogenesis in a pathway independent of the VAB-1 Eph receptor." Development **129**(23): 5499-5510.
Chisholm, A. D. a. H., J. (December 01, 2005). Epidermal morphogenesis.
Colavita, A. and J. G. Culotti (1998). "Suppressors of ectopic UNC-5 growth cone steering identify eight genes involved in axon guidance in Caenorhabditis elegans." Dev Biol **194**(1): 72-85.
Cowan, C. R. and A. A. Hyman (2004). "Asymmetric cell division in C. elegans: cortical polarity and spindle positioning." Annu Rev Cell Dev Biol **20**: 427-453.
Curtin, J. A., E. Quint, et al. (2003). "Mutation of Celsr1 disrupts planar polarity of inner ear hair cells and causes severe neural tube defects in the mouse." Curr Biol **13**(13): 1129-1133.
Davies, J. Q., G. W. Chang, et al. (2007). "The role of receptor oligomerization in modulating the expression and function of leukocyte adhesion-G protein-coupled receptors." J Biol Chem **282**(37): 27343-27353.
Dent, J. A., M. W. Davis, et al. (1997). "avr-15 encodes a chloride channel subunit that mediates inhibitory glutamatergic neurotransmission and ivermectin sensitivity in Caenorhabditis elegans." EMBO J **16**(19): 5867-5879.
Desai, C., G. Garriga, et al. (1988). "A genetic pathway for the development of the Caenorhabditis elegans HSN motor neurons." Nature **336**(6200): 638-646.
Desai, C. and H. R. Horvitz (1989). "Caenorhabditis elegans mutants defective in the functioning of the motor neurons responsible for egg laying." Genetics **121**(4): 703-721.
Deshpande, R., T. Inoue, et al. (2005). "lin-17/Frizzled and lin-18 regulate POP-1/TCF-1 localization and cell type specification during C. elegans vulval development." Dev Biol **278**(1): 118-129.
Devenport, D. and E. Fuchs (2008). "Planar polarization in embryonic epidermis orchestrates global asymmetric morphogenesis of hair follicles." Nat Cell Biol **10**(11): 1257-1268.
Dittman, J. S. and J. M. Kaplan (2008). "Behavioral impact of neurotransmitter-activated G-protein-coupled receptors: muscarinic and GABAB receptors regulate Caenorhabditis elegans locomotion." J Neurosci **28**(28): 7104-7112.
Dixon, R. A., B. K. Kobilka, et al. (1986). "Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin." Nature **321**(6065): 75-79.
Donohoe, D. R., R. A. Jarvis, et al. (2009). "Behavioral adaptation in C. elegans produced by antipsychotic drugs requires serotonin and is associated with calcium signaling and calcineurin inhibition." Neurosci Res **64**(3): 280-289.
Downing, A. K., V. Knott, et al. (1996). "Solution structure of a pair of calcium-binding epidermal growth factor-like domains: implications for the Marfan syndrome and other genetic disorders." Cell **85**(4): 597-605.
Edgley, M. L., D. L. Baillie, et al. (2006). "Genetic balancers." WormBook: 1-32.
Edgley, M. L. and D. L. Riddle (2001). "LG II balancer chromosomes in Caenorhabditis elegans: mT1(II;III) and the mIn1 set of dominantly and recessively marked inversions." Mol Genet Genomics **266**(3): 385-395.

Eisenmann, D. M. and S. K. Kim (2000). "Protruding vulva mutants identify novel loci and Wnt signaling factors that function during Caenorhabditis elegans vulva development." Genetics **156**(3): 1097-1116.

Eisenmann, D. M., J. N. Maloof, et al. (1998). "The beta-catenin homolog BAR-1 and LET-60 Ras coordinately regulate the Hox gene lin-39 during Caenorhabditis elegans vulval development." Development **125**(18): 3667-3680.

Ferguson, E. L. and H. R. Horvitz (1985). "Identification and characterization of 22 genes that affect the vulval cell lineages of the nematode Caenorhabditis elegans." Genetics **110**(1): 17-72.

Ferguson, E. L., P. W. Sternberg, et al. (1987). "A genetic pathway for the specification of the vulval cell lineages of Caenorhabditis elegans." Nature **326**(6110): 259-267.

Frokjaer-Jensen, C., K. S. Kindt, et al. (2006). "Effects of voltage-gated calcium channel subunit genes on calcium influx in cultured C. elegans mechanosensory neurons." J Neurobiol **66**(10): 1125-1139.

Frontali, N., B. Ceccarelli, et al. (1976). "Purification from black widow spider venom of a protein factor causing the depletion of synaptic vesicles at neuromuscular junctions." J Cell Biol **68**(3): 462-479.

Fukushima, Y., Y. Oshika, et al. (1998). "Brain-specific angiogenesis inhibitor 1 expression is inversely correlated with vascularity and distant metastasis of colorectal cancer." Int J Oncol **13**(5): 967-970.

Galvez, T., M. L. Parmentier, et al. (1999). "Mutagenesis and modeling of the GABAB receptor extracellular domain support a venus flytrap mechanism for ligand binding." J Biol Chem **274**(19): 13362-13369.

Gao, F. B., J. E. Brenman, et al. (1999). "Genes regulating dendritic outgrowth, branching, and routing in Drosophila." Genes Dev **13**(19): 2549-2561.

Gao, F. B., M. Kohwi, et al. (2000). "Control of dendritic field formation in Drosophila: the roles of flamingo and competition between homologous neurons." Neuron **28**(1): 91-101.

George, S. E., K. Simokat, et al. (1998). "The VAB-1 Eph receptor tyrosine kinase functions in neural and epithelial morphogenesis in C. elegans." Cell **92**(5): 633-643.

Gilleard, J. S. and J. D. McGhee (2001). "Activation of hypodermal differentiation in the Caenorhabditis elegans embryo by GATA transcription factors ELT-1 and ELT-3." Mol Cell Biol **21**(7): 2533-2544.

Gitai, Z., T. W. Yu, et al. (2003). "The netrin receptor UNC-40/DCC stimulates axon attraction and outgrowth through enabled and, in parallel, Rac and UNC-115/AbLIM." Neuron **37**(1): 53-65.

Gleason, J. E., E. A. Szyleyko, et al. (2006). "Multiple redundant Wnt signaling components function in two processes during C. elegans vulval development." Dev Biol **298**(2): 442-457.

Goldstein, B. (1995). "Cell contacts orient some cell division axes in the Caenorhabditis elegans embryo." J Cell Biol **129**(4): 1071-1080.

Goldstein, B., H. Takeshita, et al. (2006). "Wnt signals can function as positional cues in establishing cell polarity." Dev Cell **10**(3): 391-396.

Grace, C. R., M. H. Perrin, et al. (2004). "NMR structure and peptide hormone binding site of the first extracellular domain of a type B1 G protein-coupled receptor." Proc Natl Acad Sci U S A **101**(35): 12836-12841.

Gracheva, E. O., G. Hadwiger, et al. (2008). "Direct interactions between C. elegans RAB-3 and Rim provide a mechanism to target vesicles to the presynaptic density." Neurosci Lett **444**(2): 137-142.

Gray, J. X., M. Haino, et al. (1996). "CD97 is a processed, seven-transmembrane, heterodimeric receptor associated with inflammation." J Immunol **157**(12): 5438-5447.

Green, J. L., T. Inoue, et al. (2008). "Opposing Wnt pathways orient cell polarity during organogenesis." Cell **134**(4): 646-656.

Greenwald, I. (1987). "The lin-12 locus of Caenorhabditis elegans." Bioessays **6**(2): 70-73.

Greenwald, I. and G. Seydoux (1990). "Analysis of gain-of-function mutations of the lin-12 gene of Caenorhabditis elegans." Nature **346**(6280): 197-199.
Greenwald, I. S., P. W. Sternberg, et al. (1983). "The lin-12 locus specifies cell fates in Caenorhabditis elegans." Cell **34**(2): 435-444.
Guest, M., K. Bull, et al. (2007). "The calcium-activated potassium channel, SLO-1, is required for the action of the novel cyclo-octadepsipeptide anthelmintic, emodepside, in Caenorhabditis elegans." Int J Parasitol **37**(14): 1577-1588.
Hajdu-Cronin, Y. M., W. J. Chen, et al. (1999). "Antagonism between G(o)alpha and G(q)alpha in Caenorhabditis elegans: the RGS protein EAT-16 is necessary for G(o)alpha signaling and regulates G(q)alpha activity." Genes Dev **13**(14): 1780-1793.
Hallam, S., E. Singer, et al. (2000). "The C. elegans NeuroD homolog cnd-1 functions in multiple aspects of motor neuron fate specification." Development **127**(19): 4239-4252.
Hamann, J., W. Eichler, et al. (1995). "Expression Cloning and Chromosomal Mapping of the Leukocyte Activation Antigen Cd97, a New 7-Span Transmembrane Molecule of the Secretin Receptor Superfamily with an Unusual Extracellular Domain." Journal of Immunology **155**(4): 1942-1950.
Hamann, J., B. Vogel, et al. (1996). "The seven-span transmembrane receptor CD97 has a cellular ligand (CD55, DAF)." J Exp Med **184**(3): 1185-1189.
Hamdan, F. F., M. D. Ungrin, et al. (1999). "Characterization of a novel serotonin receptor from Caenorhabditis elegans: cloning and expression of two splice variants." J Neurochem **72**(4): 1372-1383.
Hargrave, P. A., J. H. McDowell, et al. (1983). "The structure of bovine rhodopsin." Biophys Struct Mech **9**(4): 235-244.
Harris, T. W., E. Hartwieg, et al. (2000). "Mutations in synaptojanin disrupt synaptic vesicle recycling." J Cell Biol **150**(3): 589-600.
Hatanaka, H., Y. Oshika, et al. (2000). "Vascularization is decreased in pulmonary adenocarcinoma expressing brain-specific angiogenesis inhibitor 1 (BAI1)." Int J Mol Med **5**(2): 181-183.
Heid, P. J., W. B. Raich, et al. (2001). "The zinc finger protein DIE-1 is required for late events during epithelial cell rearrangement in C. elegans." Dev Biol **236**(1): 165-180.
Herman, R. K. and E. M. Hedgecock (1990). "Limitation of the size of the vulval primordium of Caenorhabditis elegans by lin-15 expression in surrounding hypodermis." Nature **348**(6297): 169-171.
Hilliard, M. A. and C. I. Bargmann (2006). "Wnt signals and frizzled activity orient anterior-posterior axon outgrowth in C. elegans." Dev Cell **10**(3): 379-390.
Hlubek, M. D., E. L. Stuenkel, et al. (2000). "Calcium-independent receptor for alpha-latrotoxin and neurexin 1alpha [corrected] facilitate toxin-induced channel formation: evidence that channel formation results from tethering of toxin to membrane." Mol Pharmacol **57**(3): 519-528.
Hobson, R. J., J. Geng, et al. (2003). "SER-7b, a constitutively active Galphas coupled 5-HT7-like receptor expressed in the Caenorhabditis elegans M4 pharyngeal motorneuron." J Neurochem **87**(1): 22-29.
Hopkins, A. L. and C. R. Groom (2002). "The druggable genome." Nat Rev Drug Discov **1**(9): 727-730.
Hosono, R., S. Hekimi, et al. (1992). "The unc-18 gene encodes a novel protein affecting the kinetics of acetylcholine metabolism in the nematode Caenorhabditis elegans." J Neurochem **58**(4): 1517-1525.
Hutter, H. and R. Schnabel (1995). "Specification of anterior-posterior differences within the AB lineage in the C. elegans embryo: a polarising induction." Development **121**(5): 1559-1568.
Ichtchenko, K., M. Khvotchev, et al. (1998). "alpha-latrotoxin action probed with recombinant toxin: receptors recruit alpha-latrotoxin but do not transduce an exocytotic signal." EMBO J **17**(21): 6188-6199.

Iguchi, T., K. Sakata, et al. (2008). "Orphan G protein-coupled receptor GPR56 regulates neural progenitor cell migration via a G alpha 12/13 and Rho pathway." J Biol Chem **283**(21): 14469-14478.
Inoue, T., M. Wang, et al. (2005). "Transcriptional network underlying Caenorhabditis elegans vulval development." Proc Natl Acad Sci U S A **102**(14): 4972-4977.
Jin, Z., I. Tietjen, et al. (2007). "Disease-associated mutations affect GPR56 protein trafficking and cell surface expression." Hum Mol Genet **16**(16): 1972-1985.
Kanazawa, T., M. D. Zappaterra, et al. (2008). "The C. elegans Opa1 homologue EAT-3 is essential for resistance to free radicals." PLoS Genet **4**(2): e1000022.
Keane, J. and L. Avery (2003). "Mechanosensory inputs influence Caenorhabditis elegans pharyngeal activity via ivermectin sensitivity genes." Genetics **164**(1): 153-162.
Kemphues, K. J., M. Kusch, et al. (1988). "Maternal-effect lethal mutations on linkage group II of Caenorhabditis elegans." Genetics **120**(4): 977-986.
Kimble, J. (1981). "Alterations in cell lineage following laser ablation of cells in the somatic gonad of Caenorhabditis elegans." Dev Biol **87**(2): 286-300.
Kobe, B. and A. V. Kajava (2001). "The leucine-rich repeat as a protein recognition motif." Curr Opin Struct Biol **11**(6): 725-732.
Kobilka, B. K., C. MacGregor, et al. (1987). "Functional activity and regulation of human beta 2-adrenergic receptors expressed in Xenopus oocytes." J Biol Chem **262**(32): 15796-15802.
Krasnoperov, V., Y. Lu, et al. (2002). "Post-translational proteolytic processing of the calcium-independent receptor of alpha-latrotoxin (CIRL), a natural chimera of the cell adhesion protein and the G protein-coupled receptor. Role of the G protein-coupled receptor proteolysis site (GPS) motif." J Biol Chem **277**(48): 46518-46526.
Krasnoperov, V. G., M. A. Bittner, et al. (1997). "alpha-Latrotoxin stimulates exocytosis by the interaction with a neuronal G-protein-coupled receptor." Neuron **18**(6): 925-937.
Lagerstrom, M. C. and H. B. Schioth (2008). "Structural diversity of G protein-coupled receptors and significance for drug discovery." Nat Rev Drug Discov **7**(4): 339-357.
Lang, J., Y. Ushkaryov, et al. (1998). "Ca2+-independent insulin exocytosis induced by alpha-latrotoxin requires latrophilin, a G protein-coupled receptor." EMBO J **17**(3): 648-657.
Langenhan, T., S. Promel, et al. (2009). "Latrophilin signaling links anterior-posterior tissue polarity and oriented cell divisions in the C. elegans embryo." Dev Cell **17**(4): 494-504.
Lawrence, P. A., G. Struhl, et al. (2007). "Planar cell polarity: one or two pathways?" Nat Rev Genet **8**(7): 555-563.
Lee, R. C., T. R. Clandinin, et al. (2003). "The protocadherin Flamingo is required for axon target selection in the Drosophila visual system." Nat Neurosci **6**(6): 557-563.
Lee, Y. S., Y. S. Park, et al. (2000). "Characterization of GAR-2, a novel G protein-linked acetylcholine receptor from Caenorhabditis elegans." J Neurochem **75**(5): 1800-1809.
Leemans, J. C., A. A. te Velde, et al. (2004). "The epidermal growth factor-seven transmembrane (EGF-TM7) receptor CD97 is required for neutrophil migration and host defense." J Immunol **172**(2): 1125-1131.
Lelianova, V. G., B. A. Davletov, et al. (1997). "Alpha-latrotoxin receptor, latrophilin, is a novel member of the secretin family of G protein-coupled receptors." J Biol Chem **272**(34): 21504-21508.
Li, H., L. Avery, et al. (1997). "Identification of chemical synapses in the pharynx of Caenorhabditis elegans." Proc Natl Acad Sci U S A **94**(11): 5912-5916.
Lin, H. H., M. Stacey, et al. (2001). "Molecular analysis of the epidermal growth factor-like short consensus repeat domain-mediated protein-protein interactions: dissection of the CD97-CD55 complex." J Biol Chem **276**(26): 24160-24169.
Lin, R., S. Thompson, et al. (1995). "pop-1 encodes an HMG box protein required for the specification of a mesoderm precursor in early C. elegans embryos." Cell **83**(4): 599-609.

Lin, Y. J., L. Seroude, et al. (1998). "Extended life-span and stress resistance in the Drosophila mutant methuselah." Science **282**(5390): 943-946.
Lloyd, T. E., P. Verstreken, et al. (2000). "A genome-wide search for synaptic vesicle cycle proteins in Drosophila." Neuron **26**(1): 45-50.
Lu, B., T. Usui, et al. (1999). "Flamingo controls the planar polarity of sensory bristles and asymmetric division of sensory organ precursors in Drosophila." Curr Biol **9**(21): 1247-1250.
Lundquist, E. A., R. K. Herman, et al. (1998). "UNC-115, a conserved protein with predicted LIM and actin-binding domains, mediates axon guidance in C. elegans." Neuron **21**(2): 385-392.
Lyuksyutova, A. I., C. C. Lu, et al. (2003). "Anterior-posterior guidance of commissural axons by Wnt-frizzled signaling." Science **302**(5652): 1984-1988.
Mahoney, T. R., S. Luo, et al. (2006). "Analysis of synaptic transmission in Caenorhabditis elegans using an aldicarb-sensitivity assay." Nat Protoc **1**(4): 1772-1777.
Mango, S. E., E. J. Lambie, et al. (1994). "The pha-4 gene is required to generate the pharyngeal primordium of Caenorhabditis elegans." Development **120**(10): 3019-3031.
Marston, D. J., M. Roh, et al. (2008). "Wnt signaling during Caenorhabditis elegans embryonic development." Methods Mol Biol **469**: 103-111.
Maruyama, I. N. and S. Brenner (1991). "A phorbol ester/diacylglycerol-binding protein encoded by the unc-13 gene of Caenorhabditis elegans." Proc Natl Acad Sci U S A **88**(13): 5729-5733.
Masu, Y., K. Nakayama, et al. (1987). "cDNA cloning of bovine substance-K receptor through oocyte expression system." Nature **329**(6142): 836-838.
Mathews, E. A., E. Garcia, et al. (2003). "Critical residues of the Caenorhabditis elegans unc-2 voltage-gated calcium channel that affect behavioral and physiological properties." J Neurosci **23**(16): 6537-6545.
Matsushita, H., V. G. Lelianova, et al. (1999). "The latrophilin family: multiply spliced G protein-coupled receptors with differential tissue distribution." FEBS Lett **443**(3): 348-352.
McMillan, D. R. and P. C. White (2004). "Loss of the transmembrane and cytoplasmic domains of the very large G-protein-coupled receptor-1 (VLGR1 or Mass1) causes audiogenic seizures in mice." Mol Cell Neurosci **26**(2): 322-329.
Mendel, J. E., H. C. Korswagen, et al. (1995). "Participation of the protein Go in multiple aspects of behavior in C. elegans." Science **267**(5204): 1652-1655.
Michelini, E., L. Cevenini, et al. (2010). "Cell-based assays: fuelling drug discovery." Anal Bioanal Chem **398**(1): 227-238.
Miller, K. G., M. D. Emerson, et al. (1999). "Goalpha and diacylglycerol kinase negatively regulate the Gqalpha pathway in C. elegans." Neuron **24**(2): 323-333.
Minaba, M., S. Ichiyama, et al. (2006). "Activation of nematode G protein GOA-1 by the human muscarinic acetylcholine receptor M2 subtype. Functional coupling of G-protein-coupled receptor and G protein originated from evolutionarily distant animals." FEBS J **273**(24): 5508-5516.
Mizumoto, K. and H. Sawa (2007). "Cortical beta-catenin and APC regulate asymmetric nuclear beta-catenin localization during asymmetric cell division in C. elegans." Dev Cell **12**(2): 287-299.
Moresco, J. J. and M. R. Koelle (2004). "Activation of EGL-47, a Galpha(o)-coupled receptor, inhibits function of hermaphrodite-specific motor neurons to regulate Caenorhabditis elegans egg-laying behavior." J Neurosci **24**(39): 8522-8530.
Nakayama, J., Y. H. Fu, et al. (2002). "A nonsense mutation of the MASS1 gene in a family with febrile and afebrile seizures." Ann Neurol **52**(5): 654-657.
Nathans, J. and D. S. Hogness (1983). "Isolation, sequence analysis, and intron-exon arrangement of the gene encoding bovine rhodopsin." Cell **34**(3): 807-814.
Niacaris, T. and L. Avery (2003). "Serotonin regulates repolarization of the C. elegans pharyngeal muscle." J Exp Biol **206**(Pt 2): 223-231.
Nonet, M. L., K. Grundahl, et al. (1993). "Synaptic function is impaired but not eliminated in C. elegans mutants lacking synaptotagmin." Cell **73**(7): 1291-1305.

Nordstrom, K. J., M. C. Lagerstrom, et al. (2009). "The Secretin GPCRs descended from the family of Adhesion GPCRs." Mol Biol Evol **26**(1): 71-84.
Nurrish, S., L. Segalat, et al. (1999). "Serotonin inhibition of synaptic transmission: Galpha(0) decreases the abundance of UNC-13 at release sites." Neuron **24**(1): 231-242.
Opperman, C. H. and S. Chang (1991). "Effects of Aldicarb and Fenamiphos on Acetycholinesterase and Motility of Caenorhabditis elegans." J Nematol **23**(1): 20-27.
Ota, T., Y. Suzuki, et al. (2004). "Complete sequencing and characterization of 21,243 full-length human cDNAs." Nat Genet **36**(1): 40-45.
Page, B. D., W. Zhang, et al. (1997). "ELT-1, a GATA-like transcription factor, is required for epidermal cell fates in Caenorhabditis elegans embryos." Genes Dev **11**(13): 1651-1661.
Palczewski, K., T. Kumasaka, et al. (2000). "Crystal structure of rhodopsin: A G protein-coupled receptor." Science **289**(5480): 739-745.
Park, D., A. C. Tosello-Trampont, et al. (2007). "BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module." Nature **450**(7168): 430-434.
Park, D., A. C. Tosello-Trampont, et al. (2007). "BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module." Nature **450**(7168): 430-U410.
Park, F. D. and J. R. Priess (2003). "Establishment of POP-1 asymmetry in early C. elegans embryos." Development **130**(15): 3547-3556.
Parmentier, M., F. Libert, et al. (1989). "Molecular cloning of the thyrotropin receptor." Science **246**(4937): 1620-1622.
Piao, X., R. S. Hill, et al. (2004). "G protein-coupled receptor-dependent development of human frontal cortex." Science **303**(5666): 2033-2036.
Qu, Y., D. M. Glasco, et al. (2010). "Atypical cadherins Celsr1-3 differentially regulate migration of facial branchiomotor neurons in mice." J Neurosci **30**(28): 9392-9401.
Rabinovich, G. A. (1999). "Galectins: an evolutionarily conserved family of animal lectins with multifunctional properties; a trip from the gene to clinical therapy." Cell Death Differ **6**(8): 711-721.
Rahman, M. A., A. C. Ashton, et al. (1999). "Norepinephrine exocytosis stimulated by alpha-latrotoxin requires both external and stored Ca2+ and is mediated by latrophilin, G proteins and phospholipase C." Philos Trans R Soc Lond B Biol Sci **354**(1381): 379-386.
Raizen, D. M., R. Y. Lee, et al. (1995). "Interacting genes required for pharyngeal excitation by motor neuron MC in Caenorhabditis elegans." Genetics **141**(4): 1365-1382.
Ray, P., R. Schnabel, et al. (2008). "Behavioral and synaptic defects in C. elegans lacking the NK-2 homeobox gene ceh-28." Dev Neurobiol **68**(4): 421-433.
Ribases, M., R. Bosch, et al. (2009). "Case-control study of six genes asymmetrically expressed in the two cerebral hemispheres: association of BAIAP2 with attention-deficit/hyperactivity disorder." Biol Psychiatry **66**(10): 926-934.
Ribases, M., J. A. Ramos-Quiroga, et al. (2011). "Contribution of LPHN3 to the genetic susceptibility to ADHD in adulthood: a replication study." Genes Brain Behav **10**(2): 149-157.
Richmond, J. (2005). "Synaptic function." WormBook: 1-14.
Robatzek, M., T. Niacaris, et al. (2001). "eat-11 encodes GPB-2, a Gbeta(5) ortholog that interacts with G(o)alpha and G(q)alpha to regulate C. elegans behavior." Curr Biol **11**(4): 288-293.
Robbins, M. J., D. Michalovich, et al. (2000). "Molecular cloning and characterization of two novel retinoic acid-inducible orphan G-protein-coupled receptors (GPRC5B and GPRC5C)." Genomics **67**(1): 8-18.
Robitaille, R., E. M. Adler, et al. (1993). "Calcium channels and calcium-gated potassium channels at the frog neuromuscular junction." J Physiol Paris **87**(1): 15-24.
Robitaille, R., M. L. Garcia, et al. (1993). "Functional colocalization of calcium and calcium-gated potassium channels in control of transmitter release." Neuron **11**(4): 645-655.
Rogers, S., R. Wells, et al. (1986). "Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis." Science **234**(4774): 364-368.

Roth, A. F., D. M. Sullivan, et al. (1998). "A large PEST-like sequence directs the ubiquitination, endocytosis, and vacuolar degradation of the yeast a-factor receptor." J Cell Biol **142**(4): 949-961.
Russ, A. P. and S. Lampel (2005). "The druggable genome: an update." Drug Discov Today **10**(23-24): 1607-1610.
Sassa, T., S. Harada, et al. (1999). "Regulation of the UNC-18-Caenorhabditis elegans syntaxin complex by UNC-13." J Neurosci **19**(12): 4772-4777.
Schlesinger, A., C. A. Shelton, et al. (1999). "Wnt pathway components orient a mitotic spindle in the early Caenorhabditis elegans embryo without requiring gene transcription in the responding cell." Genes Dev **13**(15): 2028-2038.
Schmucker, D. and S. L. Zipursky (2001). "Signaling downstream of Eph receptors and ephrin ligands." Cell **105**(6): 701-704.
Schwarz, E. M. and S. Benzer (1997). "Calx, a Na-Ca exchanger gene of Drosophila melanogaster." Proc Natl Acad Sci U S A **94**(19): 10249-10254.
Segalat, L., D. A. Elkes, et al. (1995). "Modulation of serotonin-controlled behaviors by Go in Caenorhabditis elegans." Science **267**(5204): 1648-1651.
Sekhar, K. R. and M. L. Freeman (1998). "PEST sequences in proteins involved in cyclic nucleotide signalling pathways." J Recept Signal Transduct Res **18**(2-3): 113-132.
Senti, K. A., T. Usui, et al. (2003). "Flamingo regulates R8 axon-axon and axon-target interactions in the Drosophila visual system." Curr Biol **13**(10): 828-832.
Seydoux, G., C. Savage, et al. (1993). "Isolation and characterization of mutations causing abnormal eversion of the vulva in Caenorhabditis elegans." Dev Biol **157**(2): 423-436.
Shima, Y., M. Kengaku, et al. (2004). "Regulation of dendritic maintenance and growth by a mammalian 7-pass transmembrane cadherin." Dev Cell **7**(2): 205-216.
Shiratsuchi, T., H. Nishimori, et al. (1997). "Cloning and characterization of BAI2 and BAI3, novel genes homologous to brain-specific angiogenesis inhibitor 1 (BAI1)." Cytogenet Cell Genet **79**(1-2): 103-108.
Shyn, S. I., R. Kerr, et al. (2003). "Serotonin and Go modulate functional states of neurons and muscles controlling C. elegans egg-laying behavior." Curr Biol **13**(21): 1910-1915.
Sieburth, D., Q. Ch'ng, et al. (2005). "Systematic analysis of genes required for synapse structure and function." Nature **436**(7050): 510-517.
Siehler, S. (2008). "Cell-based assays in GPCR drug discovery." Biotechnol J **3**(4): 471-483.
Sigurdson, D. C., G. J. Spanier, et al. (1984). "Caenorhabditis elegans deficiency mapping." Genetics **108**(2): 331-345.
Simske, J. S. and J. Hardin (2001). "Getting into shape: epidermal morphogenesis in Caenorhabditis elegans embryos." Bioessays **23**(1): 12-23.
Skradski, S. L., A. M. Clark, et al. (2001). "A novel gene causing a mendelian audiogenic mouse epilepsy." Neuron **31**(4): 537-544.
Stacey, M., G. W. Chang, et al. (2003). "The epidermal growth factor-like domains of the human EMR2 receptor mediate cell attachment through chondroitin sulfate glycosaminoglycans." Blood **102**(8): 2916-2924.
Stacey, M., H. H. Lin, et al. (2001). "Human epidermal growth factor (EGF) module-containing mucin-like hormone receptor 3 is a new member of the EGF-TM7 family that recognizes a ligand on human macrophages and activated neutrophils." J Biol Chem **276**(22): 18863-18870.
Sternberg, P. W. (1988). "Lateral inhibition during vulval induction in Caenorhabditis elegans." Nature **335**(6190): 551-554.
Sternberg, P. W. and H. R. Horvitz (1986). "Pattern formation during vulval development in C. elegans." Cell **44**(5): 761-772.
Stiernagle, T. (2006). "Maintenance of C. elegans." WormBook: 1-11.

Sugita, S., K. Ichtchenko, et al. (1998). "alpha-Latrotoxin receptor CIRL/latrophilin 1 (CL1) defines an unusual family of ubiquitous G-protein-linked receptors. G-protein coupling not required for triggering exocytosis." J Biol Chem **273**(49): 32715-32724.
Sulston, J. E. and H. R. Horvitz (1977). "Post-embryonic cell lineages of the nematode, Caenorhabditis elegans." Dev Biol **56**(1): 110-156.
Sulston, J. E. and H. R. Horvitz (1981). "Abnormal cell lineages in mutants of the nematode Caenorhabditis elegans." Dev Biol **82**(1): 41-55.
Sulston, J. E., E. Schierenberg, et al. (1983). "The embryonic cell lineage of the nematode Caenorhabditis elegans." Dev Biol **100**(1): 64-119.
Sulston, J. E. and J. G. White (1980). "Regulation and cell autonomy during postembryonic development of Caenorhabditis elegans." Dev Biol **78**(2): 577-597.
Sundaram, M. and I. Greenwald (1993). "Genetic and phenotypic studies of hypomorphic lin-12 mutants in Caenorhabditis elegans." Genetics **135**(3): 755-763.
Sze, J. Y., M. Victor, et al. (2000). "Food and metabolic signalling defects in a Caenorhabditis elegans serotonin-synthesis mutant." Nature **403**(6769): 560-564.
Tanis, J. E., A. Bellemer, et al. (2009). "The potassium chloride cotransporter KCC-2 coordinates development of inhibitory neurotransmission and synapse structure in Caenorhabditis elegans." J Neurosci **29**(32): 9943-9954.
Tanis, J. E., J. J. Moresco, et al. (2008). "Regulation of serotonin biosynthesis by the G proteins Galphao and Galphaq controls serotonin signaling in Caenorhabditis elegans." Genetics **178**(1): 157-169.
Taylor, C. W., R. Taufiq Ur, et al. (2009). "Targeting and clustering of IP3 receptors: key determinants of spatially organized Ca2+ signals." Chaos **19**(3): 037102.
Thomas-Virnig, C. L., P. A. Sims, et al. (2004). "The inositol 1,4,5-trisphosphate receptor regulates epidermal cell migration in Caenorhabditis elegans." Curr Biol **14**(20): 1882-1887.
Thorpe, C. J., A. Schlesinger, et al. (1997). "Wnt signaling polarizes an early C. elegans blastomere to distinguish endoderm from mesoderm." Cell **90**(4): 695-705.
Tissir, F., I. Bar, et al. (2005). "Protocadherin Celsr3 is crucial in axonal tract development." Nat Neurosci **8**(4): 451-457.
Tokumaru, H. and G. J. Augustine (1999). "UNC-13 and neurotransmitter release." Nat Neurosci **2**(11): 929-930.
Towers, P. R., B. Edwards, et al. (2005). "The Caenorhabditis elegans lev-8 gene encodes a novel type of nicotinic acetylcholine receptor alpha subunit." J Neurochem **93**(1): 1-9.
Tyndall, J. D. and R. Sandilya (2005). "GPCR agonists and antagonists in the clinic." Med Chem **1**(4): 405-421.
Usui, T., Y. Shima, et al. (1999). "Flamingo, a seven-pass transmembrane cadherin, regulates planar cell polarity under the control of Frizzled." Cell **98**(5): 585-595.
Vakonakis, I., T. Langenhan, et al. (2008). "Solution structure and sugar-binding mechanism of mouse latrophilin-1 RBL: a 7TM receptor-attached lectin-like domain." Structure **16**(6): 944-953.
Vazquez-Manrique, R. P., A. I. Nagy, et al. (2008). "Phospholipase C-epsilon regulates epidermal morphogenesis in Caenorhabditis elegans." PLoS Genet **4**(3): e1000043.
Vinson, C. R., S. Conover, et al. (1989). "A Drosophila tissue polarity locus encodes a protein containing seven potential transmembrane domains." Nature **338**(6212): 263-264.
Walker, D. S., N. J. Gower, et al. (2002). "Regulated disruption of inositol 1,4,5-trisphosphate signaling in Caenorhabditis elegans reveals new functions in feeding and embryogenesis." Mol Biol Cell **13**(4): 1329-1337.
Walker, D. S., S. Ly, et al. (2004). "IRI-1, a LIN-15B homologue, interacts with inositol-1,4,5-triphosphate receptors and regulates gonadogenesis, defecation, and pharyngeal pumping in Caenorhabditis elegans." Mol Biol Cell **15**(7): 3073-3082.
Walston, T., C. Tuskey, et al. (2004). "Multiple Wnt signaling pathways converge to orient the mitotic spindle in early C. elegans embryos." Dev Cell **7**(6): 831-841.

Walston, T. D. and J. Hardin (2006). "Wnt-dependent spindle polarization in the early C. elegans embryo." Semin Cell Dev Biol **17**(2): 204-213.
Wang, X., P. J. Roy, et al. (1999). "Multiple ephrins control cell organization in C. elegans using kinase-dependent and -independent functions of the VAB-1 Eph receptor." Mol Cell **4**(6): 903-913.
Weston, M. D., M. W. Luijendijk, et al. (2004). "Mutations in the VLGR1 gene implicate G-protein signaling in the pathogenesis of Usher syndrome type II." Am J Hum Genet **74**(2): 357-366.
White, G. R., J. M. Varley, et al. (1998). "Isolation and characterization of a human homologue of the latrophilin gene from a region of 1p31.1 implicated in breast cancer." Oncogene **17**(26): 3513-3519.
Williams-Masson, E. M., P. J. Heid, et al. (1998). "The cellular mechanism of epithelial rearrangement during morphogenesis of the Caenorhabditis elegans dorsal hypodermis." Dev Biol **204**(1): 263-276.
Willson, J., K. Amliwala, et al. (2004). "Latrotoxin receptor signaling engages the UNC-13-dependent vesicle-priming pathway in C. elegans." Curr Biol **14**(15): 1374-1379.
Winnier, A. R., J. Y. Meir, et al. (1999). "UNC-4/UNC-37-dependent repression of motor neuron-specific genes controls synaptic choice in Caenorhabditis elegans." Genes Dev **13**(21): 2774-2786.
Withee, J., B. Galligan, et al. (2004). "Caenorhabditis elegans WASP and Ena/VASP proteins play compensatory roles in morphogenesis and neuronal cell migration." Genetics **167**(3): 1165-1176.
Wobus, M., B. Vogel, et al. (2004). "N-glycosylation of CD97 within the EGF domains is crucial for epitope accessibility in normal and malignant cells as well as CD55 ligand binding." Int J Cancer **112**(5): 815-822.
Wood, W. B., R. Hecht, et al. (1980). "Parental effects and phenotypic characterization of mutations that affect early development in Caenorhabditis elegans." Dev Biol **74**(2): 446-469.
Xu, J. W. and M. M. Slaughter (2005). "Large-conductance calcium-activated potassium channels facilitate transmitter release in salamander rod synapse." J Neurosci **25**(33): 7660-7668.
Xu, Q., Y. Wang, et al. (2004). "Vascular development in the retina and inner ear: control by Norrin and Frizzled-4, a high-affinity ligand-receptor pair." Cell **116**(6): 883-895.
Yook, K. J., S. R. Proulx, et al. (2001). "Rules of nonallelic noncomplementation at the synapse in Caenorhabditis elegans." Genetics **158**(1): 209-220.
Yoshikawa, S., R. D. McKinnon, et al. (2003). "Wnt-mediated axon guidance via the Drosophila Derailed receptor." Nature **422**(6932): 583-588.
Yu, S., K. Hackmann, et al. (2007). "Essential role of cleavage of Polycystin-1 at G protein-coupled receptor proteolytic site for kidney tubular structure." Proc Natl Acad Sci U S A **104**(47): 18688-18693.

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