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Phenotype analysis of *Caenorhabditis elegans* lacking the intestinal peptide transporter

Barbara Meissner

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1 Introduction

1.1 Proton-coupled peptide transporters

In the mid-1970s, the active absorption of short chain peptides in the mammalian gut epithelium was shown for the first time (Addison et al. 1972; Matthews et al. 1974). During the 1980s, the intestinal di- and tripeptide transport system was characterized functionally and evidence accumulated that similar uptake processes are also present in epithelial cells of the kidney tubule (Ganapathy and Leibach 1982). 1994, an intestinal peptide transporter was cloned (Boll et al. 1994; Fei et al. 1994) followed by other mammalian species, identified by homology screening and cloning (Liang et al. 1995; Saito et al. 1995; Rubio-Aliaga et al. 2000). The intestinal isoform was designated PEPT1, the renal isoform PEPT2. Both were the first mammalian nutrient transporters to be identified that use an electrochemical proton gradient as their driving force thus, substrate uptake depends strongly on extracellular pH and membrane potential (Ganapathy and Leibach 1983).

Peptide transporters are integral plasma membrane proteins that mediate the cellular uptake of di- and tripeptides and a variety of peptidomimetics. The transporter proteins contain 12 predicted transmembrane domains (TMD), the N- and C-termini in the cytosol (Fig. 1.1). The peptide transporters in mammals - like in most other species - are proton-dependent rheogenic carriers (Daniel 1996) and have been grouped into the Proton-coupled Oligopeptide Transporter (POT) superfamily which is also called Peptide TRansporter (PTR) family (Steiner et al. 1995). The transport process is associated with proton translocation and movement of positive charges and, thus, transport is always electrogenic, irrespective of the substrate's net charge. Substrate uptake at the intestinal brush border membrane causes proton influx that in turn leads to increased proton efflux back to the lumen by the apical sodium-proton exchanger NHE3 (Thwaites et al. 2002). Sodium influx via NHE3 is compensated by export through the basolateral sodium-potassium ATPase and potassium ions taken up leave the cell by the potassium channels.

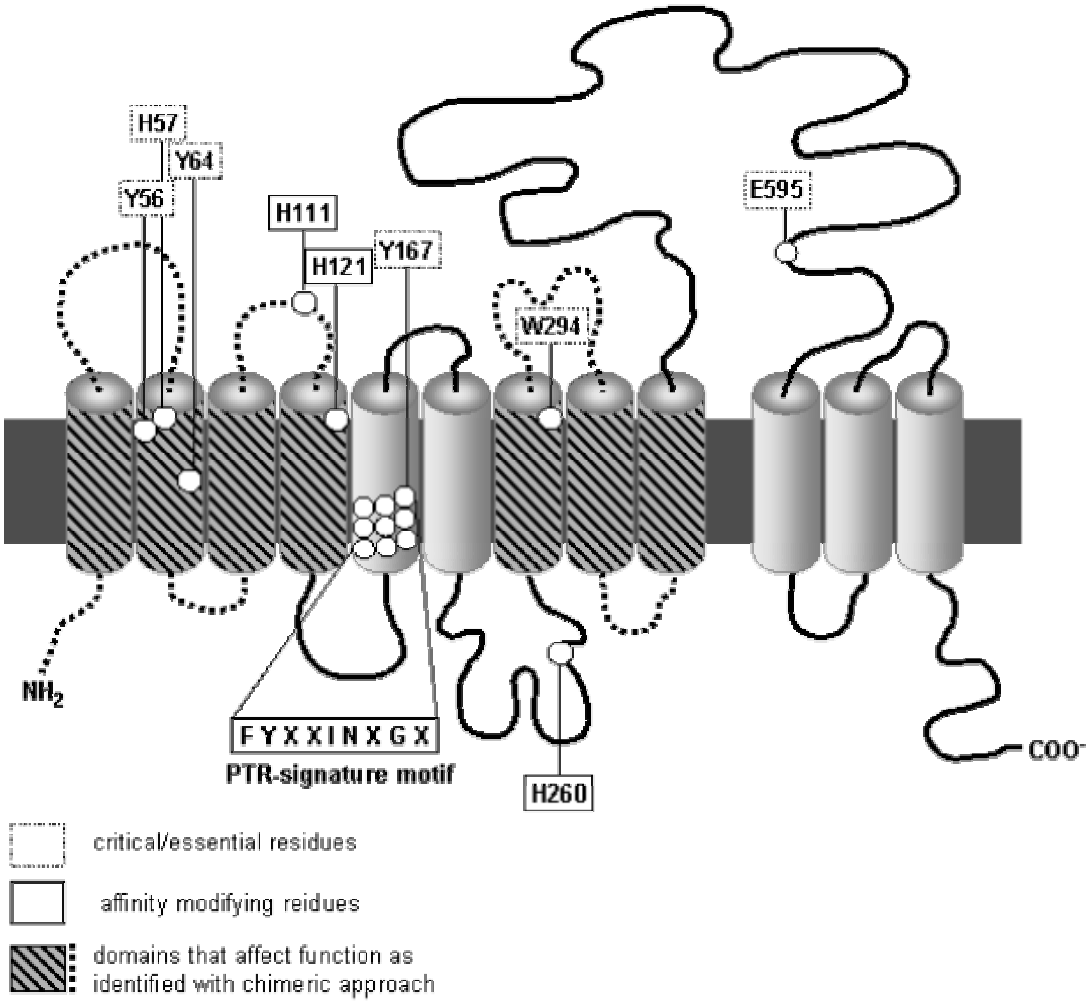

Fig. 1.1 Membrane topoloty model of PEPT1 (modified from Daniel and Kottra 2004). Domains and individual amino acid residues that have been identified as relevant in determining the functional characteristics of the protein are marked. The PTR-signature motif as highly conserved sequence stretch is found in all POT family members.

PEPT1 was first identified in the brush-border membrane of the epithelial cells of the small intestine and cloned from an intestinal cDNA library (Boll et al. 1994; Fei et al. 1994). In addition, it was found in the brush-border membrane of epithelial cells in the kidney proximal tubule (Shen et al. 1999) and more recently it has been shown to be expressed in the apical membrane of bile duct epithelial cells (Knutter et al. 2002). The main physiological function of the peptide transporter PEPT1 is the absorption of bulk quantities of amino acids in their peptide-bound form in the small intestine. PEPT2 shows much broader expression and tissue distribution. It was initially cloned from a renal cDNA library (Boll et al. 1996) and was shown to be expressed in the epithelial cells of the kidney (Shen et al. 1999). More recently it has been identified in the PNS (Groneberg et al. 2001b), CNS (Berger and Hediger 1999; Novotny et al. 2000), mammary gland (Groneberg et al. 2002) and lung (Groneberg et al. 2001c). In

the kidney, the peptide transporters contribute to the homeostasis of amino acids in the organism along with several classes of amino acid transporters located as well in the apical membrane of tubular cells (for a review, see Palacin et al. 1998). In brain astrocytes, PEPT2 was proposed to contribute to brain glutathione metabolism by providing cysteinyl-glycine derived from extracellular glutathione for glial glutathione resynthesis (Dringen et al. 1998) or for removing neuroactive peptides such as Kyotorphin (Hussain et al. 2001). The physiological role of PEPT2 in lung and mammary gland epithelium is not yet known.

The substrate specificity for PEPT1 and PEPT2 has been investigated mainly in competition studies with selected peptides and derivatives. It was shown that neither amino acids nor tetra- or oligopeptides exhibit an inhibitory effect (Ganapathy and Leibach 1991; Daniel et al. 1992). In addition to the 400 different dipeptides and 8000 different tripeptides derived from the 20 proteinogenic L-α-amino acids, a number of drugs and prodrugs have been reported to be recognized by the transporters. They include β-lactam antibiotics such as penicillins and cephalosporins (Bretschneider et al. 1999), ACE-inhibitors (Fig. 1.2) (Boll et al. 1994; Moore et al. 2000), renin inhibitors (Kramer et al. 1990), thrombin inhibitors (Walter et al. 1995) and the dipeptide-like antineoplastic drug bestatin (Inui et al. 1992).

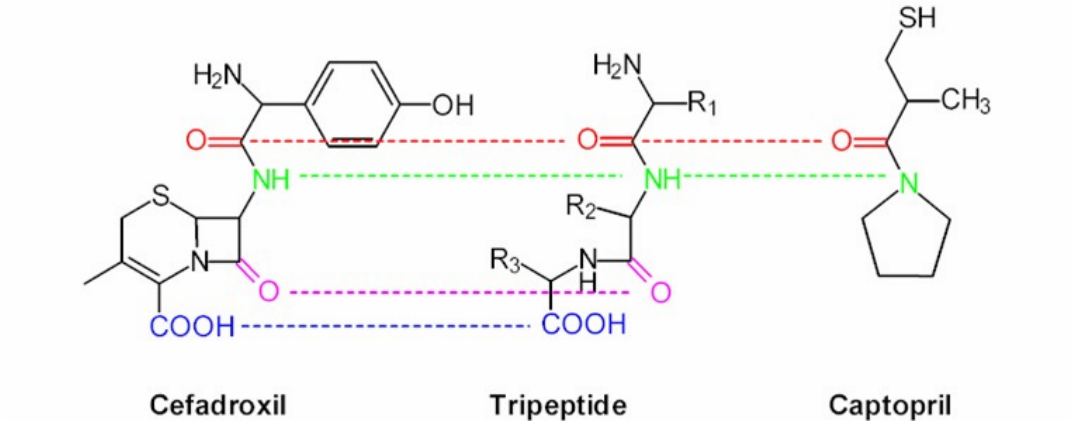

Fig. 1.2 Minimal structural similarities between the different classes of substrates are shown for cefadroxil (cephalosporin), a tripeptide and captopril (ACE inhibitor). (Rubio-Aliaga et al. 2002)

Prodrugs of acyclovir, ganciclovir and L-Dopa are also recognized and transported by PEPT1 (Hu et al. 1989; Balimane et al. 1998; Tamai et al. 1998). The knowledge about substrate specificity and the transport capacities of PEPT1 and PEPT2 enables the rational design of pharmacological compounds that possess good oral availability by delivery via PEPT1. PEPT2, the renal tubular peptide transporter may

contribute to the renal clearance of drugs by efficient reabsorption, thus affecting their pharmacokinetics.

Although similar in function, both proteins display major differences in substrate affinity and transport capacity. PEPT1 can be considered as a low-affinity, high-capacity system with apparent affinity constants (Km values) ranging form 200 µM to 10 mM, depending on the substrate. Under identical experimental conditions, the affinity of PEPT2 is higher for most, but nor all, substrates. PEPT2 can be considered as a high-affinity, low-capacity transport system with Km values of 5-500 µM, depending on the substrate (Daniel and Herget 1997).

The transport activity of PEPT1 can be modulated to meet physiological needs either by regulation at the transcriptional level or by the translocation of preformed transporter proteins to the cell surface. Dietary regulation of the intestinal peptide transporter has been demonstrated in several studies (Erickson et al. 1995; Walker et al. 1998; Shiraga et al. 1999). The translocation of preformed transporter proteins to the cell surface was shown to occur in response to acute insulin or leptin treatment (Buyse et al. 2001) or prolonged exposure to dipeptides in cell culture (Walker et al. 1998; Shiraga et al. 1999). In addition, a number of *in vivo* studies have shown that a dietary protein load causes an increase in di- and tripeptide transport in rat (Erickson et al. 1995) and mice small intestine (Ferraris et al. 1988). More recently, evidence was presented that PEPT1 in rat intestine is upregulated after a short fast via an increase in gene expression (Thamotharan et al. 1999; Ihara et al. 2000) and rats made diabetic by streptozotocin showed increased PEPT1 activity and increased protein levels most likely by enhanced stabilization of its mRNA (Gangopadhyay et al. 2002).

Much is known about transport properties and substrate recognition of peptide transporters, but their contribution to amino acid nitrogen absorption in concert with amino acid transporter systems and their relevance for protein nutrition *in vivo* is not known. *Caenorhabditis elegans* could provide a useful model system to investigate the physiological importance of peptide transporters *in vivo*.

1.2 The model organism *Caenorhabditis elegans*

In 1965, Sydney Brenner chose the free-living nematode *Caenorhabditis elegans* (*C. elegans*) as a promising animal model for a concerted genetic, ultrastructural, and behavioural investigation of the development and function of a simple nervous system. *C. elegans* is a small (~ 1 mm), non parasitic, free-living soil nematode found commonly in many parts of the world. *C. elegans* is easily maintained in the laboratory, where it can be grown on nematode growth media (NGM) agar plates or in liquid culture with *Escherichia coli* as a food source. It can also be grown axenically in liquid media. Under standard laboratory conditions, growth of *C. elegans* is rapid. The entire life cycle, from an egg to an adult that produces progeny, takes just about three days at 20°C (Byerly et al. 1976). Post-embryonic development involves growth through four larval stages (L1 to L4) before the final molt to produce the adults (Fig. 1.3). In the absence of food and/or at a high population density, an alternative stage, the dauer, is formed at the second molt instead of the normal L3. Dauer larvae can survive for several months and, once food becomes available, they molt to develop into normal L4 larva (Fig. 1.3).

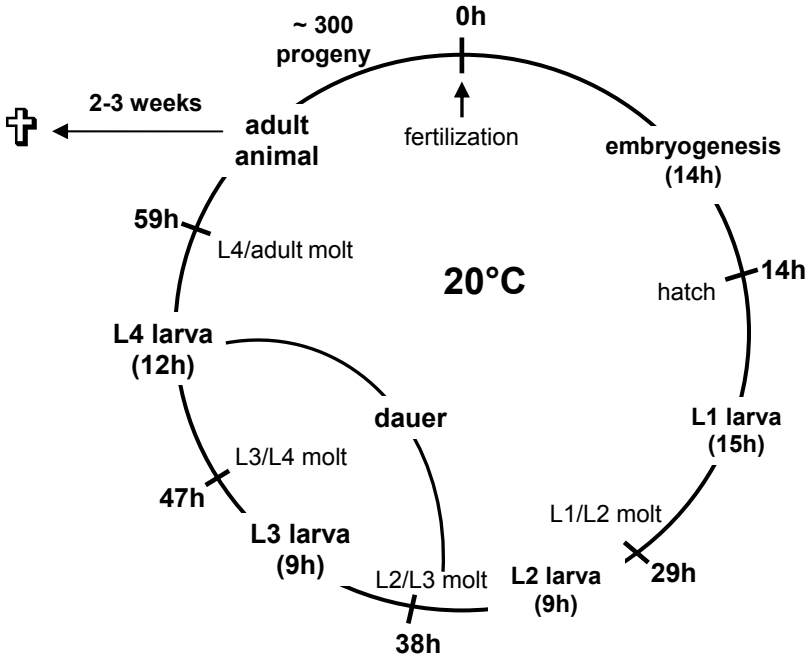

Fig. 1.3 Diagrammatic representation of the *C. elegans* life cycle, showing durations of developmental stages (modified from Schierenberg and Cassada 1986). Numbers on the outside of the circle indicate hours after fertilization at 20°C, the four larval molts are indicated inside the circle.

C. elegans is diploid and has five pairs of autosomal chromosomes (I, II, III, IV and V) and one pair of sex chromosomes (X). There are two sexes, male (X0) and hermaphrodite (XX). The hermaphrodite produces both sperm and oocytes and can reproduce by self-fertilization, without mating. Males naturally appear with a frequency of ~ 0.2 % but can be generated experimentally, for example by heat shock.

Further advantages for using *C. elegans* as a model organism, in addition to the short generation time and self reproducing hermaphrodites, are:

- the complete genome is sequenced since 1998 (Consortium 1998)
- animals have a transparent body, visible under a light microscope (Fig. 1.4)
- the cell lineage is completely known; hermaphrodites are composed of 959, males of 1031 somatic cells
- animals (L1-larvae) can be frozen at -80°C or liquid nitrogen for several years
- many molecular biology techniques are applicable, for example:
    - construction of transgenic animals by microinjection
    - RNA Interference (RNAi)
    - chemical mutagenesis for establishing deletion libraries (reverse genetic approaches)
- a large variety of genetic phenotypes and markers are characterized
- a wide spectrum of behaviour patterns is analyzed

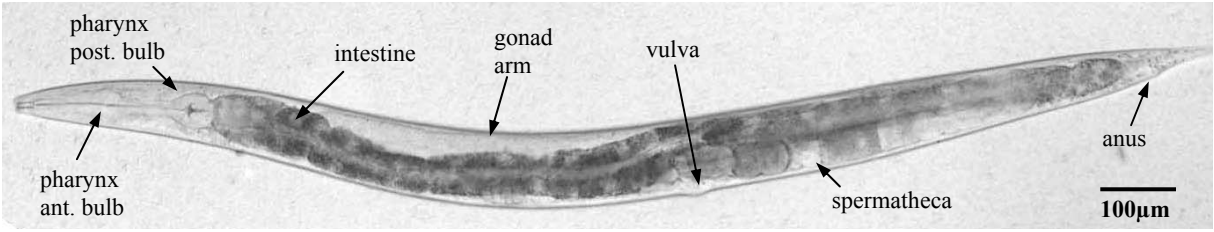

Fig. 1.4 Transmission light microscopic image of a young adult, wild type hermaphrodite

1.3 The alimentary tract and peptide transporters in *C. elegans*

The alimentary system is divided into the foregut (the pharynx), the midgut (intestine), and hindgut (rectum and anus) and contains 127 cells. The pharynx is a self-containing system of muscles, epithelial cells, and nerves, bounded by a basement membrane, which functions to ingest, concentrate, and process food before pumping it into the gut (Seymour et al. 1983). In the terminal bulb of the pharynx, the grinder squelches the food by muscle contraction and the debris is passed to the intestine through the pharyngeal-intestinal valve, the connection between the pharynx and the intestine.

The intestine itself consists of a tube of 20 cells (Fig. 1.5) and is devoid of any muscular structure in *C. elegans*. During defecation, the body wall muscles also contribute to the control of internal pressure and concentration of the gut contents before the expulsion of waste material.

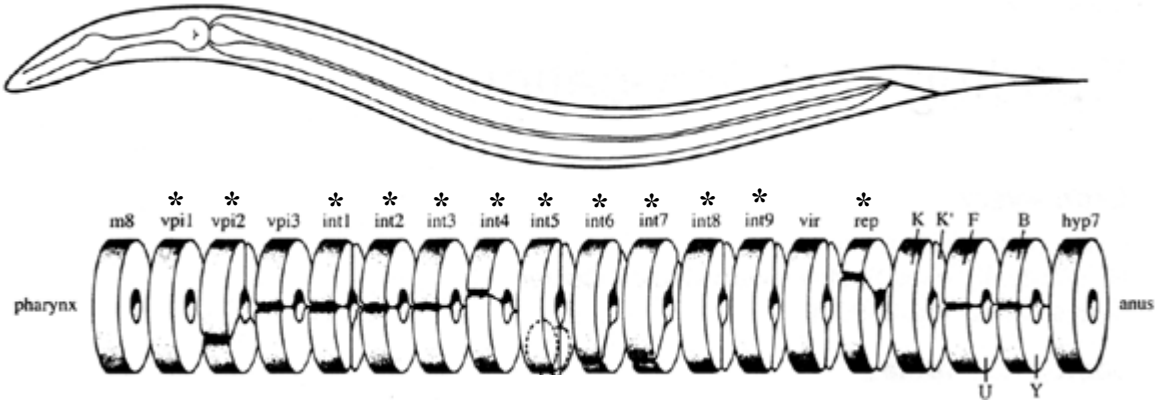

Fig. 1.5 The *C. elegans* digestive tract (Avery and Thomas 1997). (**Top**) Schematic overview. The intestine connects to the posterior end of the pharynx and is a tube made up of a single layer of cells. (**Bottom**) Intestinal cells in the mid embryo (430 minutes). The bulk of the intestine is formed by the cell layers called int1 through int9. The four most anterior layers include the most posterior muscle of the pharynx (m8) and the pharyngeal-intestinal valve (vpi). At the posterior end of the intestine is a single layer that forms the intestinal-rectal valve (vir), followed by five layers of rectal epithelium (rep through hyp7). *cells with microvilli.

The intestinal cells are very large and many microvilli extend into the lumen from the apical face, forming a brush border. In *C. elegans,* intestine carries out multiple functions that are executed by distinct organs of higher eukaryotes. The primary function of intestinal cells seems to be digestive since they secrete digestive enzymes (e.g. cysteine protease, Britton et al. 1998) into the lumen and take up processed material and nutrients. The intestine also seems to be a large storage organ since it contains a large number of assorted storage granules (White 1988).

These granules change in size, shape and number during various developmental stages of the animal. In hermaphrodites, it is also involved in synthesis and secretion of yolk material, which is then transported to the oocytes through the body cavity (Kimble and Sharrock 1983). Along with muscle, intestine is thought to be the major organ where fatty acid metabolism takes place. Through the function of a glyoxylate cyclase (SRH-1) yolk fatty acid-derived acetyl-coA is converted to succinate from which carbohydrates are synthesized (Liu et al. 1995).

Nutrients must be absorbed with astonishing speed: Tracers such as mineral oil or iron particles remain in the intestine for only a few minutes (Avery and Thomas 1997). A powerful muscular pump at the anterior end, the pharynx, is needed to force food against pressure into the intestine. Smaller muscles at the posterior end of the intestine control the opening of the anus, which facilitates the expulsion of intestinal contents by the high internal pressure. Defecation is achieved by periodically activating a stereotyped sequence of muscle contractions (Croll and Smith 1978). Until now, nothing is known about active nutrient uptake in the *C. elegans* intestine via transporters like PEPT1, amino acid or glucose transporters.

Three *C. elegans* oligopeptide transporters *pep-1* (*opt-1*), *pep-2* (*opt-2*) and *opt-3* have been cloned and characterized by functional expression in *Xenopus* oocytes (Fei et al. 1998; Fei et al. 2000). Full-length cDNA clones were expressed in *Xenopus laevis* oocytes to assess the transport by measurement of [^{14}C]-glycylsarcosine (Gly-Sar) uptake and by determining peptide-induced transport currents electrophysiologically. The kinetic analysis revealed PEP-1 as the high affinity, low capacity, and PEP-2 as the low affinity, high capacity isoform (Fei et al. 1998), but also a transporter which functions predominantly as a H^+ channel. The H^+ channel activity of OPT-3 is ~3-4-fold greater than the H^+/peptide cotransport (Fei et al. 2000).

PEP-1, the high-affinity isoform in *C. elegans* is encoded by *pep-1*, also known as *opt-1* or C06G8.2 on chromosome IV (+ 4.71). The gene produces a single transcript containing 17 exons (Fig. 1.6) with a corresponding protein consisting of 785 amino acids. PEP-2, the low-affinity isoform in *C. elegans* is encoded by *pep-2*, also known as *opt-2* or K04E7.2 on chromosome X (- 2.94). The gene produces a single transcript containing 14 exons (Fig. 1.6), the corresponding protein consists of 835 amino acids.

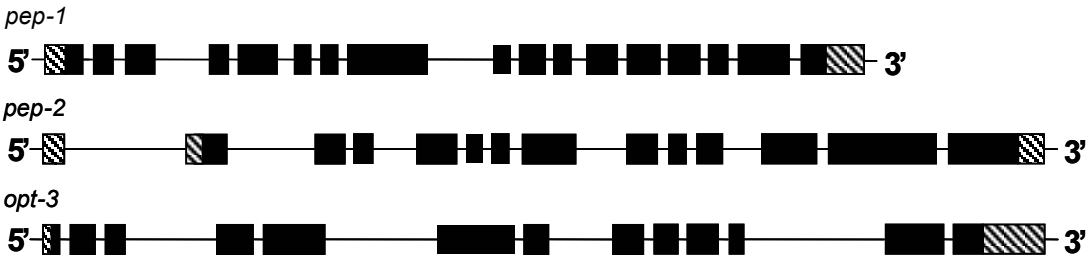

Fig. 1.6 Genomic organization of the *C. elegans* peptide transporter genes. Exon are indicated by filled boxes, introns are indicated by horizontal lines. The untranslated regions in exons are indicated by striated boxes.

opt-3, also known as F56F4.5 maps at chromosome I (+ 0.78), contains 13 exons (Fig. 1.6) and is predicted to encode a single protein consisting of 701 amino acids.

All members of the POT superfamily show characteristic protein signatures (PTR-2) assigned by Steiner and Becker in the Pfam database, which can be accessed at the Sanger Center website (http://www.sanger.ac.uk/Software/Pfam/). All three *C. elegans* peptide transporters include these motifs. One high conserved signature motif of the PTR-family is found in the central part of TMD 5 (Fig. 1.1) and comprises a stretch of nine amino acid residues (marked in the sequence alignment shown in Fig. 1.7).

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PEP-2     1    MGYSESRSESVSSKGKTSYGHELETVPLPEKKIYTTWPDMIRHWPKTTLCIVSNEFCERFSYYGMRTVLTFYLLNVLKFT
PEP-1     1    MGASHLHDDPRPGS..............PVDHQPTTWGGIIKKWPKQTFLIVGNELCERFSFYGMRAVLTLYFFNILNFS
OPT-3     1    ................................MEEKSLLQKLRSYPPAVFFMLGNEFCERFSFYGMKTILFIYLITEHEFS
hPEPT1    1    ..MG.MSKSHS...FFG..............................YPLSIFFIVVNEFCERFSYYGMRAILILYFTNFISWD
hPEPT2    1    ..MNPFQKNESKETLFSPVSIEEVPPRPESPPKKPSPTICGSNYPLSIAFIVVNEFCERFSYYGMKAVLILYFLYFLHWN

PEP-2     81   DSQSTIFFNGFTVLCYTTPLLGSIVADGYIGKFWTIFSVSILYAIGQVVLALASTKNFQSS..........VHPWMDLSG
PEP-1     67   QSFSTVLFHAFTVICYSSPLLGSILADGYIGKFWTIFFISIFYACGQILLAFSSIAPSGSS..........HHPLLDLLG
OPT-3     50   PSKATFIYHLFTCIAYLTPLIGSIMADSVFGRFKVILYGSSIYVVGHVLLSLGAVPFLSYP..........IFSSLDFSG
hPEPT1    49   DNLSTAIYHTFVALCYLTPILGALLADSWLGKFKTIVSLSIVYTIGQAVTSVSSINDLTDHNHDGTPDSLPVHVVLSLIG
hPEPT2    79   EDTSTSIYHAFSSLCYFTPILGAALADSWLGKFKTIIYLSLVYVLGHVIKSLGALPILGGQ.........VVHTVLSLIG

                                                FYXXINXGX
PEP-2     151  LLIIAFGTGGIKPCVSAFGGDQFELGQERMLSLFFSMFYFSINAGSMISTFISPIFR.SQPCLG.QDSCYPMAFGIPAIL
PEP-1     137  LLIVGLGTGGIKPCVSAFGGDQFPAHYTRMISLFFSMFYFSINAGSLISMWLTPYFR.SMSCFG.HDSCYPLAFGIPAIL
OPT-3     120  LFVIAFATGCIKPCVSAFAADQFTEDQKDLRSQFFSFFYFAINGGSLFAIIITPILRGRVQCFG.NAHCFPLAFGVPGVL
hPEPT     129  LALIALGTGGIKPCVSAFGGDQFEECQEKQRNRFFSIFYLAINAGSLLSTIITPMLRVQQCGIHSKQACYPLAFGVPAAL
hPEPT2    150  LSLIALGTGGIKPCVAAFGGDQFEEKHAEERTRYFSVFYLSINAGSLISTFITPMLRGDVQCFG..EDCYALAFGVPGLL

PEP-2     229  MIVATLVFMGGSFWYKKNPP.KDNVFCEVSRLMFRAVGNKMKSGS..TPK.EHWLLHYLTTHDCALDAKCLELQAEKRNK
PEP-1     215  MIVATLVFMAGSFWYKKVPP.KENIIFKVIGTITTALRKKASSSS..THQRSHWLEYSLDGHDCALSTECKNLHGN....
OPT-3     199  MLIAALIQFLMGWSMYKKHPPSKENVGSKVVAVIYTSLRKMVGGASR.DKPVTHWLDHAAPEHS.................
hPEPT1    209  MAVALIVFVLGSGMYKKFKP.QGNIMGKVAKCIGFAIKNRFRHRSKAFPKREHWLDWAKEKYD.................
hPEPT2    228  MVIALVVFAMGSKTYNKPPP.EGNIVAQVFKCIWFAISNRFKNRSGDIPKRQHWLDWAAEKYP.................

PEP-2     305  NLCQKKKFIDDVRSLLRVLVMFLPVPMFWALYDQQGSVWLIQAIQMDCRLSDTLLLLPDQMQTLNAVLILLFIPLFQVII
PEP-1     288  ..CAQRRYIEDIKRLFRVIVMMIPVPMFWALYDQQGSTWVLQAVGMDAKVFG.FEILPDQMGVLNAFLILLFIPIFQSIV
OPT-3     261  .....QKMIDSTRGLLNVAVIFCPLIFFWALFDQQGSTWVLQARRLDGRVGH.FSILPEQIHAINPVCVLILVPIFEGWV
hPEPT1    271  .....ERLISQIKMVTRVMFLYIPLPMFWALFDQQGSRWTLQATTMSGKICA.LEIQPDQMQTVNAILIVIMVPIFDAVL
hPEPT2    290  .....KQLIMDVKALTRVLFLYIPLPMFWALLDQQGSRWTLQAIRMNRNLCF.FVLQPDQMQVLNPLLVLIFIPLFDFVI
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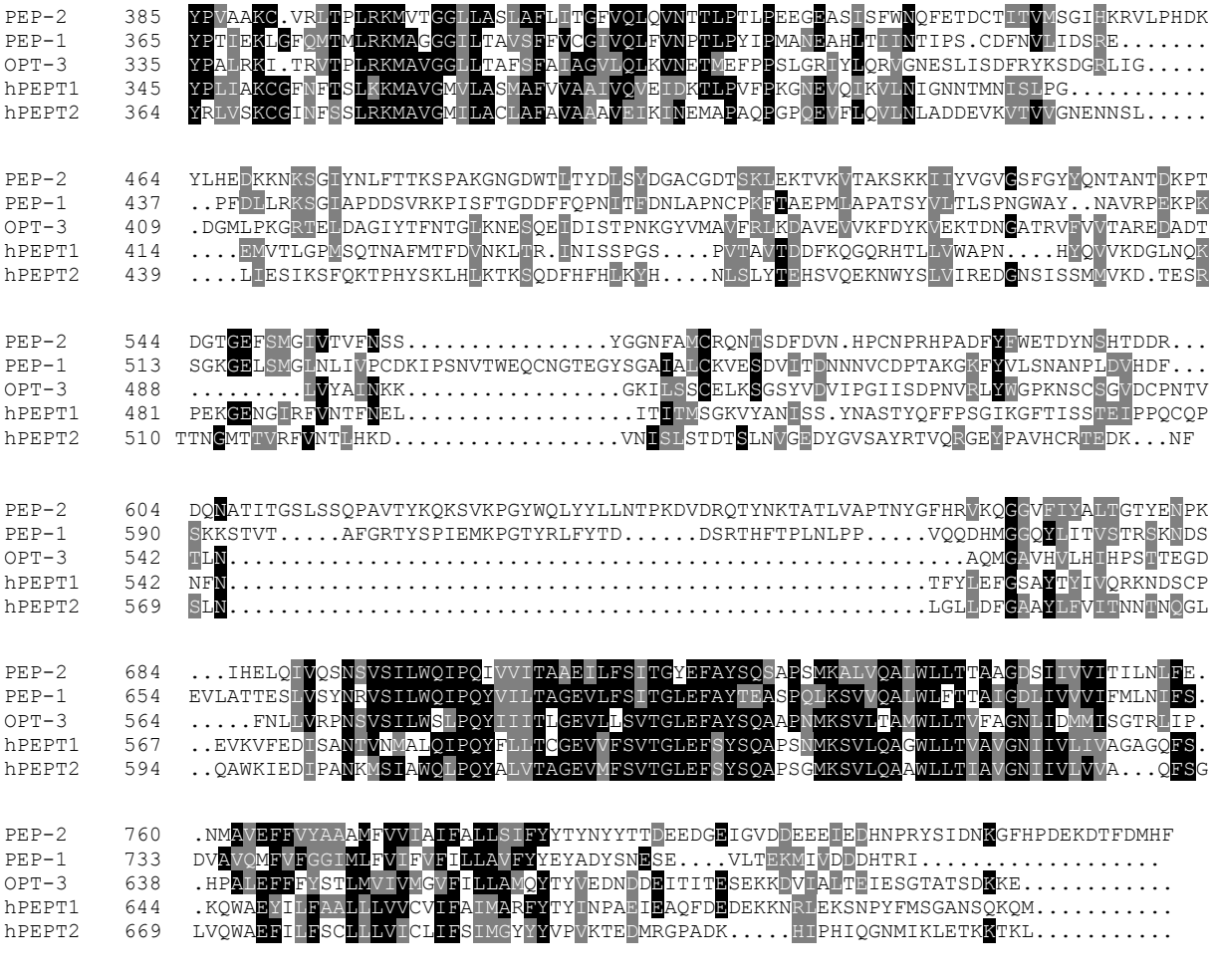

Fig. 1.7 Sequence alignment of all known peptide transporters from the *C. elegans* genome (PEP-2, PEP-1 and OPT-3) compared to the human isoforms PEPT1 and PEPT2. Identical amino acids are indicated by black boxes, similar amino acids are indicated by grey boxes. The high conserved PTR-signature motif, FYXXINXGX, located from Phe-188 to Ser-196, is highlighted in the sequence.

The transport activity of PEP-1 and PEP-2 is H^+-dependent and Na^- and Cl^--independent. Both transporter proteins display a broad substrate specificity. They recognize di- and tripeptides and, to a minor extend, tetrapeptides but none of the tested amino acids was found to be a substrate. Additionally it was shown that *N*-acetyl-aspartylglutamate (NAAG), an endogenous peptide found in mammalian brain and PEPT2 substrate (Wang et al. 1998), is a preferred substrate for PEP-1, with a transport efficiency which is ~10-fold higher than that of the prototype substrate Gly-Sar. PEP-1 and PEP-2 are reported to be expressed during the entire life-cycle of *C. elegans*, shown by stage-specific RT-PCR (Fei et al. 1998). By expression of a transcriptional *opt-3::gfp* reporter construct, OPT-3 was shown to be expressed exclusively in neurons (Fei et al. 2000).

1.4 Metabolism, ageing and development in *C. elegans*

In *C. elegans* there are two pathways known to act in parallel to regulate *C. elegans* metabolism and development, the DAF-2 (insulin/IGF receptor-like) (Kimura et al. 1997) and the DAF-7 (TGF-β-like) pathways (Ren et al. 1996; Schackwitz et al. 1996). A decrease in either of the signals causes dauer arrest, indicating that both pathways are required for reproductive growth. Many mutants are known to be either dauer-defective (Daf-d) or dauer-constitutive (Daf-c). Daf-d mutants are not able to enter the dauer stage even in unfavourable conditions like high concentrations of pheromone, absence of food and high temperature. In contrast, Daf-c mutants enter the dauer stage even in the abundance of food and low pheromone concentrations. The dauer larva exhibits a metabolism that is consistent with long-term survival in the absence of food. They have reduced TCA cycle activity but high phosphofructokinase activity relative to adults, indicating that dauer larvae have a greater capacity to metabolize glycogen (O'Riordan and Burnell 1989). The decreased TCA cycle activity relative to the glyoxylate cycle in dauer larvae indicates the importance of lipid storage as an energy reserve in the dauer stage (Wadsworth and Riddle 1989; O'Riordan and Burnell 1990; Kimura et al. 1997).

DAF-7, a TGF-β homologue, is secreted by the ASI neurons and signals through the heteromeric TGF-β receptor complex of DAF-1 and DAF-4 (Georgi et al. 1990; Estevez et al. 1993; Ren et al. 1996; Schackwitz et al. 1996). *daf-7* loss-of-function mutants exhibit a strong Daf-c phenotype, thus *daf-7* function is necessary for proper development (Swanson and Riddle 1981). Cell ablation and genetic studies suggest that the ASI neurons have a basal level of DAF-7-secreting activity in the absence of sensory inputs (Bargmann and Horvitz 1991). Dauer pheromone is thought to inhibit this basal activity, allowing dauer formation, whereas attractive food cues might antagonize pheromone action by stimulating ASI activity and DAF-7 release. A *daf-7::GFP* fusion gene is predominately expressed in the ASI sensory neurons in well fed animals, and the level of expression is downregulated by dauer pheromone and upregulated by food signals (Ren et al. 1996; Schackwitz et al. 1996).

Dauer formation in *C. elegans* is also regulated by the DAF-2 insulin-like signalling pathway, which is orthologous to the mammalian insulin and insulin-like growth factor 1 signalling (IGF-1) cascade. The *daf-2* gene encodes an insulin/IGF receptor (Kimura et al. 1997), the downstream components include the AGE-1/PI3 kinase (Morris et al. 1996), PDK-1/PDK1 (Paradis et al. 1999), AKT-1 and AKT-2/Akt/PKB (Paradis and Ruvkun 1998), DAF-18/PTEN (Ogg and Ruvkun 1998), and the DAF-16/forkhead transcription factor (Lin et al. 1997; Ogg et al. 1997) (Fig. 1.8). Recently, *daf-28* was shown to encode an insulin-like protein which directly affects the *daf-2* signalling pathway (Li et al. 2003). Down-regulation of DAF-2 results in nuclear localization and thus activation of the DAF-16 transcription factor (Henderson and Johnson 2001; Lee et al. 2001; Lin et al. 2001) regulating genes involved in metabolism and ageing (Fig. 1.8).

In wild type animals, DAF-2 signalling activates reproductive growth, which is associated with utilization of food for growth in cell number and size, and small stores of fat. In *daf-2* mutant animals, metabolism is shifted to the production of fat and glycogen (Kimura et al. 1997) in intestinal and hypodermal cells. Even when a temperature-sensitive *daf-2* mutant allele is shifted to the nonpermissive temperature at L4 or adult stage, metabolism is shifted toward storage of fat, similar to the metabolic shift seen in *daf-7* mutants (Kimura et al. 1997). In support of this metabolic shift, in dauer larvae, enzymes that regulate glycolysis are down-regulated while those that regulate glycogen and fat synthesis are up-regulated, and there is ultrastructural evidence for increased lipid and glycogen storage (Popham and Webster 1978; Wadsworth and Riddle 1989).

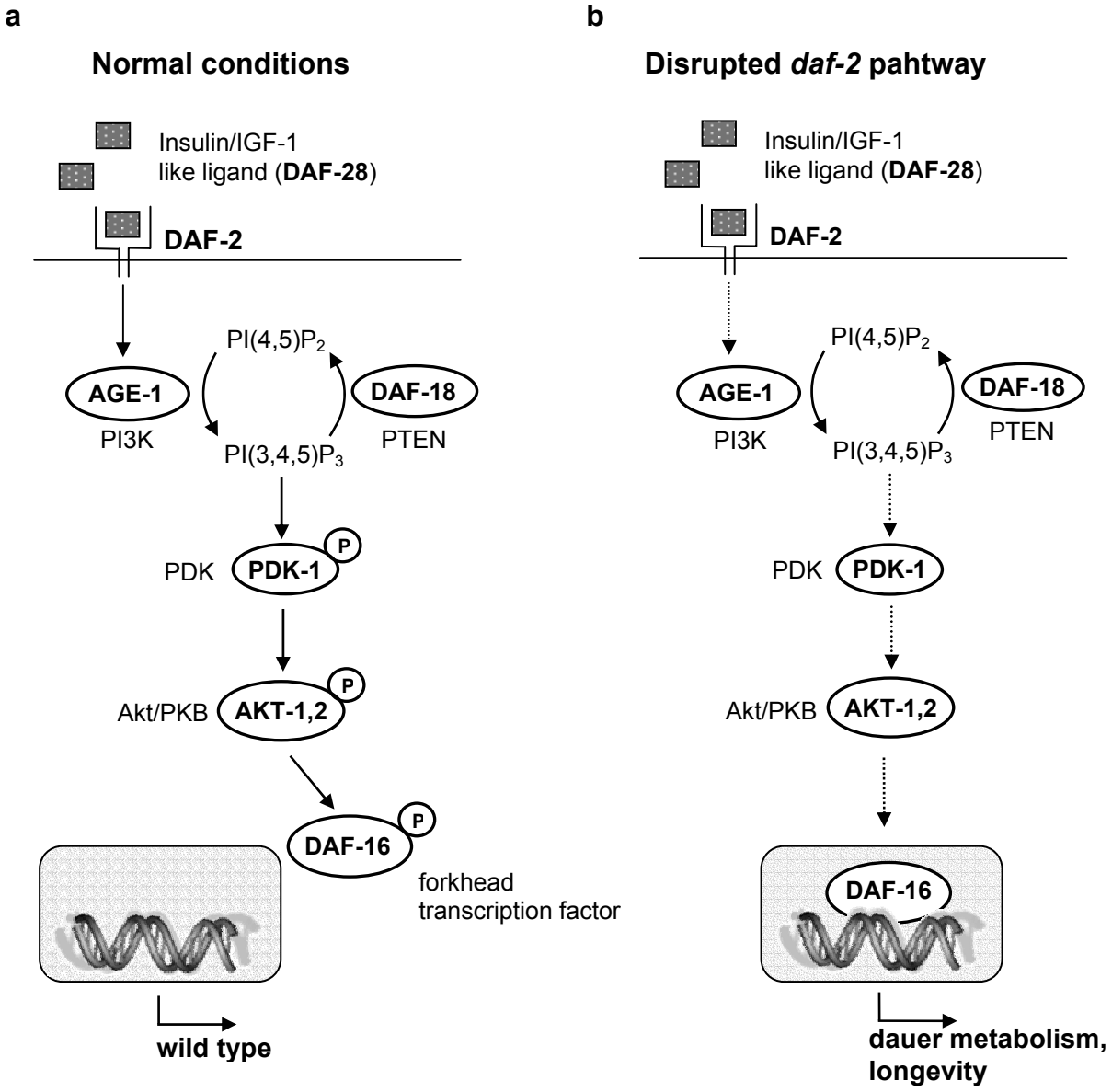

Fig. 1.8 ***C. elegans daf-2* pathway (modified from Carter et al. 2002).** (**a**) Under permissive growth conditions, an insulin-like substrate (DAF-28) binds to the *daf-2*-encoded receptor, initiating a cascade of events, including activation of the *age-1*-encoded homologue of mammalian phosphoinositide-3-OH-kinase (PI3K). PI3K activates the akt-encoded protein kinase B (PKB), which phosphorylates the DAF-16 transcription factor, preventing its translocation to the nucleus. (**b**) Disruption of the *daf-2* pathway (including mutations in *daf-2*, *age-1* or *akt* genes) prohibits the phosphorylation of DAF-16, permitting its translocation to the nucleus, thus regulating gene expression involved in metabolism and life span.

Both, the TGF-β-like neuroendocrine and the insulin-like signalling pathway regulate metabolism and dauer arrest but only the *daf-2* insulin-like pathway has effects on the stress resistance, fertility and longevity of reproductively growing adults. Components of the insulin/IGF-1 pathway also regulate life span in flies, yeast, and mice, suggesting that this system arose early in evolution (Kenyon 2001; Bluher et al. 2003; Holzenberger et al. 2003). In *C. elegans*, the life span in *daf-2* and *age-1* mutant animals is known to be significantly increased (Friedman and Johnson 1988; Kenyon et al. 1993), but neither fat accumulation nor reduced fertility is required for longevity (Kenyon et al. 1993; Wolkow et al. 2000). The longevity phenotype in *daf-2* loss-of-function mutants was shown to be dependent on a functional DAF-16 transcription factor. Furthermore it was shown that the insulin-like signalling acts in parallel to the caloric restriction, which is also known to enhance life span in *C. elegans* like in many other organisms (Lakowski and Hekimi 1998).

daf-2 and *age-1* adult animals exhibit high levels of catalase and superoxide dismutase (SOD) activity, that may further increase with age (Larsen 1993; Vanfleteren 1993; Vanfleteren and De Vreese 1995), and most consistently, these mutants exhibit an increased resistant to oxygen (Adachi et al. 1998), hydrogen peroxide (Larsen 1993) and the superoxide generator paraquat (methylviologen) (Vanfleteren 1993) as compared to wild type animals. In contrast, *mev-1* mutant animals, in which the Cu/ZnSOD activity is reduced by 30-50%, are more sensitive to paraquat and they have a life span that is reduced by 30% (Ishii et al. 1990; Ishii et al. 1994; Adachi et al. 1998). In addition it was shown, that the accumulation of age pigments and protein carbonyls, resulting from oxidative damage, is correlated positively with ageing (Hosokawa et al. 1994; Adachi et al. 1998; Yasuda et al. 1999).

1.5 Aim of the work

Since 1968, when it was shown for the first time that amino acids offered as small peptides can be absorbed in the gut much faster than a corresponding amino acid mixture (Adibi 1968), the function and regulation of the peptide transporters have been studied extensively *in vivo* and *in vitro* (for reviews see (Daniel 1996; Adibi 1997; Meredith and Boyd 2000). Nevertheless, nothing is known about the physiological importance of intestinal peptide uptake in connection with the uptake of free amino acids by a large number of different amino acid transporter systems.

Recently two *PEPT2*-deficient mice lines have been reported (Rubio-Aliaga et al. 2003; Shen et al. 2003). They were found to be viable and displayed no obvious abnormalities in kidney or brain. Functional deficiencies were shown by the almost abolished uptake of model peptides in the choroid plexus (Shen et al. 2003) and the kidney (Rubio-Aliaga et al. 2003). For PEPT1, no strains deficient in this protein have been reported so far.

In this work, *C. elegans* was used to analyze the expression and function of PEP-1 and PEP-2 transporter proteins *in vivo*, with a main focus on the *pep-2* gene.

2 Materials

2.1 Instruments

The following instruments were used in addition to the lab standard:

Tab. 2.1 Instruments

Camera Axio Cam HRc	Zeiss (Jena)
Fluorescence- and light microscope AxioPlan2	Zeiss (Jena)
Microinjector 5242	Eppendorf (Hamburg)
Micromanipulator 5171	Eppendorf (Hamburg)
Stereomicroscope MZ8	Leica (Wetzlar)
Peltier Thermal Cycler PTC-200	MJ Research (Waltham, USA)
MultiScreen multiwell filter plates (96-well)	Millipore (Billerica, USA)
Wallac1450 MicroBeta Trilux	PerkinElmer (Wellesley, USA)

2.2 Chemicals and consumable supplies

The following chemicals and consumable supplies were used beside the lab-usual:

Tab. 2.2 Chemicals

2,4-Dinitrophenylhydrazine	Sigma (Deisenhofen)
5-Fluoro-2'-deoxyuridine	Sigma (Deisenhofen)
Bacto-Pepton	Difco (Detroit, USA)
Bacto-Trypton	Difco (Detroit, USA)
Bacto-Yeast Extract	Difco (Detroit, USA)
Complete,Mini (Protease-Inhibitor-Cocktail-Tablets)	Roche (Mannheim)
MEM Mino Acids W/O Glut (50x)	Invitrogen (Karlsruhe)
MEM Non Essential Amino Acids (100x)	Invitrogen (Karlsruhe)
Nystatin	Sigma (Deisenhofen)
Platinum wire	Merck (Darmstadt)
Sodium Azide	Sigma (Deisenhofen)
β-Ala-Lys-AMCA	Biotrend (Köln)
L-Leucine, [3,4,5-^{3}H]	ICN Biomedicals (California, USA)
L-Lysine Monohydrochloride, [U-^{14}C]	ICN Biomedicals (California, USA)

Enzymes like restrictionendonucleases, *Taq* DNA-Polymerases, T4-DNA-Ligase, Proteinase K, and Alkaline Phosphatase were obtained from MBI Fermentas (Vilnius, Litauen), New England Biolabs (Beverly, MA, USA) or Roche (Mannheim).

2.3 Buffer and Media

LB Media
10 g/l Bacto-Trypton
5 g/l Bacto-Yeast Extract
10 g/l NaCl
for plates add:
15g/l agar

10x TBE
890 mM TRIS
890 mM Boric Acid
20 mM EDTA

NGM-Agar for plates
3 g/l NaCl
2.5 g/l Bacto-Pepton
17 g/l Agar
ad 967 ml H_2O
add after autoclaving:
1 ml 0.1 M $CaCl_2$
1 ml 0.1 M $MgSO_4$
25 ml 1 M KH_2PO_4 (pH 6.0)
5 ml Nystatinsolution
1 ml Cholesterol (5mg/ml in Ethanol)

for RNAi plates add:
1 ml 1M IPTG
1 ml Ampicillin (100mg/ml)

M9 Buffer
3 g/l KH_2PO_4
6 g/l NaH_2PO_4
5 g/l NaCl
add after autoclaving:
1 ml/l 1M $MgSO_4$-solution

Worm Lysis Buffer
5 ml 1M KCl
1 ml 1M TRIS (pH=8.2)
250 µl 1M $MgCl_2$
450 µl NP-40
450 µl Tween 20
200 µl 5% Gelatine
ad 100ml
before use, add
3µl 20mg/ml Proteinase K

Nystatin-solution
4 g Nystatin
200 ml Ethanol
200 ml Ammoniumacetate

Hypochlorit-Solution
200 mM NaClO
100 mM NaOH

Staining Solution (*LacZ*)
250 µl 0.8 M Na-phosphate buffer pH7.5
100 µl 100 mM Redox buffer (see below, keep stocks at -20°C)
20 µl 50 mM $MgCl_2$
15 µl 5mg/ml kanamycin
4 µl 1% SDS
2 µl 1mg/ml DAPI
8 µl 5% X-Gal in dimethyl formamide
ad 1ml H_2O
X-Gal was added last, solution was vortexed quickly to avoid precipitation
Redox buffer was made fresh each time by mixing equal volumes of the following stock solutions:
100 mM Potassium Ferricyanide
100 mM Potassium Ferrocyanide

2.4 Bacteria - and *C. elegans* strains, plasmids and primers

2.4.1 Bacteria strains

Tab. 2.3 Bacteria strains

Strain	Genotype	Reference
OP50	*ura*$^-$	Brenner, 1974
DH5α	*end*A1, *hsd*R17(r_k^-,m_k^+), *sup*E44, *thi, rec*A1, *gyr*A96, *rel*A1, Δ(*lac*ZYA-*arg*F) U169, Φ80d*lac*ZΔM15	Hanahan, 1985
HT115(DE3)	*F*$^-$*, mcr*A*, mcr*B, IN*(rrnD-rrnE)1, lambda*$^-$*, rnc14::Tn10(DE3 lysogen:*lacUV5 promoter-T7 polymerase, RNAse III minus	Takiff, 1989

2.4.2 *C. elegans* strains

All *C. elegans* strains used were received from the "*C. elegans* Genetic Center" (CGC) at the University of Minnesota, USA or from the lab in which the strain had been constructed. N2 *var. Bristol* was used as wild type strain.

Tab. 2.4 *C. elegans* strains. For crossing strategies see supplement 7.1.

Strain	Genotype	Reference
DA453	*eat-2(ad453)II*	Avery, 1997
CB1370	*daf-2(e1370)III*	Riddle, 1977
DR26	*daf-16(m26)I*	Riddle *et al*., 1981
DR1309	*daf-16(m26)I;daf-2(e1370)III*	Riddle *et al*., 1981
BR2742	*pep-2(lg601)X*	EleGene, Munich
BR2743	*pep-1(lg501)IV*	EleGene, Munich
DH1033	*bIs1[vit-2::gfp,pFR4,sqt-1(sc103)]*	Grant and Hirsh, 1999
DR1808	*mIs6[rol-6(su1006),daf-7p::GFP]*	Ren et al., 1996
BR2068	*unc-4(e120)I; fem-1(hc17ts)IV*	Eimer, 2000
NU3	*dbl-1(nk3)V*	Morita, 1999
BW1940	*ctIs40[dbl-1(+);sur-5::gfp]*	Suzuki, 1999

Strains obtained by injections

Strain	Genotype	Injected plasmids
BR3117	N2;*byEx230*	50ng/µl pBY1510, 50ng/µl salmon
BR3116	N2;*byEx231*	70ng/µl pBY1511, 30ng/µl pRF4
BR2895	N2;*byIs105*	BR3117 strain integrated

BR3124	N2;byEx408	50ng/µl pBY1510, 50ng/µl salmon
BR2875	N2;*byEx347*	10ng/µl pBY1510, 90ng/µl pRF4
BR2876	N2;*byEx346*	10ng/µl pBY1510, 90ng/µl pRF4
BR2747	N2;*byEx352*	70ng/µl pBY1635, 30ng/µl pRF4
BR2893	*pep-2(lg601); byEx232*	70ng/µl pBY1512, 30ng/µl pRF4
BR2894	*pep-2(lg601);byEx351*	70ng/µl pBY1512, 30ng/µl pRF4

Strains obtained by crossings

BR2751	*dpy-1(e1)III; pep-2(lg601)X*
BR2513	*dpy-5(e61) daf-16(m26) unc-75(e950)I; pep-2(lg01)X*
BR2688	*daf-2(e1370)III; pep-2(lg601)X*
BR2689	*daf-16(m26)I; pep-2(lg601)X*
BR3061	*daf-16(m26)I; daf-2(e1370)III; pep-2(lg601)X*
BR2746	*pep-2(lg601)X; bIs1*[*vit-2::GFP*,pFR4,*sqt-1(sc103)*]
BR3062	*pep-2(lg601)X; mIs6*[*rol-6(su1006)*,*daf-7p::GFP*]
BR2744	*pep-1(501)IV; pep-2(lg601)X*
BR3059	*dbl-1(nk3)V; pep-2(lg601)X*
BR3060	*ctIs40[dbl-1(+);sur-5::gfp];pep-2(lg601)*

2.4.3 Plasmids

Tab. 2.5 Plasmids

Name	Description	Reference
pPD95.75	promoterless *gfp* vector, ampicillin resistance	Fire Vector Kit 1995
pPD95.03	promoterless *lacZ* vector, ampicillin resistance	Fire Vector Kit 1995
pPD129.36	Two T7 promoters flanking the MCS, for transcription of dsRNA in *E. coli* HT115, ampicillin resistance	Fire Vector Kit 1999
pRF4	*rol-6(su1006)*	Mello *et al.* 1991

Plasmids constructed in this work

pBY1510	*pep-2* promoter sequence 2.4 kb upstream the coding region cloned into pPD95.75 (*Hind*III;*BamH*I)
pBY1511	*pep-2* promoter sequence 2.4 kb upstream the coding region cloned into pPD95.03 (*HindIII*;*BamHI*)
pBY1512	*pep-2* entire coding region including 2.4 kb upstream region cloned into pPD95.75 (*Hind*III;*BamH*I)

pBY1635	*pep-1* promoter sequence 3.8 kb upstream the translational start cloned into pPD95.75 (*Pst*I;*BamH*I)

2.4.4 Oligonucleotides

Tab. 2.6 Oligonucleotids

Name	Sequence in 5'-3' orientation	Use
RB2244_pep-2/1	CCCAAGCTTGGGTCTTCTATGCC ATGGAGGTCTTCG	Cloning of pBY1510, 1511 and 1512
RB2245_pep-2/2	GGAGATCTTCCATAGTGGCGATA CTGACGAGGAATGAG	Cloning of pBY1510, 1511
RB2246_pep-2/3	GGAAGATCTTCCCCAAAATGCAT ATCGAAAGTATCTTT	Cloning of pBY1512
RB1861_pep-2/4	GCAACACACTGTACGGAAC	Screening for *pep-2* deletion
RB1862_pep-2/5	CCAGTGGGTGCACCACAAGG	Screening for *pep-2* deletion
RB1863_pep-2/ 6.15	AAAAATTTGCAGCGGTCTTG	Screening for *pep-2* deletion
RB1864_pep-2/ 6.16	GTTGCCACGGTTGAAGTTCT	Screening for *pep-2* deletion
RB2247_pep-1/1	GCCTGCAGGTAGGAATGAGCAAC TCACGTGTG	Cloning of pBY1635
RB2248_pep-1/2	GCGGATCCTCCATATACGATCGC CTATACAGAAC	Cloning of pBY1635
RB2255_pep-1/3-F4771	CATAGGATTTCCAGACATGG	Screening for *pep-1* deletion
RB2256_pep-1/4-B5910	CTGCCATGAACACCACTG	Screening for *pep-1* deletion
RB2257_pep-1/5-F-2598	GACGTAAGGTTTCTGGAGGCA	Screening for *pep-1* deletion
RB2258_pep-1/6-B6297	CAGTTGGAATGGATGCAAAGG	Screening for *pep-1* deletion

3 Methods

3.1 *C. elegans* methods

3.1.1 Breeding of *C. elegans*

The animals were maintained on NGM plates seeded with *E. coli* OP50 like previously described (Brenner, 1974). Petri dishes with the diameters 3.5 cm, 5 cm and 9 cm were used in this work. Animals were kept in air permeable cardboard boxes at 15, 20 or 25°C. The basic culture methods (handling of *C. elegans*, decontamination, freezing, preparation of synchronized cultures and specific stages) were done as previously described (Lewis and Fleming 1995).

3.1.2 Genetic crosses

L4 hermaphrodites and males were placed on a small (3.5 cm) plate in a 1:3 ratio. After 24, 48 and 72 hours, the adult hermaphrodites were transferred to a fresh plate, the males were removed. Progeny laid within the first 24 hours were discarded, because they were likely to represent self progeny, instead of cross progeny. About ten of the following F1 animals were singled and their progeny (F2) were further analysed. All strains obtained by crossings in this work are listed in Tab. 2.4.

3.1.3 Mutagenesis by Trimethylpsoralen/UV treatment

The *pep-1(lg501)* and *pep-2(lg601)* mutant strains used in this work were obtained from Claudia Rudolph (EleGene). The mutants were generated by screening a deletion library constructed by UV/TMP (Trimethylpsoralen) treatment (Yandell et al. 1994).

3.1.4 Worm Lysis for Single Worm PCR (SW-PCR)

One animal was transferred into a PCR tube containing 10 µl Worm Lysis Buffer and frozen for 10 minutes at -80°C, followed by incubation at 65°C for one hour and at 95°C for 10 minutes. One microliter of the lysate were used as a template for the following PCR.

3.1.5 Transformation of *C. elegans*

DNA plasmids were transformed into the animals by microinjection as previously described (Fire 1986; Mello et al. 1991). All transgenic lines generated during this work are listed in Tab. 2.4.

3.1.6 X-ray-induced integration of extrachromosomal arrays

5 NGM plates (9 cm) with about 30 L4 larvae bearing the extrachromosomal array were irradiated with x-rays (4500 rad). 3-5 days later, 100 F1s carrying the array were singled to new plates (3.5 cm). From each F1 animal that had progeny, 4 F2s were picked onto individual plates. After the F2 animals have grown up and reproduced, the plates were examined to identify those that laid 100% transgene progeny. To confirm that lines have integrated the array and to get rid of unwanted mutations, integrated lines were crossed against wild type background.

3.1.7 Staining for *lacZ* expression

Worms were washed off 5 cm NGM plates, transferred into a 15 ml Falcon tube and spun down for 1 min at 3000 rpm. The supernatant was removed, the animals were transferred into a 1.5 ml Eppendorf tube and centrifuged for 1 min at 3000 rpm. After removing the supernatant as carefully as possible, the Eppendorf tube was capped and frozen in liquid nitrogen. Than, the caps were opened and the frozen animals were lyophilized for about 30 minutes (speed-vac). 250 µl cold acetone was added and the sample was incubated at -20°C for three minutes. The acetone was removed and the probe was dried in the speed vac. After adding 200 µl of the staining solution and incubation at 37°C (5 min to 1 hr), the staining was observed with normal light microscopy using standard Nomarski optics.

3.1.8 Feeding assay with ß-Ala-Lys-AMCA

Mixed staged animals were washed off agar plates with M9 buffer. Equal amounts of worms were incubated in a 1 mM ß-Ala-Lys-AMCA solution (in M9) for two to three hours, followed by at least four washing steps with M9. As control, worms were incubated in M9 buffer for the same time.

3.1.9 Assays for developmental and behavioral phenotypes

Body length measurements: Synchronized animals were collected at the L1 stage (t = 0) and 20 individual worms were analyzed every day until adulthood (for body length during larval development) or 0 – 5 days after L4 moulting (for body length during adulthood). Pictures were taken with Axioplan 2 (Zeiss) and the precise body length was measured with Axio Vision 3.0 software.

Postembryonic development: To determine the generation time of worms, 5-15 adult hermaphrodites were placed on fresh plates for egg-laying. Measurements were started after two to three hours. The P0 generation was removed and 10 to 50 worms of the progeny were singled. After two or more days (depending on the strain), the F1 animals were monitored every 2 hours until they laid the first egg.

Embryonic development: Adult hermaphrodites were placed in a drop of water and cut open with a razor blade. Eggs at the 2-cell stage were picked, placed individually onto fresh plates and monitored every 30 min until hatching.

Self-brood size: Individual L4 larvae were placed onto fresh plated and incubated at 20°C until they had laid the first few eggs. Then, the hermaphrodites were transferred onto fresh plates daily to prevent overcrowding. The progeny was counted two to three days after removal of the P0.

Eggs per hour: Five hermaphrodites that had reached adulthood one day (wild type) or two days (*pep-2* mutant) before were allowed to lay eggs for 5 hours. Then, P0 were removed and the progeny (F1) was counted two to four days after removal of the parents.

Yolk protein distribution: The distribution of yolk protein was analysed by visual inspection of GFP expression in DH1033 *bIs1*[*vit-2::gfp*,pFR4,*sqt-1(sc103)*] (Grant and Hirsh 1999) and BR2746 *bIs1*[*vit-2::GFP*,pFR4,*sqt-1(sc103)*]; *pep-2(lg601)*X.animals.

Male mating test: *unc-4(e120); fem-1(hc17ts)* double mutants (BR2068) were grown at 15°C, the permissive temperature. Eggs, collected by hypochlorite treatment, were placed on fresh plates and incubated at 25°C, the restrictive temperature. Three young adult *unc-4(e120); fem-1(hc17ts)* double mutants grown at 25°C were used for the mating with either ten wild type or ten *pep-2* mutant males per plate. After 14

hours, the males were removed from the plates and the progeny laid by the *unc-4(e120); fem-1(hc17ts)* was scored.

Pharyngeal pumping: Pharyngeal pumping was visualized through the dissection microscope. The animals were scored for 30 sec on OP50 and each animal was scored twice.

Defecation: Hermaphrodites were maintained at 20°C and the length of their defecation cycle was measured. Defecation cycle length was defined as the duration between the first muscular contraction and the expulsion of one defecation. Ten defecation cycles were measured for each animal.

Lifespan: For the lifespan analysis, 5 to 10 adult hermaphrodites were transferred onto fresh plates for egg laying and after 8-10 hours, the starting point for life span measurements, the parents were removed. Animals were cultured at 20°C and examined every day until death. They were scored death when they did no longer move in response to prodding them with a platinum pick. Each day, any dead worms were removed from the plates and the deaths were recorded. Experiments were started with 100 worms per genotype (10 per plate) and the wild type (N2) was always included as a control. For the assays at 25°C, the animals were grown at 15°C until the L4 molt, then they were shifted to 25°C. The L4 molting was used as a starting point for the measurement of adult life span at 25°C.

Heat tolerance: Thermotolerance of various strains was measured using young adults at 35°C. Synchronously cultured animals were kept on NGM plates with OP50 at 20°C until the young adult stage. At the start of the thermotolerance assay, at least 20 worms from each strain were transferred to a fresh NGM plate with OP50, and shifted to 35°C. Numbers of surviving and dead animals were scored every 2h.

3.1.10 Amino acid supplementation

For the experiments with amino acid supplementation, 300 µl amino acids (mixture 1:1 of MEM (100x) non-essential amino acids and MEM amino acids (50x) without L-glutamine were added on the top of agar plates (35 mm) seeded with *E.coli*. Fresh plates were prepared each day during the experiment.

3.1.11 Culturing aged, synchronized animals for protein carbonyl determination

Hermaphrodites of wild type and the different mutants were grown on NGM agar plates seeded with *E.coli* OP50. Eggs were collected by using sodium hypochlorite and allowed to hatch by incubating them overnight at 20°C in M9 buffer. Newly hatched L1 larvae were cultured on 15 cm agar plates seeded with OP50. To prevent reproduction, 5-fluoro-2'deoxyuridine (dFUR; Sigma Chemical, St. Louis, MO) was added to the agar to a final concentration of 40 µM after animals had reached adulthood.

3.1.12 Protein extraction from *C. elegans*

For protein extraction and carbonyl measurements, the animals were washed off the agar plates with M9 buffer. Living animals were collected in 15 ml tubes by floating on sucrose followed by four washing steps with M9 buffer. After washing, the "wormpellet" was resuspended in 5 mM EDTA, centrifuged, and the supernatant was discarded. The resulting "wormpellet" was frozen in liquid nitrogen and stored at -80°C until use. The frozen pellet was allowed to thaw in the 15 ml tube on ice and filled up to a total volume of 1.5 ml with 5 mM EDTA. 10 µl of a tablet with protease-inhibitors (complete Mini, Roche) solved in 1 ml 5 mM EDTA were added to the probe. A sterile mortar was cooled down with liquid nitrogen and the suspension was dropped into the liquid nitrogen to get small frozen particles, followed by strong pounding until the particles liquefied again. The suspension was quickly transferred to a fresh 15 ml tube, kept on ice and sonified three times for one minute. Finally the probe was spun down for 15 minutes with 4500 rpm at 4°C and the supernatant was transferred to a fresh tube. Protein concentration was determined by the Bio-Rad Protein Assay (BIO-RAD Laboratories GmbH, München)

3.1.13 Protein carbonyl measurement

Protein carbonyl content was measured by the method of Levine and colleagues (Levine et al. 1990) with slight modifications. One ml of 20 % trichloroacetic acid (TCA) was added to a 1 ml aliquot of the protein homogenate, and the mixture was allowed to stand for 15 minutes at 4°C for protein precipitation. After centrifugation for 15 minutes at 12,000 *g*, the supernatant was discarded, and 500 µl of 10 mM 2,4-

dinitrophenylhydrazine (DNPH) dissolved in 2 M HCl was added to the pelleted protein. In parallel, a blank was prepared by treatment with 2 M HCl without DNPH. After 60 min incubation at 20°C, 500 µl of TCA was added to the sample and kept 15 minutes at 4°C. After centrifugation at 12,000 g for 15 minutes, the supernatant was discarded and the pellets were washed three times by centrifugation with 1 ml of a mixture (1:1 v/v) of 40% TCA with 100% ethanol and ethyl acetate to remove free reagent. Before each centrifugation step, the samples were allowed to stand 15 minutes at 4°C. The precipitated protein was redissolved in 800 µl of 6 M guanidine hydrochloride solution. After incubation for 3 hours at 37°C, the samples were centrifuged at 600 g for 10 minutes. The amount of DNPH bound to protein was quantified by absorbance at 380 nm and converted to nmoles DNPH using the molar absorption coefficient of 22 $mM^{-1}cm^{-1}$. Three to nine separate determinations were used to calculate the mean ± SEM in each different age group.

3.1.14 RNA Interference (RNAi) experiments

For RNAi-through-feeding experiments the partial cDNA from yk18c10 was cloned into Vector pPD129.36. The resulting plasmid was a gift from A. Gartner, Munich, and was used in RNAi feeding experiments like previously described (Kamath et al. 2001). L4-stage worms were placed on RNAi-producing plates and were allowed to produce progeny. Adults were removed or transferred to new RNAi-producing plates. First and third generation progeny grown on RNAi plates were scored for a TOR phenotype and yielded identical results.

3.1.15 Uptake assay with radioactive labelled amino acids

For the measurements of ^{3}H-Leucine and ^{14}C-Lysine uptake, the animals have been synchronized by hypochlorite treatment (3.1.11). For the uptake studies, the animals were washed off the agar plates as young adults with M9 buffer, collected in 15 ml tubes and washed two more times with M9 buffer. The wormpellet was diluted with M9 buffer to get 100 respectively 200 animals in 100 µl wormsuspension. The uptake of the labeled amino acids was performed in 96-well filter plates with 0.5 and 0.25 µCi/well for ^{3}H-Leucine (specific activity: 170 Ci/mmol) and 0.1 µCi/well for ^{14}C-Lysine (specific activity: 322 mCi/mmol). To show inhibition of radioactive substrate uptake, 20 mM Leucine or 8 mM Lysine, respectively, were added to the incubation mixture. After two hours of incubation with slightly shaking, the solution was sucked of and the

animals remaining on the filter were washed three times with M9 buffer. Finally the radioactivity of each well was measured in a β-counter type 1450 MicroBeta Trilux.

3.2 Molecular biology techniques

Following methods were applied as previous described:

- Preparation of competent *E. coli* (DH5α, HT115) (Maniatis et al. 1982)
- Transformation of *E. coli* (Hanahan 1985)
- Plasmid preparation from *E. coli* (QIAgen, Hilden)
- Digestion of DNA with Restriction Endonucleases (Sambrook et al., 1989)
- Gel-Electrophoresis (Agarose) (Maniatis et al. 1982)
- Ligation of DNA fragments (Maniatis et al. 1982)
- Polymerase Chain Reaction (PCR) (Maniatis et al. 1982)

  A representative PCR Mixture (50µl) contained:

  - 10-100 ng template-DNA (*C. elegans* genomic DNA, cDNA, Plasmid-DNA or Worm-Lysate)
  - 5 µl 10x Polymerase buffer mix with $MgCl_2$ (accordingly to the used Taq-Polymerase)
  - 10 pmol sense Oligonucleotide
  - 10 pmol antisense Oligonucleotide
  - 10 µl dNTP-Mix (each 2mM dATP, dCTP, dGTP, dTTP)
  - 0.3-1µl Taq-Polymerase (2.5U)

  Temperature Cycling:

  Denaturation: 2 min at 94°C

  30-35 cycles under following conditions:

  - Primer annealing: 40 sec at 45-58°C depending on the melting temperature of the oligonucleotides
  - Extending step: 1-3 min (depending on fragment size) at 68 or 72°C (depending on Taq DNA polymerase)

  Final extending: 5 min at 68 or 72°C

4 Results

4.1 Analysis of PEP-2, the low-affinity, high capacity peptide transporter in *C. elegans*

4.1.1 Identification of *pep-2* gene expression with GFP and lacZ reporter constructs

For the examination of the *pep-2* gene expression pattern, reporter constructs were generated to express the green fluorescence protein (*gfp*) or β-galactosidase (*lacZ*) under the control of the *pep-2* promoter. A PCR product containing 2.4 kb upstream of the transcriptional start was cloned into the expression vectors pPD95.75 and pPD95.03 resulting in the plasmids pBY1510 (gfp) and pBY1511 (lacZ) respectively (Fig. 4.1).

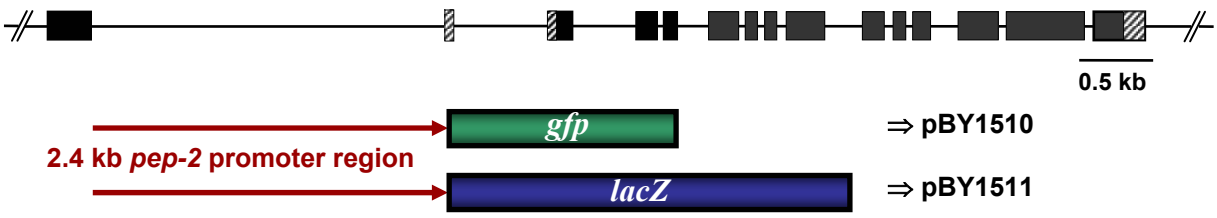

Fig. 4.1 Reporter gene fusions under *pep-2* promoter control.

The plasmids were injected into wild type worms to obtain stable lines of transgenic animals. Four independent lines for pBY1510 (BR3124, BR3117, BR2875, BR2876) and one line for pBY1511 (BR3116) were analyzed. In addition, the extrachromosomal array in strain BR3117 was integrated into the genome by X-ray treatment, resulting in strain BR2895. The expression pattern of the *pep-2* promoter driven reporter constructs is shown in Fig. 4.2. All transgenes are expressed exclusively in the intestinal cells and in a pair of sensory neurons in the head, which were identified as ASI, by the disposition of the surrounding neurons ASK and ADL (Fig. 4.2f). The strong expression starts in embryogenesis (Fig. 4.2a) and is maintained throughout the development to adulthood (Fig. 4.2e).

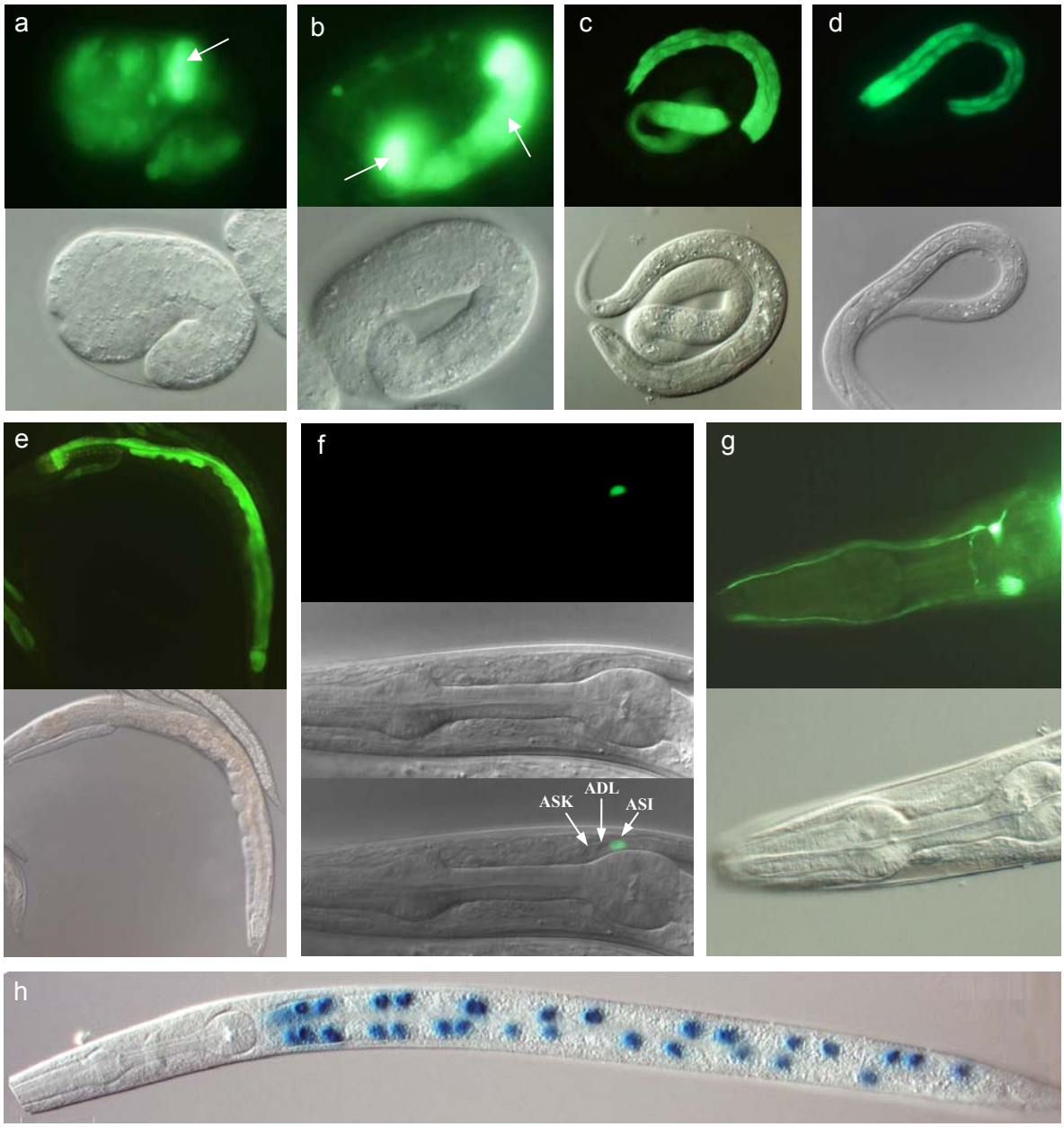

Fig. 4.2 ***pep-2* promoter::GFP expression pattern.** The expression starts in embryogenesis (**a** and **b**) and is maintained throughout the larval development (**c**, **d**, **f**) until adulthood (**e**). The reporter constructs are expressed in the intestinal cells (**b**, **c**, **d** and **e**) and a pair of sensory neurons in the head, identified as ASI (**f** and **g**). (**a**) 1.5-fold embryo (arrow indicates the pharyngeal-intestinal valve), (**b**) embryo before hatching (arrows indicate the intestine), (**c**) L1 larva surrounding an hatching embryo, (**d**) L2 larva and (**e**) an adult hermaphrodite showing strong intestinal GFP-expression. (**f**) anterior end of an adult animal, localization of the GFP-expressing neuron, (**g**) head of an adult animal, ASIL and ASIR with neuronal processes. Corresponding Nomarski pictures are shown below. (**h**) galactosidase staining of a *pep-2p::*NLS-*lacZ* fusion gene in a L3-larva; the intestinal nuclei are stained blue.

4.1.2 Identification of the mutant allele *pep-2(lg601)*

The *pep-2(lg601)* deletion mutant, generated by TMP/UV mutagenesis (3.1.3), was obtained from EleGene (Munich). A 1.7 kb deletion eliminates 257 bp of the promoter sequence, the translational start codon and the exons two to seven (bp 5788 to 7486 on cosmid K04E7) (Fig. 4.3), the coding sequence for the first six transmembrane domains of the protein. In human orthologue PEPT1, this region is known to be important for substrate binding and translocation (Doring et al. 2002; Fig. 1.1). Thus, *pep-2(lg601)* most likely represents a strong loss-of-function or even a null allele. To verify this deletion, a Single-Worm-PCR (SW-PCR) with *pep-2* specific primers was performed. One set of primers anneals in the genomic region outside of the deletion (RB1861 and RB1862 for PCR 1) to get a wild type fragment (2.1 kb) and a *pep-2* deletion fragment (0.5 kb) (Fig. 4.3). To recognize the difference between homo- and heterozygote animals, a second set of primers was used that is located in the deletion (RB1863 and RB1864 for PCR 2) (Fig. 4.3), to get a 0.4 kb fragment if there is a copy of the wt *pep-2* gene.

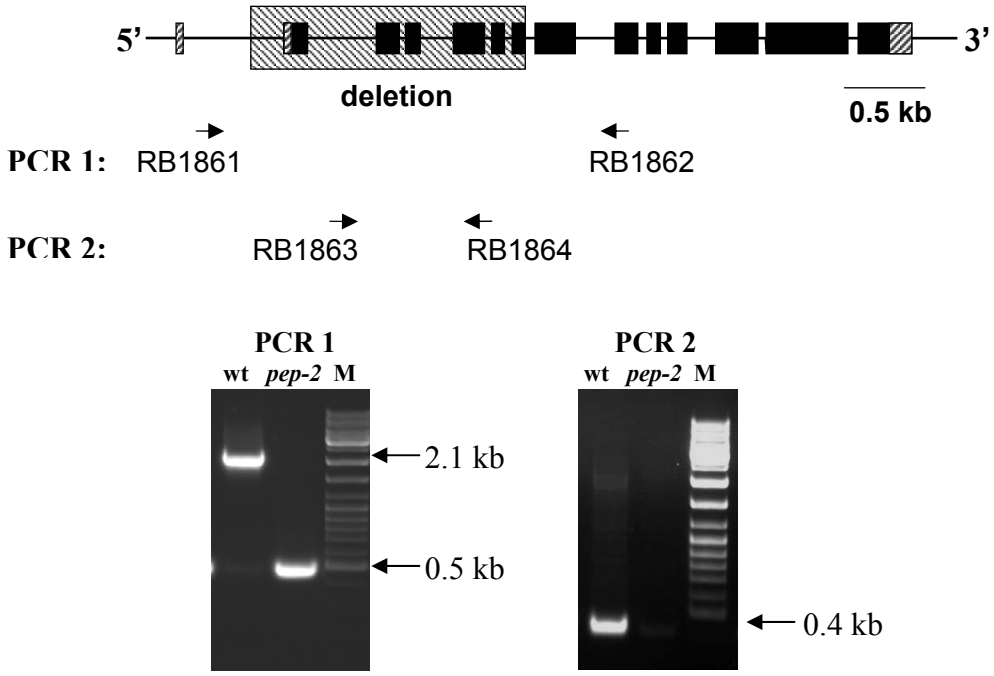

Fig. 4.3 Localisation and confirmation of the deletion in *pep-2(lg601)* homozygote animals by PCR. Top: genomic organisation of the *pep-2* gene and region which is deleted in the mutant allele *lg601* (hatched box). The position of the two primer sets is indicated by arrows. **Bottom:** Result of SW-PCRs with a wild type (wt) and *pep-2* mutant (*pep-2*) animal. M: Marker (GeneRuler^{TM} DNA Ladder Mix, MBI Fermentas, Vilnius, Litauen)

To eliminate background mutations in the *pep-2(lg601)* deletion mutant, the strain was crossed against N2 wild type animals seven times. After the last crossing step, a homozygote mutant was verified by SW-PCR again.

To demonstrate that the *pep-2(lg601)* mutants have lost their capability to transport peptides, worms were exposed to the fluorescent dipeptide conjugate ß-Ala-Lys-AMCA previously shown to be a representative substrate for PEPT1 (Groneberg et al. 2001a). Efficient uptake of the reporter molecule into intestinal epithelial cells of wild type animals was indicated by a strong fluorescence staining of all intestinal cells (Fig. 4.4), whereas the gut lumen lacked staining suggesting complete and rapid intestinal peptide absorption. When *pep-2(lg601)* mutant animals were exposed to ß-Ala-Lys-AMCA, the fluorescence was detectable only in the gut lumen indicative of a normal ingestion. However, fluorescence inside epithelial cells other than the normal gut epifluorescence was never observed (Fig. 4.4).

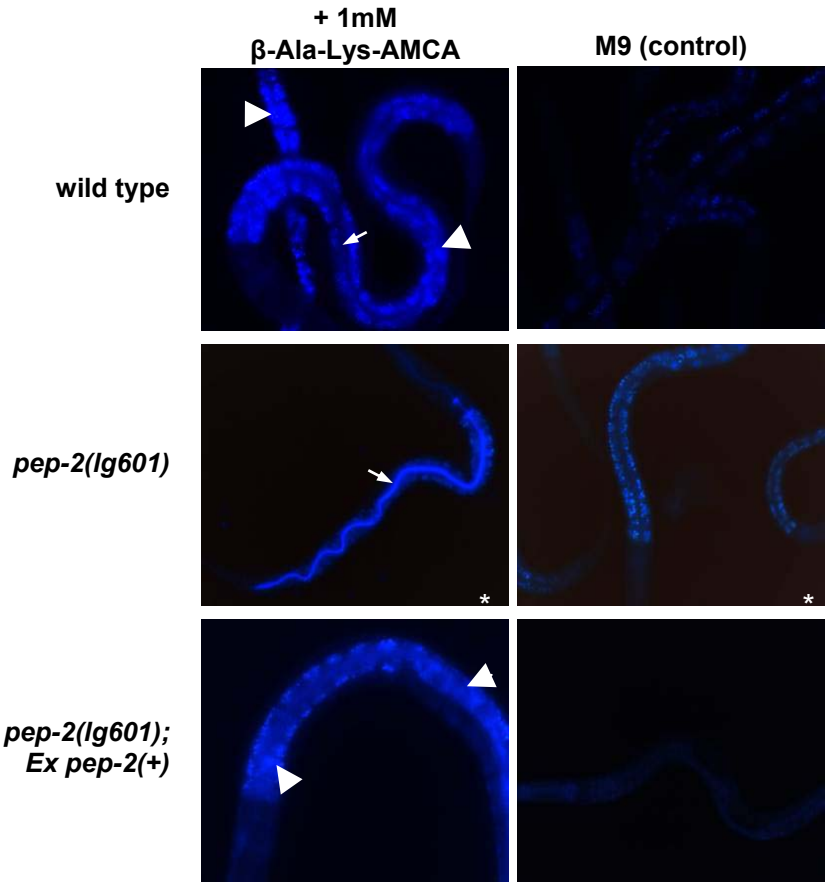

Fig. 4.4 Uptake of the fluorescent dipeptide conjugate ß-Ala-Lys-AMCA in wild type, *pep-2(lg601)* and *pep-2(lg601);Ex pep-2(+)* animals. Left column: in wild type animals the intestinal cells stain (arrowheads), no fluorescence is visible in the gut lumen (arrow); in *pep-2* mutants, fluorescence is restricted to the gut lumen (arrow) and *in pep-2;Ex pep-2(+)* animals, intestinal cells staine like in wild type animals. Right column: autofluorescence of worms treated with M9 buffer; * note extended exposure time: 174 ms compared to 10ms in wild type.

A plasmid containing the entire predicted coding region of the *pep-2* gene and the 2.4 kb upstream region (pBY1512) was injected into *pep-2* mutant animals. Two independent lines carrying this transgene (*pep-2(lg601);byEx232 and pep-2(lg601);byEx351*, *pep-2;Ex pep-2(+)* in Fig. 4.4) were also tested for β-Ala-Lys-AMCA uptake. The staining in the intestinal cells in 28% of the transgenic animals indicates that the expression of the wild type gene in the *pep-2* mutant is sufficient to restore intestinal peptide uptake. Thus the *pep-2* deletion is responsible for the loss of β-Ala-Lys-AMCA uptake.

4.1.3 Analysis of *pep-2(lg601)* mutant phenotype

pep-2(lg601) homozygous mutant animals are viable but display strong developmental defects. During a first visual inspection, it became obvious that the mutant animals grow much slower, have less progeny and are smaller compared to wild type animals. For a detailed phenotypic description, the following assays were performed.

4.1.3.1 Measurement of body size

In *C. elegans*, there are different mutants known with a variable body size (Brenner 1974). On the one hand dumpy (Dpy) and small (Sma) mutants are shorter than wild type, on the other hand, long (Lon) mutants are longer (and thinner) compared to wild type animals. To measure the body length of *pep-2(lg601)* mutant animals during larval development, worms have been synchronized by hypochlorite treatment (Lewis and Fleming 1995). The measurements were started in L1 larvae (t = 0) and continued until the animals reached adulthood. In another experiment, the measurements were started with L4 animals (t = 0) and continued through adulthood. *pep-2(lg601)* mutant animals are significant shorter at all stages of larval development (Fig. 4.5a) and adulthood (Fig. 4.5b).

In addition to the decrease in body length, *pep-2(lg601)* mutants appear thinner compared to wild type animals. The pictures in Fig. 4.6 show wild type and mutant animals, reaching adulthood just one day before. Both carry eggs in the uterus, but in contrast to the wild type animal, in *pep-2(lg601)* only a few eggs are squeezed in the thin *pep-2* mutant uterus (Fig. 4.6).

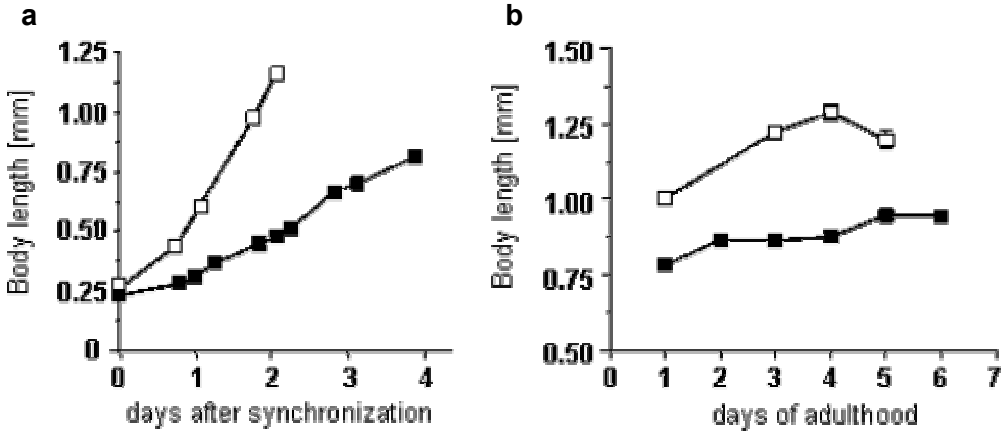

Fig. 4.5 Comparison of the body length in wild type and *pep-2(lg601)* animals. (a) Body length measurement during larval development in wild type (□) and *pep-2(lg601)* (■) animals. (**b**) Measurement of the body length in adult wild type (□) and *pep-2(lg601)* (■) animals.

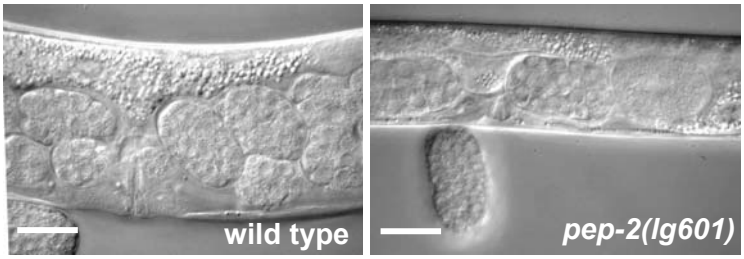

Fig. 4.6 Comparison of wild type and *pep-2* mutants 24 hours after reaching adulthood. The *pep-2(lg601)* mutant animals are significant thinner and carry fewer eggs in the uterus compared to wild type animals. Scale bars 50µm.

So, *pep-2(lg601)* mutant animals exhibit a Sma phenotype, affecting both body length and width. Recent studies have shown that some *C. elegans* Sma mutants affect genes that encode components of a transforming growth factor-β (TGF-β) signalling pathway (Patterson and Padgett 2000). DBL-1 has been identified as the TGF-β ligand that triggers the *sma* signalling pathway (Suzuki et al. 1999). A *dbl-1* overexpressing strain *ctIs40* (*dbl-1(++)*), containing multiple copies of the *dbl-1* genomic fragment, exhibits a Lon phenotype (Suzuki et al. 1999) and, in contrast, the *dbl-1(nk3)* loss-of-function mutant displays a Sma phenotype (Morita et al. 1999). In a cDNA array screen for genes regulated by *dbl-1*, the cDNA clone yk225d6 (matching the *pep-2* cDNA sequence), was found to be upregulated in *dbl-1(++)* animals, compared to the *dbl-1(nk3*) loss of function mutants (Morita et al. 2002). Since the *pep-2(lg601)* mutants also show a Sma phenotype, double mutants were constructed to investigate a potential direct function of the *pep-2* gene in this TGF-β signalling pathway for the regulation of body size. *dbl-1(nk3)* and *dbl-1(++)* were crossed into the *pep-2* mutant background and the resulting double mutants were

analyzed for Sma or Lon phenotype. All strains had been synchronized by hypochlorite treatment and pictures were taken from animals which reached adulthood about two days before (Fig. 4.7).

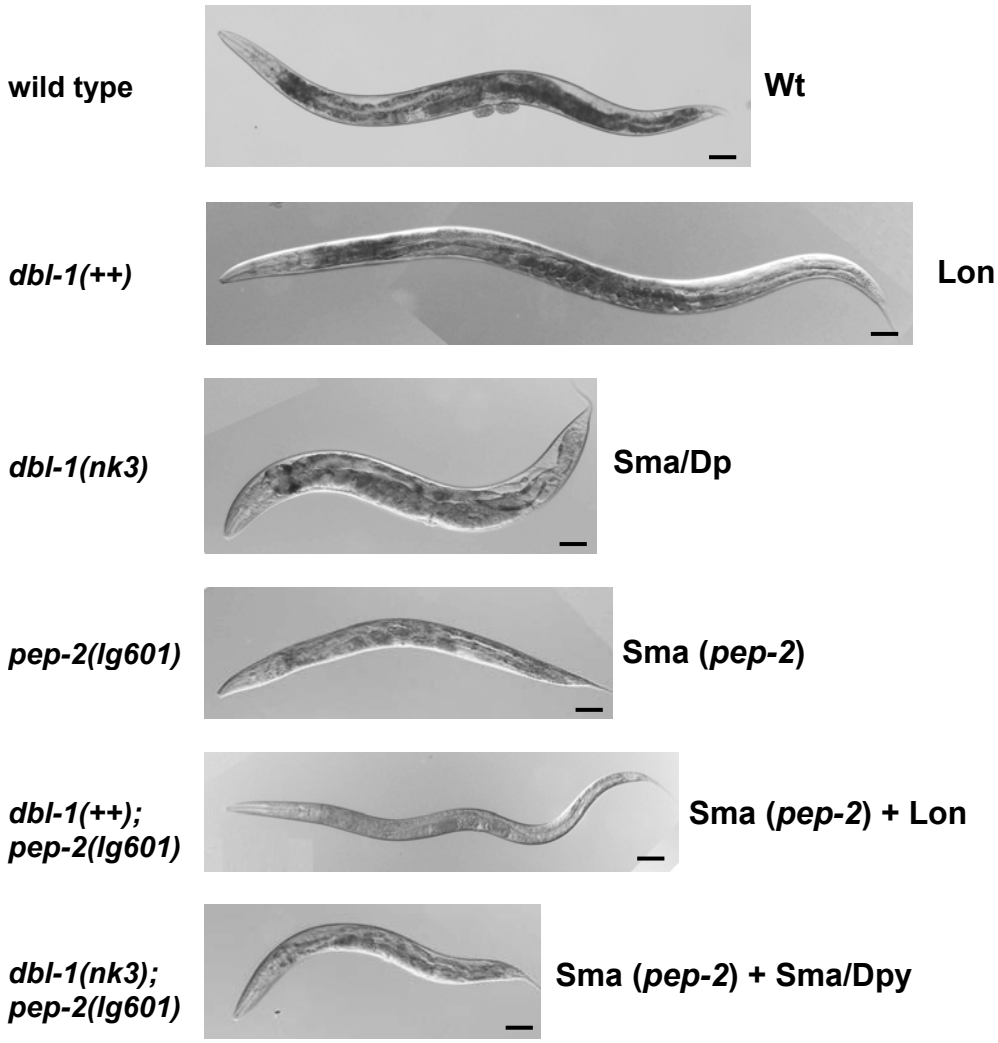

Fig. 4.7 Different Lon and Sma mutants in the *pep-2(lg601)* mutant background. Pictures were taken from two days old adult animals. Scale bars 0.1mm.

Compared to wild type animals, the *dbl-1(++)* show a clear Lon phenotype and the *dbl-1(nk3)* animals exhibit a strong Sma phenotype, reminiscent of a Dpy phenotype, as described previously. The animals are shorter but, unlike the *pep-2(lg601)* mutant animals, they are not thinner. The *dbl-1(++); pep-2(lg601)* double mutant exhibit a clear Lon phenotype compared to the *pep-2* single mutant. They are longer than *pep-2* mutant animals, but shorter and thinner compared to the *dbl-1(++)* single mutant. Finally, the *dbl-1(nk3); pep-2(lg601)* double mutants are even shorter than the *pep-2* single mutant, but not thinner.

Thus, *pep-2(lg601)* cannot suppress the Lon phenotype in *dbl-1(++)* mutant animals moreover the phenotype of the double mutants represents a clear additive effect of each single mutant phenotype. This results leads to the conclusion that *pep-2* is not directly involved in the regulation of body size by the DBL-1/TGF-β signalling pathway.

4.1.3.2 Analysis of embryonic and postembryonic development in *pep-2(lg601)* mutants

To analyze the postembryonic (larval) development, *pep-2* mutant and wild type animals have been synchronized by hypochlorite treatment. L1 larvae were grown at 20°C and were observed for up to seven days. At 13 time points between 7.5 and 168 hours after L1 synchronization the animals were analyzed for developmental alterations and pictures were taken. The development of *pep-2(lg601)* mutants was delayed in every stage of postembryonic development (Fig. 4.8a). Mutant animals took 95 hours to reach adulthood as compared to 56 hours in wild type animals.

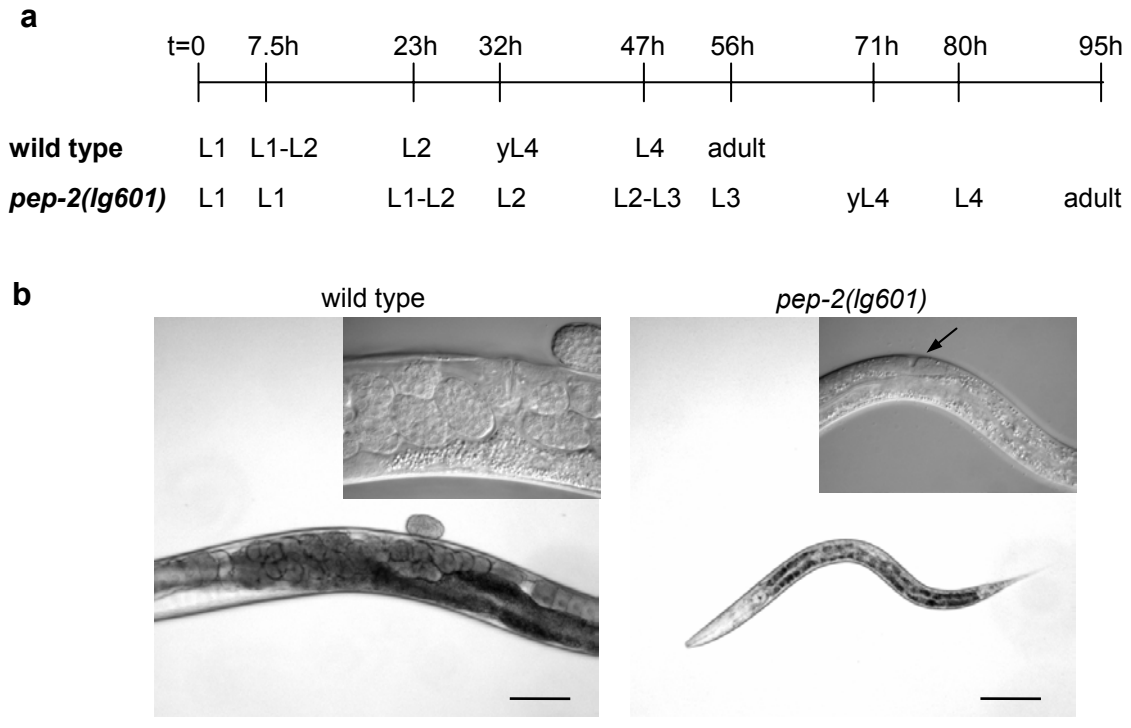

Fig. 4.8 **Delayed larval development in *pep-2* mutant animals. (a)** Schematic representation of the retarded development *in pep-2(lg601)* animals, starting with L1 larvae after synchronization (t=0) at 20°C. The developmental stages are assigned to the corresponding time points on an axis for wild type and *pep-2* mutants (*pep-2(lg601)*). L1-L4: different larval stages form 1 to 4; yL4: young L4 larva. (**b**) Pictures of a wild type and *pep-2(-)* animal, 71 hours after synchronisation. The insert shows the vulva region in a higher magnification. Arrow indicates the L4-typical vulva structure. Scale bars 0.1mm.

As a consequence, at any given time point during larval development, *pep-2(lg601)* animals are significantly smaller as a consequence of retarded development (Fig. 4.8b) and reach the reproductive stage on average 40 hours later than wild type.

In a second approach, the development was measured as generation time from “egg to egg” at 15 and 20°C. Fresh laid eggs were observed until they get adult worms and laid their first egg. At 20°C the generation time in *pep-2* mutants was extended to 118.1 ± 3.1 hours (n = 49) compared to 65.3 ± 1.2 hours in wild type (n = 47) (Fig 4.9a). At 15°C, the generation time in *pep-2* mutants was extended to 207.7 ± 8.3 hours (n = 27) compared to 114.7 ± 0.8 hours in wild type (n = 10) (Fig. 4.9b). Thus, the *pep-2* deletion causes a 1.8-fold increase in generation time at both tested temperatures. In addition, the rescue lines BR2893 and BR2894 were tested for generation time at 15°C. They both exhibited a significant reduction of generation time (135.9 ± 6.2 (n=25) and 138.0 ± 5.6 (n=25) respectively) compared to *pep-2(lg601)* mutants (Fig. 4.9b), demonstrating that the expression of the *pep-2* wild type gene is sufficient to rescue the delayed postembryonic development. In contrast, the embryonic development, which takes place in a protective eggshell, measured from the two-cell stage to hatching, was just slightly enhanced from 12.8 ± 0.7 hours in wild type to 14.9 ± 0.9 in *pep-2* mutants (Fig. 4.9c).

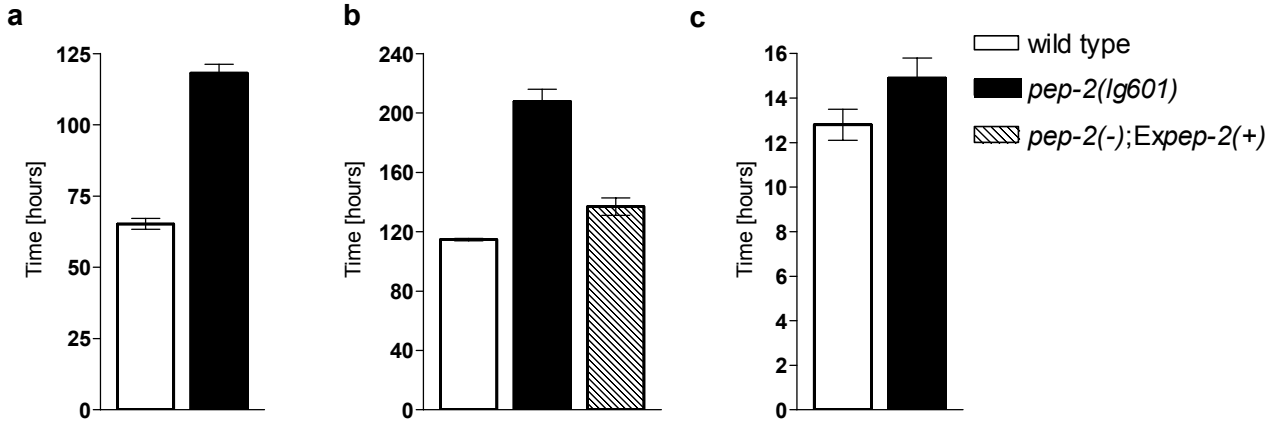

Fig. 4.9 Generation time and embryonic development in *pep-2* mutant animals. (**a**) Extended generation time of wild type and *pep-2(lg601)* animals at 20°C. (**b**) Generation time of wild type, *pep-2(lg601)* and *pep-2(-)*;Ex *pep-2(+)* (mean of BR2893 and BR2894) animals at 15°C. (**c**) Embryonic development of pep-2 mutants is comparable to wild type.

4.1.3.3 Investigation of the fertility of *pep-2(lg601)* mutants compared to wild type animals

To measure the fertility of the mutant strain, the self brood size of hermaphrodites was determined. While wild type hermaphrodite produces 316.6 ± 21.5 (n = 54) self-progeny, in *pep-2* mutant animals the production of progeny was strongly decreased to 109.3 ± 13.5 (n = 79) animals per hermaphrodite (Fig. 4.10a). Not only the production of progeny was significantly reduced, in addition the egg-laying period was extended from five days in wild type to nine days in *pep-2* mutants (Fig. 4.10b). The brood size experiments also suggested that *pep-2(lg601)* is a recessive allele. Heterozygote *pep-2(+/-)* animals (*pep-2*/+ in Fig. 4.10a) did not show a decrease in progeny production (331.9 ± 22.5; n = 19) compared to wild type. Homozygous *pep-2(lg601)* mutants originating from heterozygous animals did not show a significant change in brood size (122.7 ± 11.9; n = 19) compared to the *pep-2* homozygote mutant strain (Fig. 4.10a), thus there is no maternal contribution to this phenotype.

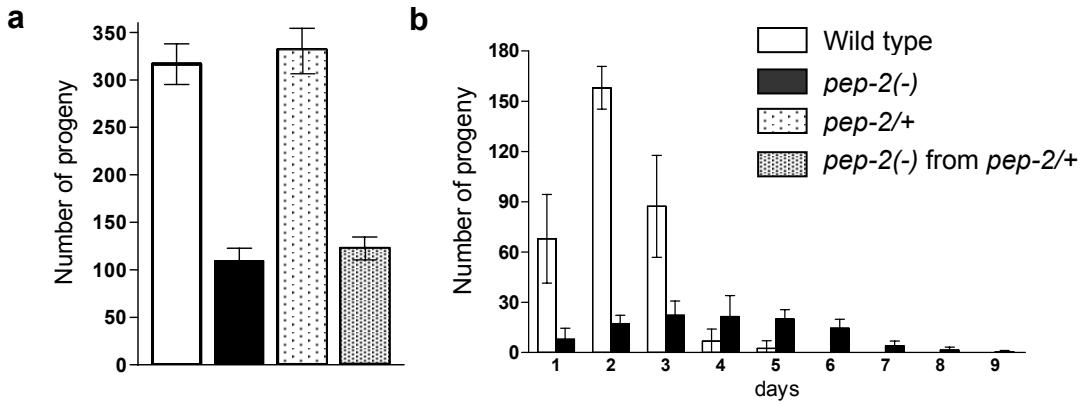

Fig. 4.10 Self brood size and egg-laying period in *pep-2* homozygous mutants. (a) The total brood size is significantly reduced in homozygous *pep-2(lg601)* mutant animals, but not in heterozygote (*pep-2/*+). *pep-2* homozygous mutants produced by *pep-2* heterozygous animals (*pep-2(-)* from *pep-2/*+) produce as many progeny as the *pep-2(lg601)* mutant strain. (**b**) Extended egg-laying period in *pep-2* mutant animals.

Another experiment was performed to show the delayed production of progeny in the *pep-2* mutant animals. For this purpose, the number of eggs laid per hour was counted. Whereas wild type animals produced on average 5.1 progeny per hour (128 ± 4 for five hermaphrodites in five hours; n = 3), in *pep-2(lg601)* animals the production of progeny was reduced to 25 % compared to wild type (on average 1.3 progeny per hour; 32 ± 2 for five hermaphrodites in five hours; n = 3).

The eggs were laid at approximately the same developmental stage as in wild type animals (28-56 cell stage) therefore the production of eggs rather than their retention in the uterus appears to be the limiting factor in *pep-2(lg601)* animals. In *C. elegans*, the yolk protein is produced in the intestine and transported into the oocyte by endocytosis (Kimble and Sharrock 1983). In order to monitor this process, a mutant strain (*bIs1[vit-2::gfp,pFR4,sqt-1(sc103)]* expressing a vitellogenin::green fluorescent protein fusion (YP170::GFP) was used (Grant and Hirsh 1999). In this strain, the accumulation of yolk protein in the eggs can be followed by the corresponding GFP expression. *pep-2(lg601)* was crossed into the bIs1*[vit-2::gfp,pFR4,sqt-1(sc103)]* background and the double mutant was analyzed for GFP expression. Fertilized eggs in the uterus of *pep-2(lg601)* displayed the same level of YP170::GFP fluorescence as those of wild type animals (Fig. 4.11) indicative of a similar yolk concentration in mature eggs.

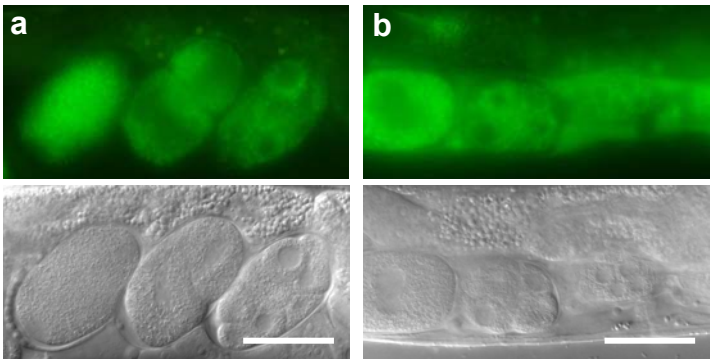

Fig. 4.11 Expression of YP170::GFP in (a) wild type and (b) *pep-2(lg601)* background. The pictures were taken at the same exposure time, corresponding Nomarski pictures see below. Scale bars 50µm.

To investigate if spermatogenesis is also affected in *pep-2(lg601)* mutant animals, a male mating test was performed. The temperature sensitive allele *fem-1(hc17ts)* was used for this experiment. At the restrictive temperature of 25°C, XX animals are functionally female rather than hermaphroditic due to the absence of spermatogenesis. The mutant was crossed into an *unc-4(e120)* background to facilitate the mating test. *unc-4(e120); fem-1(hc17ts)* double mutants (BR2068) were grown at 25°C and young adult animals were used for the mating with either wild type or *pep-2* mutant males. Three *unc-4(e120); fem-1(hc17ts)* were allowed to mate over night with ten wild type males, resulting in 940.4 ± 73.3 progeny (313.5 ± 24.4 per *unc-4(e120); fem-1(hc17*)) and ten *pep-2* males, resulting in 585.8 ± 80.5

progeny (195.3 ± 26.8 per *unc-4(e120); fem-1(hc17ts)*) (Fig. 4.12). Thus, the fertility of *pep-2* males is reduced to 62.3 %.

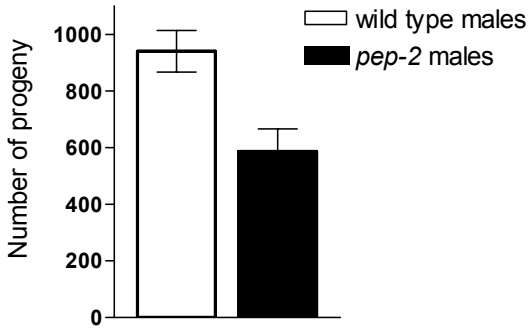

Fig. 4.12 Reduced fertility of *pep-2* males. In a male mating test with five *unc-4(e120);fem-1(hc17ts)* per plate, *pep-2* males produces only 62.3% of the progeny compared to wild type males.

4.1.3.4 Amino acid supplementation

To examine if the phenotype in *pep-2(lg601)* mutant animals is caused by a limited availability of amino acid nitrogen, worms were supplemented with a mix of free amino acids (3.1.10), the substrate for amino acid transporters. Self-brood size and postembryonic development (generation time) were measured for supplemented (+ aas) and non-supplemented (- aas) worms. In wild type animals, the brood size was not affected by amino acid supplementation (Fig. 4.13a) (wild type - aas: 298.7 ± 18.3; wild type + aas: 296.3 ± 22.3; p = 0.94). In contrast, the production of progeny in *pep-2(lg601)* mutants was enhanced significantly ($p < 0.05$) from 100.7 ± 11.6 without additional amino acids to 150.1 ± 16.4 with the amino acid supplementation (Fig. 4.13a). Therefore, an increased offer of free amino acids can attenuate the phenotype concerning the production of progeny, but not restore the wild type situation.

The generation time of wild type animals was also not affected by amino acid supplementation (Fig. 4.13b) (wild type – aas: 115.0 ± 1.6 h (n = 34); first generation + aas: 115.8 ± 1.5 h (n = 33); third generation + aas: 115.7 ± 1.8 h (n = 29)). In *pep-2(lg601)* mutants the generation time is slightly reduced by amino acid supplementation, but even in the third generation of supplemented animals, this alteration is not statistically significant (p = 0.125) (*pep-2(lg601)* – aas: 185.3 ± 9.2 h (n = 29); first generation + aas: 171.5 ± 7.3 h (n = 29); third generation + aas: 166.6 ± 7.4 h (n = 28)).

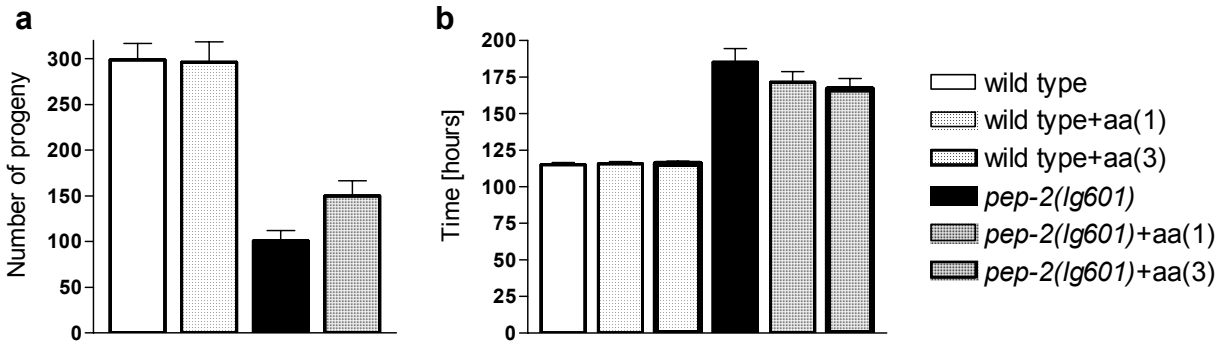

Fig. 4.13 Brood size and postembryonic development with amino acid supplementation. The brood size (**a**) and the generation time (**b**) are not affected by amino acid supplementation in wild type animals. In *pep-2(lg601)* mutant animals, the number of progeny can be significantly increased (**a**), but the postembryonic development (**b**) is slightly reduced by additional availability of free amino acids. aa(1): first generation of supplemented animals; aa(3): third generation of supplemented animals.

Therefore, the strong phenotype observed in *pep-2(lg601)* mutant animals can only be partially rescued by an increased supply of free amino acids that may serve as an additional source for amino acid nitrogen.

4.1.3.5 Measurement of parameters for feeding and digestion

Pharyngeal pumping and defecation are two important parameters to investigate the feeding behaviour in *C. elegans*. Rapid pharyngeal pumping is one of the behavioural responses of a wild type worm to food and ensures a sufficient supply of nutrient to the animal. Defecation is achieved through rhythmic activation of a stereotyped cycle of muscle contractions and occurs approximately every 45 seconds at variable temperatures (Liu and Thomas 1994; Iwasaki et al. 1995). The length of the defecation cycle is responsible for the resting time of nutrients in the intestine. To investigate the feeding behaviour and food consumption in *pep-2(lg601)* mutant animals, pharyngeal pumping and defecation cycle were measured in mutant and wild type animals. The pharyngeal pumping was counted as pumps per minute and was slightly reduced in *pep-2(lg601)* mutant animals (255.1 ± 14.7; n = 19) compared to wild type control (271.3 ± 13.9; n = 20, p = 0.43) (Fig. 4.14a). The frequency of defecation in *pep-2(lg601)* animals was not significantly changed compared to wild type animals (46.3 ± 3.5 and 47.9 ± 2.1 respectively, p = 0.71; Fig. 4.14b).

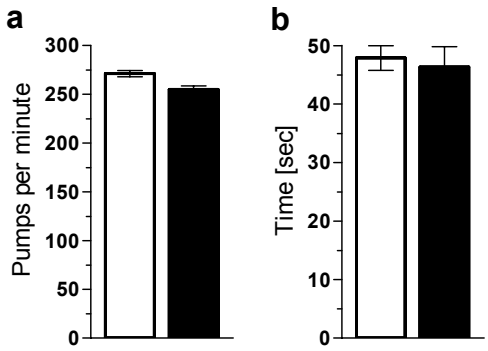

Fig. 4.14 Measurement of pharyngeal pumping and defecation cycle in mutant and wild type animals. (**a**) The pharyngeal pumping in *pep-2(lg601)* mutants (black bars) is slightly reduced compared to wild type (white bars). (**b**) The defecation cycle in *pep-2(lg601)* mutant animals (black bars) do not show any significant deviation compared to wild type (white bars).

4.1.3.6 Life span analysis

Like in many other organisms, in *C. elegans* it is known that a caloric restriction (CR) can lengthen the mean and maximal life span of the animals. For example, the mutant allele *eat-2(ad453)*, showing a reduced pharyngeal pumping rate (Avery 1993) and as a consequence an impaired food uptake, exhibit a life span extension of about 29% (Lakowski and Hekimi 1998). Life span experiments were performed to investigate whether the reduced dietary availability of amino acids in *pep-2(lg601)* mutants may be interpreted as a reduced intake of calories. As a control for caloric restricted mutants, *eat-2(ad452)* was included into the experiment. The mean life span of *pep-2(lg601)* animals was slightly enhanced from 16.3 ± 0.4 days in wild type to 18.8 ± 1.4 days in the mutant, but it is shorter than *eat-2(ad452)* animals (21.3 days) (Fig. 4.15a). The weak life span extension in the *pep-2(lg602)* mutants may reflect the observed delay in postembryonic development. For this reason, the adult mean life span was determined (Fig. 4.15b). In this experiment, the adult mean life span of *pep-2(lg601)* mutant animals (14.2 ± 1.4 days) was not significantly enhanced compared to the wild type control (13.6 ± 0.5 days; $p = 0.7$) in contrast to the adult life span in *eat-2(ad453)* mutant animals (18.3 days).

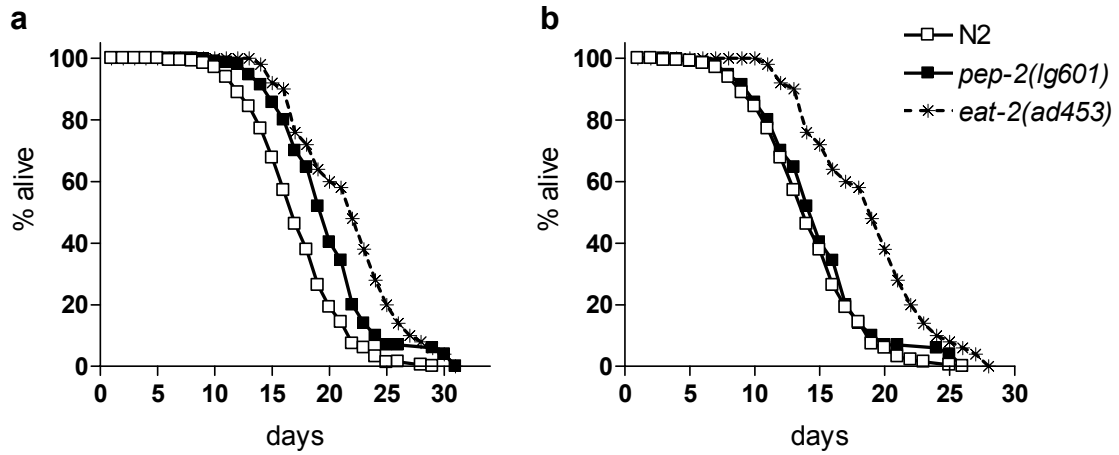

Fig. 4.15 Measurement of survival in wild type, *pep-2(lg601)* and *eat-2(ad453)* mutant animals at 20°C. Total life span is shown in (**a**) and adult life span in (**b**).

Therefore, the deletion in the *pep-2(lg601)* mutant does not cause a caloric restriction, which is severe enough to lengthen life span.

4.2 Analysis of PEP-1, the high-affinity, low capacity peptide transporter of *C. elegans*

4.2.1 Localisation of *pep-1* gene expression with a GFP reporter construct

For the examination of the *pep-1* expression pattern, a reporter construct was generated to express the green fluorescence protein (*gfp*) under the control of the *pep-1* promoter. A PCR product containing 3.8 kb upstream the transcriptional start was cloned into the expression vector pPD95.75 resulting in the plasmid pBY1635 (Fig. 4.16).

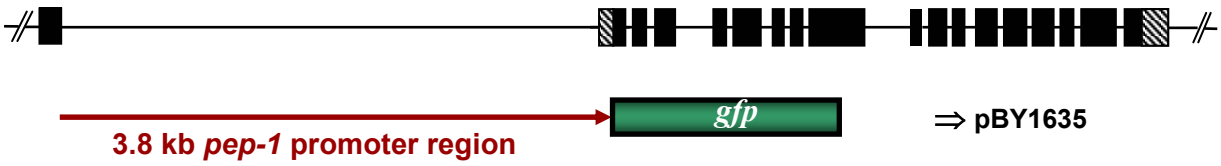

Fig. 4.16 Reporter gene fusion under *pep-1* promoter control.

For expression pattern analysis, a transgenic line (BR2747) was constructed by injecting the plasmid pBY1635 (together with pRF4). *pep-1* expression starts in embryogenesis (Fig. 4.17a) and is maintained throughout the development until adulthood (Fig. 4.17b and d). The reporter construct was expressed in a variety of different cells and tissues like vulva (Fig. 4.17c), anal muscles (Fig. 4.17e), ventral and dorsal nerve cord (Fig. 4.17c and f) and some neurons in the head of the animal (Fig. 4.17g). No intestinal expression was detectable.

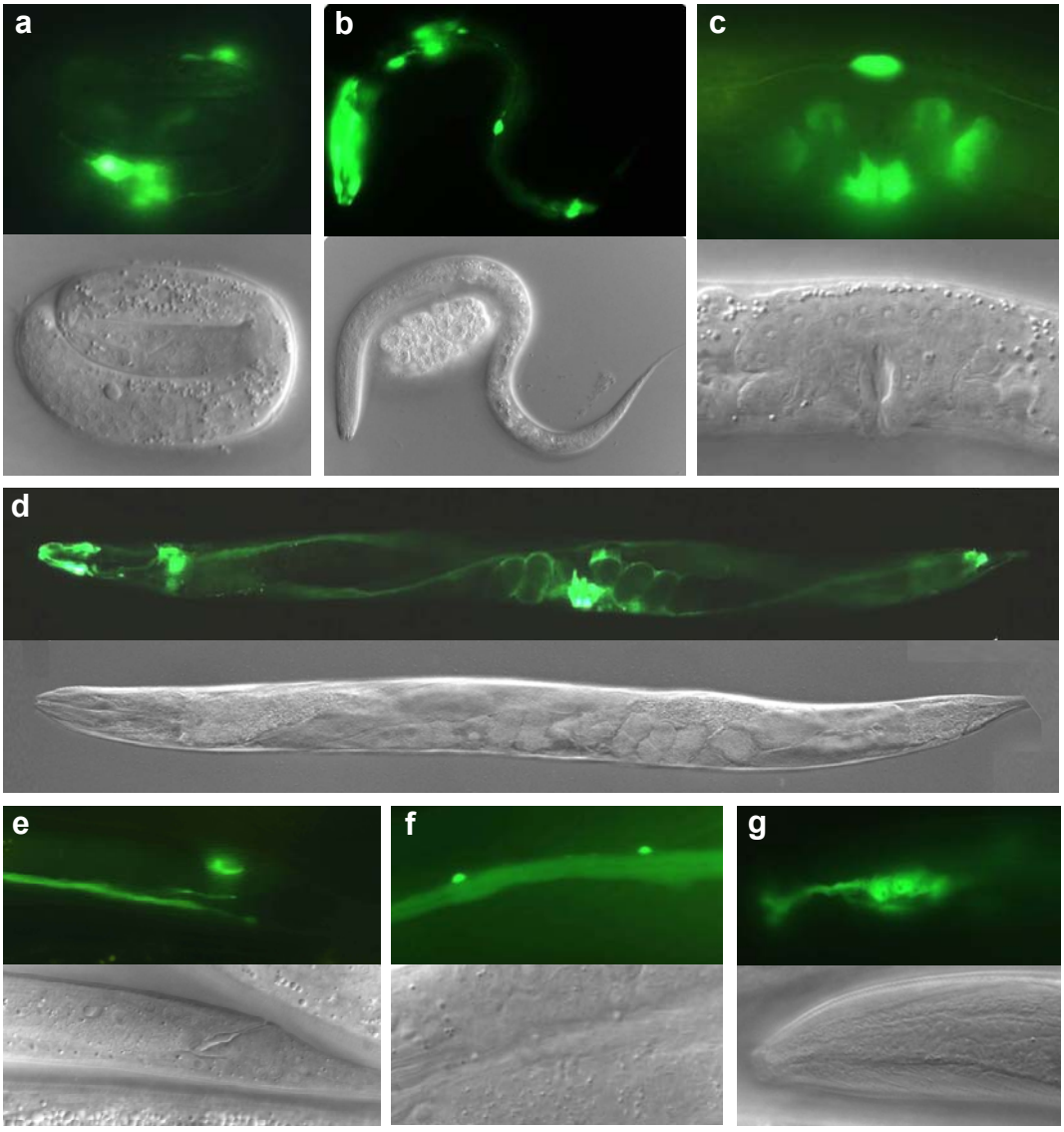

Fig. 4.17 ***pep-1*promoter::GFP expression pattern.** The expression starts in embryogenesis (**a**) and is maintained throughout the larval development (**b**) until adulthood (**d**). The reporter construct is expressed in variety of tissues like vulva (**c**), anal muscles (**e**), ventral and dorsal nerve cord (c, d, e, f) and different head neurons (**g**).

4.2.2 Identification of the *pep-1(lg501)* mutant allele

The *pep-1(lg501)* deletion mutant, generated by TMP/UV mutagenesis (3.1.3), was obtained from EleGene (Munich). A 2.5 kb deletion eliminates 1269 bp of the promoter sequence, the translational start codon and the first six exons (bp18289 to 20838 on cosmid C06G8) (Fig. 4.18). Thus, *pep-1(lg501)* is missing the most important domains for function and most likely represents a null allele. To verify this deletion, a SW-PCR with *pep-1* specific primers was performed. One set of primers anneals in the genomic region outside of the deletion (RB2257 and RB2258 for PCR 1) a results in wild type PCR fragment (3.7 kb) and a *pep-1* deletion PCR fragment (1.4 kb) (Fig. 4.18). To recognize the difference between homo- and heterozygote animals, a second set of primers was used that is located in the deletion, (RB2255 and RB2256 for PCR 2) (Fig. 4.18) to get a 1.2 kb fragment if there is a copy of the wt *pep-1* gene.

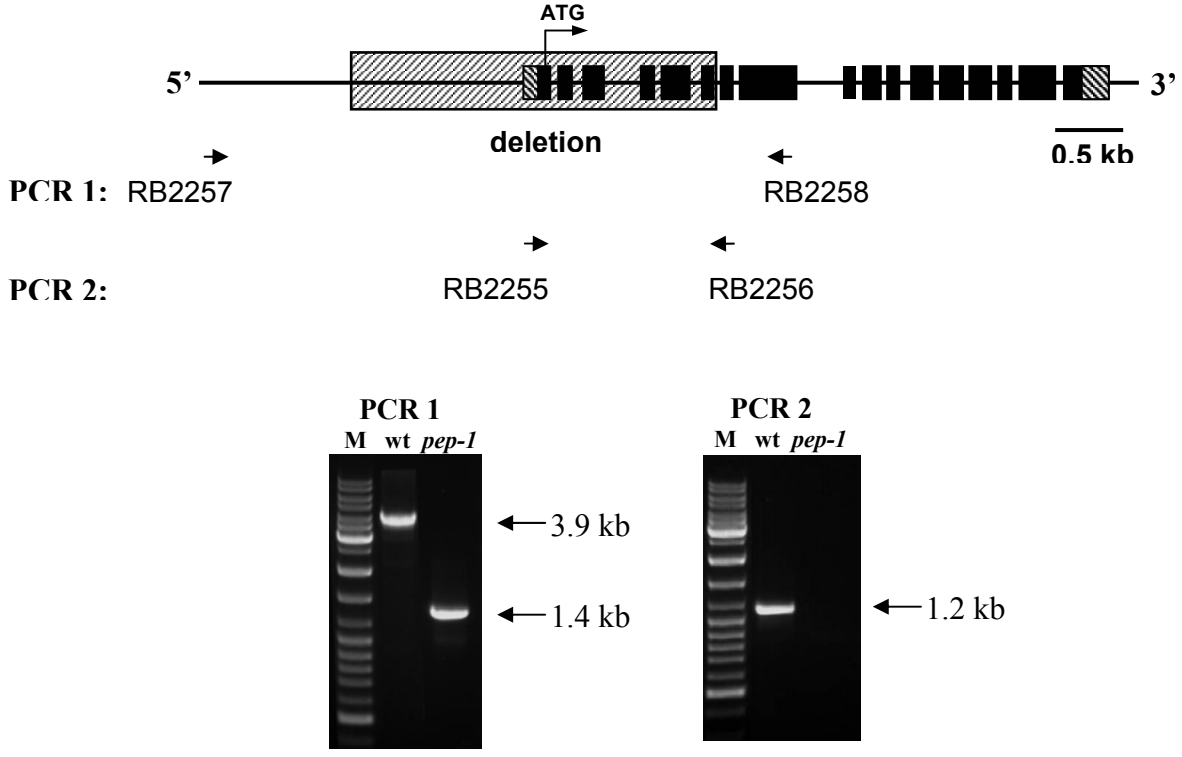

Fig. 4.18 Localisation and confirmation of deletion in *pep-1(lg501)* homozygote animals by PCR. Top: genomic organisation of the *pep-1* gene and region which is deleted in the mutant allele *lg501* (hatched box). The position of the two primer sets is indicated by arrows. **Bottom**: Result of SW-PCRs with a wild type (wt) and *pep-1* mutant (*pep-1*) animal. M: Marker (GeneRuler™ DNA Ladder Mix, MBI Fermentas, Vilnius, Litauen)

To eliminate background mutations in the *pep-1(lg501)* deletion mutant, the strain was crossed against N2 wild type animals five times. After the last crossing step, a homozygote mutant was verified by SW-PCR again.

To test redundancy of PEP-1 and PEP-2 in intestinal peptide transport, *pep-1(lg501)* homozygote mutants were also tested for the uptake of the fluorescent dipeptide conjugate ß-Ala-Lys-AMCA. The animals were exposed to the substrate like *pep-2(lg601)* mutants and the straining of intestinal cells in *pep-1(lg501)* mutant animals (Fig. 4.19), confirm a wild type like uptake of the fluorescent model substrate in spite of the missing PEP-1 transporter function.

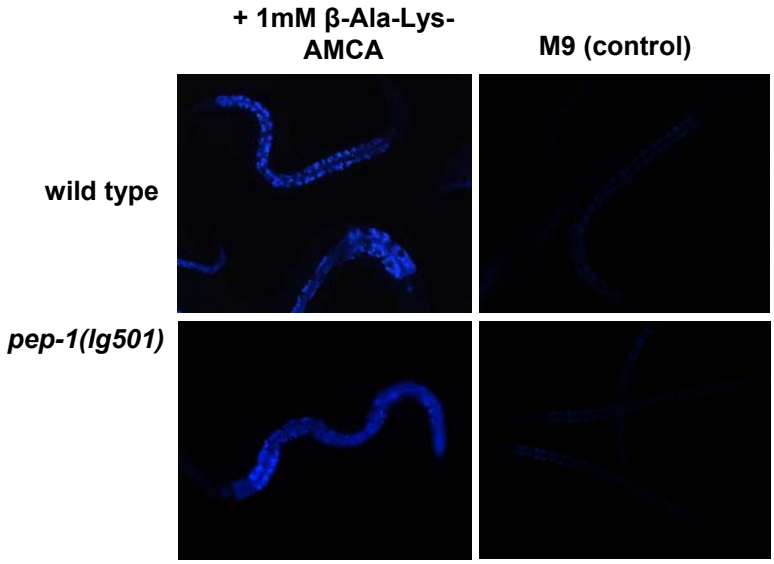

Fig. 4.19 Uptake of the fluorescent dipeptide conjugate ß-Ala-Lys-AMCA in *pep-1(lg501)* mutant animals. Top: wild type staining and buffer (M9) control. **Bottom**: *pep-1(lg501)* staining and buffer (M9) control

4.2.3 Analysis of *pep-1(lg501)* and *pep-1(lg501); pep-2(lg601)* mutants

pep-1(lg501) homozygote mutant animals are viable and do not show any obvious defects like *pep-2(lg601)* mutants. Generation time and brood size have been determined for *pep-1(lg501)* and *pep-1(lg501)*; *pep-2(lg601)* to prove potential overlapping functions.

4.2.3.1 Determination of generation time

The postembryonic development (generation time) was measured as previously described. At 20°C, there was no significant difference detectable between wild type and *pep-1(lg501)* mutant animals (65.3 ± 1.9 hours and 66.3 ± 1.5 hours respectively, p = 0.74; Fig. 4.20). In addition, *pep-1(lg501), pep-2(lg601)* double mutants did not show a more severe phenotype than *pep-2(lg601)* single mutants (111.9 ± 1.8 hours (n=7) and 118.1± 3.1 hours, respectively; p = 0.46; Fig. 4.20).

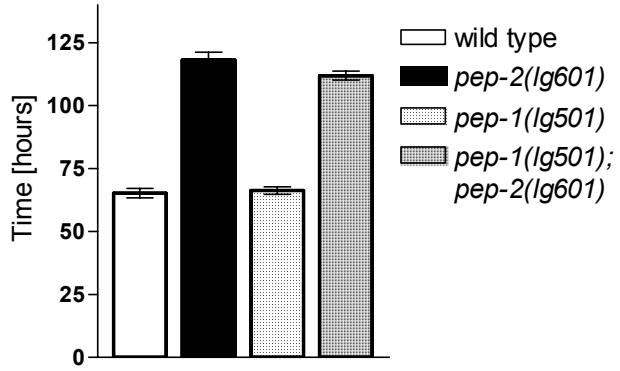

Fig. 4.20 Generation time of *pep-1(lg501)* and *pep-1(lg501);pep-2(lg601)* mutant animals. There was no significant difference between the generation times of *pep-1(lg501)* mutants and wild type animals, and between *pep-1(lg501), pep-2(lg601)* double and *pep-2(lg601)* single mutants.

4.2.3.2 Measurement of brood size

The progeny of *pep-1(lg501)* homozygote animals were counted. There was no significant difference (p = 0.63) in brood size comparing wild type and *pep-1(lg501)* mutant animals (316.6 ± 21.5; n = 54 and 289.1 ± 21.4; n = 8 respectively; Fig. 4.21).

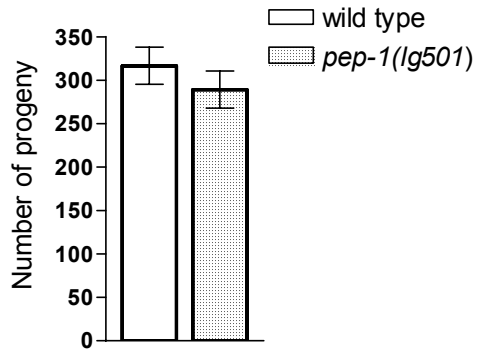

Fig. 4.21 Measurement of brood size in *pep-1(lg501)* homozygote mutant animals.

4.3 Influence of *pep-2* on the regulation of development, metabolism and ageing

4.3.1 *pep-2* and the DAF-7/TGF-β signalling pathway

Food is one of the sensory inputs that control whether wild type worms enter the metabolically shifted, non-feeding, non-reproducing dauer stage and store large amounts of fat (Kimura et al. 1997; Riddle and Albert 1997). The DAF-7 transforming growth factor (TGF)-β signalling pathway acts to regulate *C. elegans* metabolism and a disruption of the pathway is sufficient to cause constitutive arrest at the dauer stage (Riddle and Albert 1997) and a switch to fat storage metabolism (Kimura et al. 1997). The expression of DAF-7 in the ASI sensory neurons is regulated by dauer pheromone and food signals. In a *tph-1* (tryptophan hydroxylase gene) deletion mutant, which is deficient in the synthesis of serotonin, the *daf-7::GFP* expression in the ASI neurons is reduced at least six fold (Sze et al. 2000).

To investigate a possible function of *pep-2* in the ASI neurons in sensing food or other environmental cues transmitted through the DAF-7 signalling pathway, the *daf-7::GFP* expression strain (mIs6*[rol-6(su1006),daf-7p::GFP]*; Ren et al. 1996) was crossed into the *pep-2(lg601)* mutant background. The level of expression was analyzed by fluorescent microscopy. No visible change in GFP expression in the ASI neurons could be determined (Fig. 4.22).

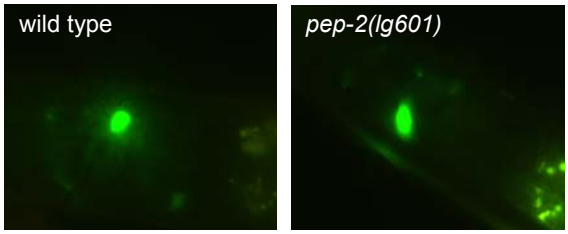

Fig. 4.22 *daf-7* TGF-β expression in wild type and *pep-2(lg601)* mutant background. *daf-7::GFP* is strongly expressed in the sensory neuron ASI in wild type and *pep-2(lg601)* mutant animals. L3 animals are shown. Exposure time was identical for both pictures (1400ms).

4.3.2 Interaction between *pep-2* and the DAF-2/insulin-like signalling pathway

In mammals, a low dietary protein intake or the dietary restriction of individual essential amino acids is known to lower plasma IGF-1 and insulin levels that in turn impair growth and development (Noguchi 2000; Takenaka et al. 2000; Gems and Partridge 2001). Moreover, in Drosophila the insulin receptor/phosphoinositide 3-kinase (Inr/PI3K) signalling is a key regulator of growth and development that was shown to be affected by the availability of dietary protein *in vivo* (Britton et al. 2002). To investigate a possible connection between the availability of amino acids and the insulin signalling in *C. elegans*, *pep-2(lg601)* was crossed into the *daf-2(e1370)* and the *daf-16(m26)* mutant backgrounds and several phenotypic analyses were performed with the corresponding double and triple mutants.

4.3.2.1 Dauer formation assays

The dauer formation was analyzed in wild type, *pep-2(lg601)*, *daf-2(e1370)* and *daf-2(e1370); pep-2(lg601*) mutant strains (Tab. 4.1). At 20°C, *pep-2(lg601)* mutants do not form dauers like wild type (0%) and the mutation in *pep-2(lg601)* is not able to enhance the *daf-2(e1370)* phenotype in the *daf-2(e1370); pep-2(lg601*) double mutant significantly (3.2 % and 1.4 % dauer formation respectively).

Tab. 4.1 Dauer formation at 20 and 25°C. Numbers of animals reaching adulthood (≥ L4 larva) or arresting at the dauer stage at 20 and 25°C.

	20°C		25°C	
Genotype	**≥ L4 larva**	**dauer**	**≥ L4 larva**	**dauer**
wild type	85	0	196	0
pep-2(lg601)	105	0	119	0
daf-2(e1370)	95	3	0	84
daf-2(e1370); pep-2(lg601)	72	1	0	96

At 25°C, the *pep-2(lg601)* single mutant behaves like wild type animals (0 % dauer) and the *daf-2(e1370); pep-2(lg601*) double mutant behaves like the *daf-2(e1370)* single mutant (100 % dauer). Thus, the *pep-2* deletion does not show any effect on dauer formation at the tested temperatures.

4.3.2.2 Determination of generation time

To examine the influence of insulin-signalling and peptide transport on the postembryonic development, generation time was measured for *daf-16(m26), daf-2(e1370), pep-2(lg601)* and the corresponding double and triple mutant animals at 20°C (Fig. 4.23; Tab. 4.2).

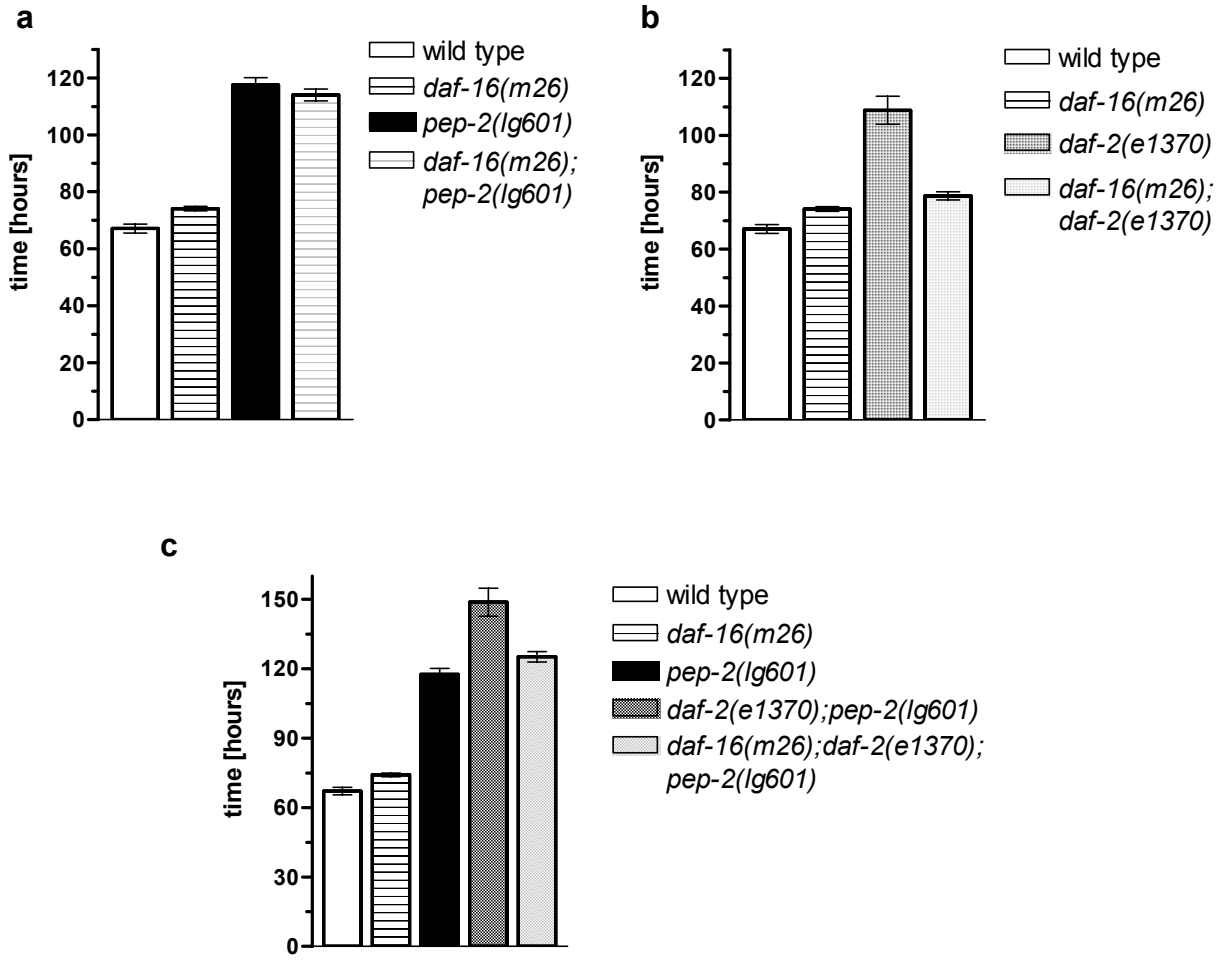

Fig. 4.23 Generation time of *pep-2, daf-2 and daf-16* single, double and triple mutants at 20°C.

The generation time of *daf-16(m26)* was slightly extended compared to the wild type control (Fig. 4.23, Tab. 4.2). In contrast, the postembryonic development in *daf-2(e1370)* and *pep-2(lg601)* mutant animals was significantly enhanced to a similar extend compared to wild type animals ($p < 0.0001$ for both mutants) (Fig. 4.23a and b; Tab. 4.2). Only the extended postembryonic development in *daf-2* mutant animals could be suppressed by an additional mutation in *daf-16* (Fig. 4.23b, Tab. 4.2). The *daf-16(m26); pep-2(lg601)* double mutant strain exhibited the same extension in

generation time like the *pep-2(lg601)* single mutant animals (Fig. 4.23a, Tab. 4.2). Thus, the delay in postembryonic development of *pep-2* mutants could not be suppressed by *daf-16(m26)*. The time required for post-embryonic development in the *daf-2(e1370); pep-2(lg601)* double mutants was even more enhanced than in the corresponding single mutants and was extended 2.3-fold compared to wild type animals (Fig. 4.23c; Tab. 4.2). The *daf-16(m26); daf-2(e1370); pep-2(lg601*) triple mutants exhibit the same generation time like *pep-2(lg601)* single mutant animals (Fig. 4.23c, Tab. 4.2), thus, only the *daf-2(-)* caused phenotype was suppressed by *daf-16(m26)*.

Tab. 4.2 Generation time of *pep-2* and insulin-like signalling mutants at 20°C.

Genotype	Generation time (hours)	% wild type
wild type	67.1 ± 1.6 (n = 48)	100
daf-16(m26)	74.1 ± 0.8 (n = 37)	110
pep-2(lg601)	117.5 ± 2.6 (n = 64)	175
daf-16(m26);pep-2(lg601)	114.0 ± 2.1 (n = 16)	170
daf-2(e1370)	108.8 ± 4.9 (n = 20)	162
daf-16(m26);daf-2(e1370)	78.7 ± 1.4 (n = 37)	117
daf-2(e1370);pep-2(lg601)	148.7 ± 6.1 (n = 21)	222
daf-16(m26);daf-2(e1370);pep-2(lg601)	125.1 ± 2.2 (n = 24)	186

4.3.2.3 Brood size measurements

The production of progeny was shown to be reduced in *daf-2(e1370*) mutants at 15 and 25.5°C (Larsen et al. 1995). To examine a possible interaction between *pep-2* function and the *daf-2* signalling pathway concerning the fertility, the brood size was determined for *daf-16(m26), daf-2(e1370), pep-2(lg601)* and the corresponding double and triple mutant animals at 20°C (Fig. 4.24; Tab. 4.3).

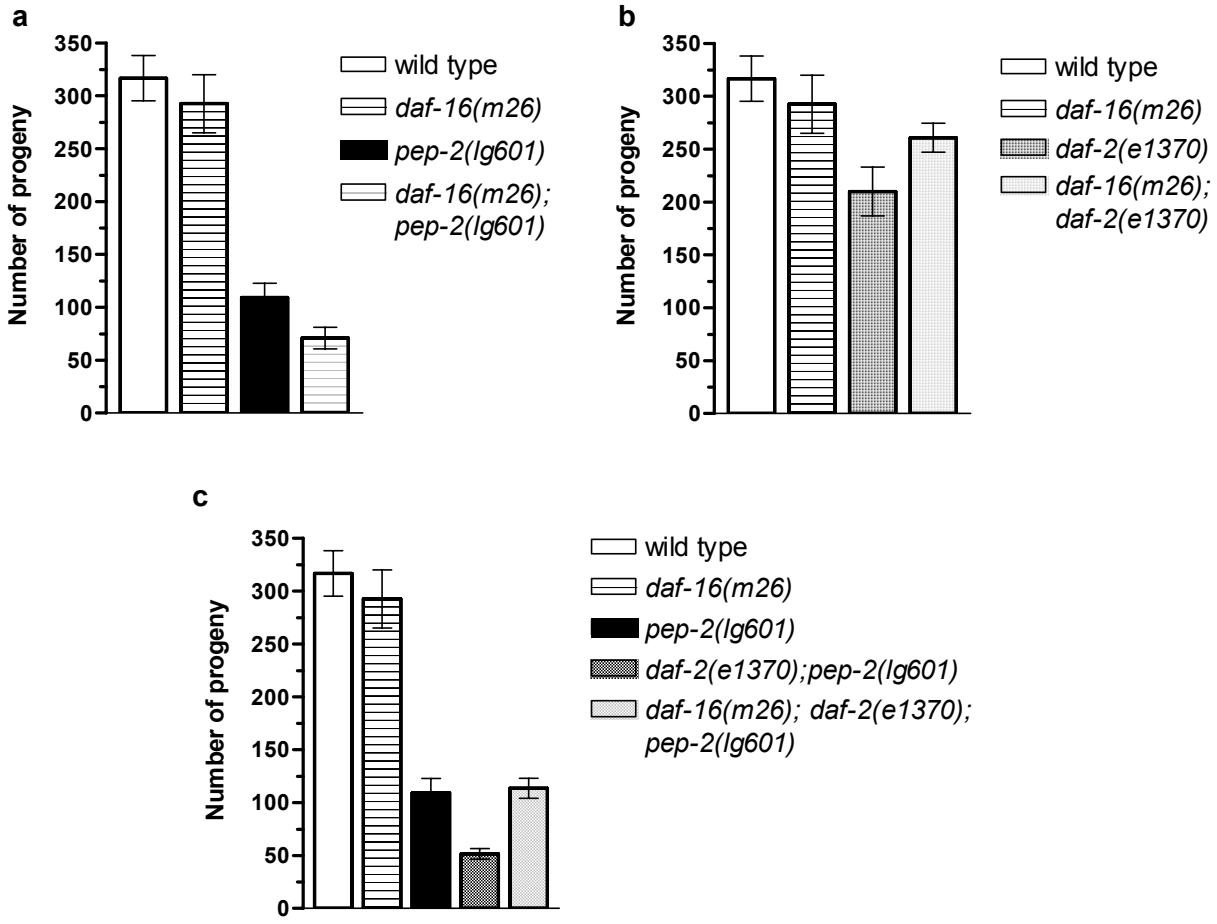

Fig. 4.24 Brood size of *pep-2*, *daf-2*, *daf-16* and corresponding double and triple mutant strains.

The brood size of *daf-16(m26)* mutants was not significantly altered compared to wild type animals (Fig. 4.24a, Tab. 4.3; p = 0.5). In a *daf-16(m26); pep-2(lg601)* double mutant strain, the brood size was slightly reduced (p = 0.32) compared to the *pep-2(lg601)* single mutant (Fig. 4.24a; Tab. 4.3). Thus, decrease in progeny production could not be suppressed by *daf-16(m26)* and the significant decreased progeny production in *pep-2(lg601)* mutant animals is not dependent on a functional *daf-16*. In contrast, the decreased brood size of *daf-2(e1370)* mutants was suppressed by *daf-16(m26)*, resulting in a higher brood size in the *daf-16(m26); daf-2(e1370)* double mutant strain compared to the *daf-2(e1370)* single mutants (Fig. 4.24b; Tab. 4.3). The number of progeny in *daf-2(e1370); pep-2(lg601)* double mutants showed a stronger decrease compared to each of the single mutants (Fig. 4.24; Tab. 4.3). Finally, the brood size of *daf-16(m26); daf-2(e1370); pep-2(lg601)* triple mutants was indistinguishable from that of *pep-2(lg601)* single mutants (Fig. 4.24c; Tab. 4.3), thus

the *daf-2(e1370)* caused phenotype in *daf-2(e1370); pep-2(lg601)* double mutants could be completely suppressed by the *daf-16(m26)* allele.

Tab. 4.3 Brood sizes and fertile periods for *pep-2, daf-2* and *daf-16* single, double and triple mutants at 20°C.

Genotype	Brood size	% wild type	Fertile period (days)
wild type	316.6 ± 21.5 (n = 54)	100	5
daf-16(m26)	292.6 ± 27.5 (n = 28)	92	4
pep-2(lg601)	109.3 ± 13.5 (n = 79)	35	9
daf-16(m26) pep-2(lg601)	70.9 ± 10.1 (n = 10)	22	9
daf-2(e1370)	210.0 ± 23.2 (n = 30)	66	8
daf-16(m26);daf-2(e1370)	260.8 ± 13.7 (n = 18)	82	5
daf-2(e1370);pep-2(lg601)	51.6 ± 5.1 (n = 32)	16	10
daf-16(m26);daf-2(e1370);pep-2(lg601)	113.6 ± 9.4 (n = 20)	36	9

In addition to the total number of progeny, the duration of the egg-laying (fertile) period was also altered in these mutants (Tab. 4.3). *daf-16(m26)* mutants had a fertile period of five days, like the wild type animals. In *daf-2(e1370)* mutants this period was extended to eight days, compared to nine days in the *pep-2(lg601)* mutant animals. The *daf-16(m26); daf-2(e1370)* double mutants exhibited an egg-laying period like wild type, indicating that the extended fertile period in *daf-2(e1370)* mutants was suppressed by *daf-16(m26)* (Tab. 4.3). In contrast, the *daf-16(m26); pep-2(lg601)* double mutant animals still exhibited a prolonged fertile period like *pep-2(lg601)* single mutants. In *daf-2(e1370); pep-2(lg601)* double mutant animals, the egg-laying period was slightly extended compared to the corresponding single mutants (Tab. 4.3), and in the *daf-16(m26); daf-2(e1370); pep-2(lg601)* triple mutant, the *pep-2(-)* egg-laying period of nine days was restored. These data indicate that *pep-2* and *daf-2* act in parallel pathways to control fertility in *C. elegans*.

4.3.2.4 Life span analyses

To investigate a possible interaction between peptide transport and insulin-signalling in the ageing process, mutants were tested for survival under normal growth conditions. First, life span experiments were performed at 20°C. *daf-2(e1370)* is known to have a longevity phenotype at 15, 20 and 25.5°C (Larsen et al. 1995; Lin et al. 2001) and *daf-16(m26)* mutant animals were described to be short-lived

(Vanfleteren and Braeckman 1999; Lin et al. 2001). The results in this work correspond to the previously published data. *daf-2(e1370)* mutant animals exhibited a 1.6-fold increase of life span and *daf-16(m26)* mutant animals were short-lived compared to wild type (Fig. 4.25; Tab. 4.4).

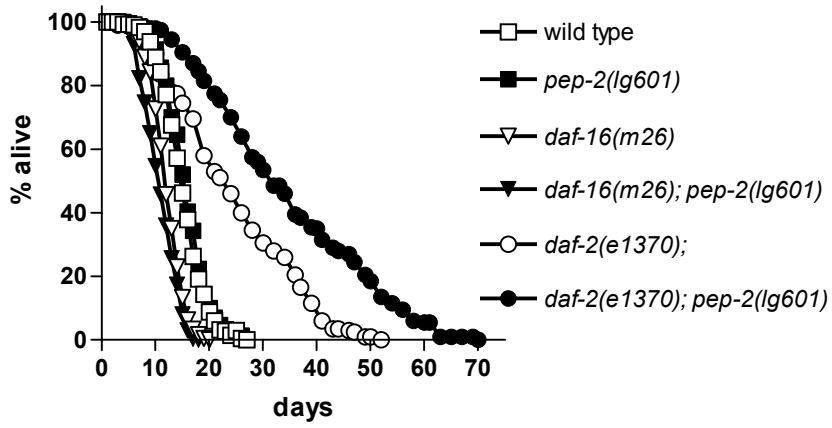

Fig. 4.25 Survival curves for *pep-2*, *daf-2*, *daf-16* and double mutant adults at 20°C.

In daf-*2(e1370); pep-2(lg601)* double mutant animals, the mean adult life span was even more enhanced than in *daf-2(e1370)* single mutants and compared to wild type animals the double mutants exhibited a 3.5-fold increase in adult life span. d*af-16(m26); pep-2(lg601)* double mutants behaved like *daf-16(m26)* single mutant animals (Fig. 4.25; Tab. 4.4), thus *pep-2(lg601*) had no effect on the reduced life span of *daf-16(m26)*.

Tab. 4.4 Adult life span of *pep-2*, *daf-2*, *daf-16* and double mutants at 20°C

Strain	Mean life span	% wild type	Maximum	N[a]
Wild type	13.6 ± 0.5	100	25	500
pep-2(lg601)	14.2 ± 1.9	104	26	250
daf-2(e1370)	21.8 ± 0.1	160	52	150
daf-16(m26)	10.4 ± 0.3	76	20	300
daf-2(e1370);pep-2(lg601)	31.9 ± 0.6	235	71	150
daf-16(m26);pep-2(lg601)	9.3 ± 0.5	68	20	300

A *daf-16(m26); daf-2(e1370); pep-2(lg601)* triple mutant was constructed to see whether the life span extension in the *daf-2(e1370); pep-2(lg601)* double mutant is dependent on a functional *daf-16* or not. Life span experiments were performed at 25°C, the restricted temperature of the *daf-2(e1370)* mutant allele. The results are shown in Fig. 4.26 and Tab. 4.5.

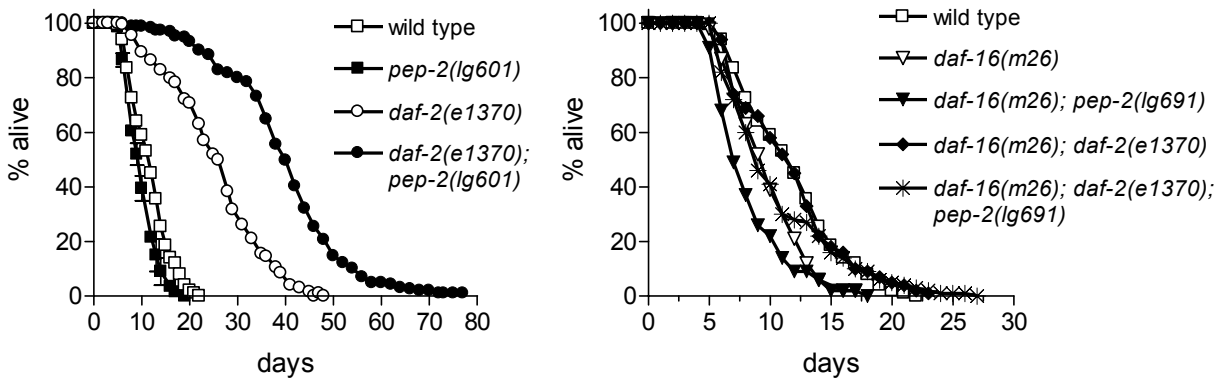

Fig. 4.26 Survival curves for *pep-2*, *daf-2*, *daf-16*, double and triple mutant adults at 25°C.

Tab. 4.5 Adult life span of *pep-2*, *daf-2*, *daf-16*, double and triple mutants at 25°C.

strain	Mean life span	% wild type	Maximum	N[a]
Wild type	11.0 ± 1.4	100	21	200
pep-2(lg601)	9.0 ± 0.2	82	18	200
daf-2(e1370)	24.3 ± 2.7	221	46	197
daf-16(m26)	8.9 ± 2.4	81	17	100
daf-16(m26);daf-2(e1370)	10.9 ± 3.6	99	23	100
daf-16(m26);pep-2(lg601)	7.4 ± 2.3	67	17	100
daf-2(e1370);pep-2(lg601)	38.3 ± 1.1	348	77	187
daf-16(m26);daf-2(e1370);pep-2(lg601)	9.8 ± 3.7	89	26	100

At 25°C, *daf-2(e1370)* mutant animals exhibit a strong longevity phenotype with a 2.2-fold increase compared to wild type. *pep-2(lg601)* mutants showed the same decrease in life span like *daf-16(m26)* mutant animals (Fig. 4.26; Tab. 4.5). In daf-*2(e1370); pep-2(lg601)* double mutants, the mean adult life span was increased 3.5-fold compared to wild type and 1.6-fold compared to the *daf-2(e1370)* single mutant. All life span extensions seen in this experiments were suppressed by *daf-16(m26)*, thus all double or triple mutants in *daf-16(m26)* mutant background show wild type-like or shortened life span (Fig. 4.26; Tab. 4.5).

4.3.2.5 Determination of heat tolerance

Ageing *daf-2* mutants have higher levels of catalase and superoxide dismutase relative to wild type, and the difference increases with age (Larsen 1993; Vanfleteren 1993; Vanfleteren and De Vreese 1995). Accordingly, they become hyperresistant to hydrogen peroxide (Larsen 1993) and the superoxide generator paraquat (Vanfleteren 1993). Subsequently, Lithgow et al. (Lithgow et al. 1994; Lithgow et al. 1995) demonstrated that *daf-2* mutant animals exhibit a significantly increased intrinsic thermotolerance and they discovered that essentially all long-lived mutants in the *daf-2* signalling pathway were hyperresistant to oxidative, heat, or ultraviolet stress. To investigate, if the longevity phenotype caused by *pep-2(lg601)* in the *daf-2(e1370)* mutant background is due to an increased stress resistance, the heat tolerance of the animals was tested. Young adults (t = 0) were incubated at 35°C and the survival of the animals was scored every two hours. The survival curve is shown in figure Fig. 4.27. Surprisingly, the *pep-2(lg601)* single mutant animals exhibited an increased heat tolerance compared to wild type even though they do not show a longevity phenotype. The increased thermo tolerance in *daf-2(e1370)* animals could be further enhanced in the *pep-2* mutant background, corresponding to the life span phenotype.

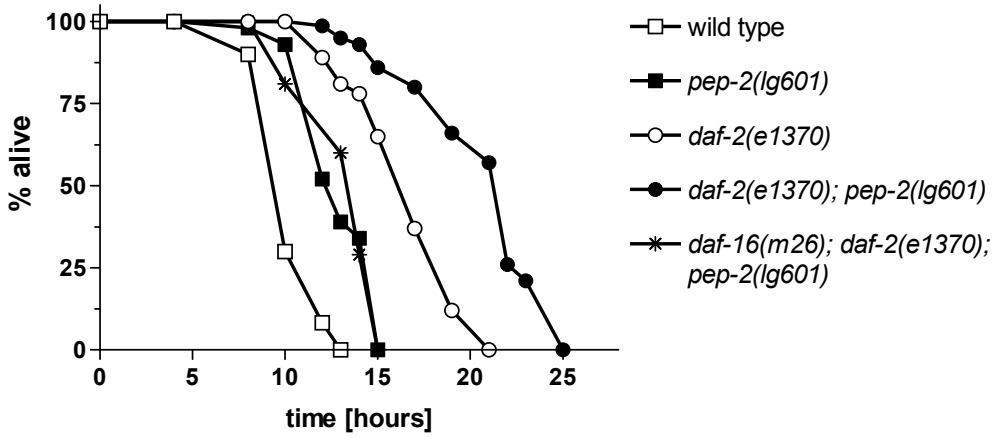

Fig. 4.27 Thermotolerance of *pep-2*, *daf-2*, *daf-2; pep-2* and *daf-16; daf-2; pep-2* mutant animals. Survival curve of young adult animals at 35°C.

After incubating the animals for 14 hours at 35°C, all wild type animals were dead, but 34% of *pep-2(lg601)*, 78% of *daf-2(e1370)*, 93% of *daf-2(e1370); pep-2(lg601)* and 29% of *daf-16(m26); daf-2(e1370); pep-2(lg601)* animals were still alive. The maximal survival of young adult wild type animals at 35°C (13h) was slightly

extended in *pep-2(lg601)* and *daf-16(m26); daf-2(e1370); pep-2(lg601)* (15 hours both; 1.17-fold increase compared to wild type). In *daf-2(e1370)* mutant animals, the maximal survival is extended 1.58-fold (21h) and in *daf-2(e1370); pep-2(lg601)* 2.92-fold (25h) compared to wild type. The results of the *daf-16(m26); daf-2(e1370); pep-2(lg601)* triple mutant reveals that only the increased heat resistance in *daf-2(e1370)* can be suppressed by *daf-16(m26)*, but not the *pep-2(lg601)* caused phenotype.

4.3.2.6 Measurements of oxidative damaged protein

Ageing was shown to be correlated with oxidatively damaged proteins in different short- and long-lived mutants in *C. elegans* including *daf-2* and *daf-16* (Yasuda et al. 1999). To test whether similar mechanisms are responsible for the pronounced life span extension observed in *daf-2(e1370); pep-2(lg601)* animals, the protein-carbonyl content in *daf-2(e1370)*, *pep-2(lg601) and daf-2(e1370); pep-2(lg601)* mutants during aging was measured (Fig. 4.28).

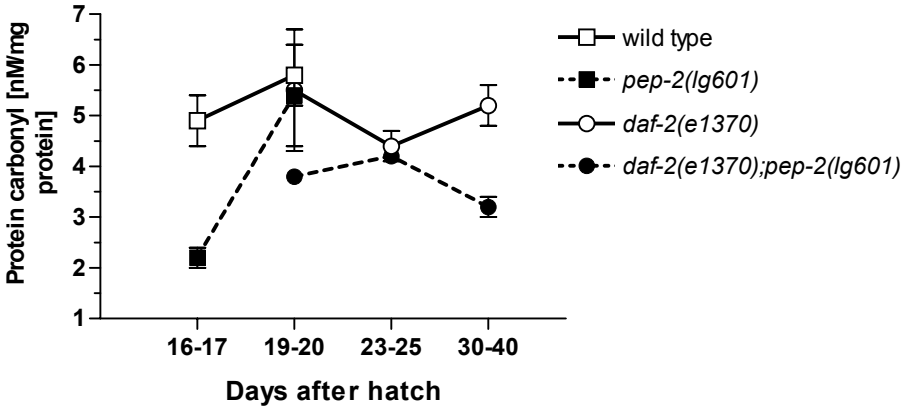

Fig. 4.28 Accumulation of oxidative damaged proteins in wild type, *pep-2*, *daf-2* and *daf-2; pep-2* mutant animals. Wild type and *pep-2(lg601)* animals were collected and measured only until day 20 after hatching, because it was not possible to get enough living animals at older stage. The data shown represent mean ± SEM from each time period.

At days 16-17 post-hatching, the protein carbonyl content in *pep-2(lg601)* mutant animals (2.2 ± 0.2 nmol/mg protein; n = 2) was significantly reduced compared to wild type (4.9 ± 0.5; n = 5). At days 19-20 post-hatching the level of oxidatively damaged protein was only slightly reduced in *pep-2(lg601)* (5.4 ± 1.0 nmol/mg protein; n = 2) and *daf-2(e1370)* (5.5 ± 1.2; n = 6) compared to wild type (5.8 ± 0.6; n = 9), but in *daf-2; pep-2* double mutant animals the accumulation of oxidative

damaged proteins was very low (3.8 ± 0.1; n = 3). *daf-2(e1370)* and *pep-2 (lg601)* animals analysed 23-25 days post-hatching showed a comparable amount of protein-carbonyl (4.4 ± 0.3 nmol/mg protein; n = 3 and 4.2 ± 0.1; n = 4 respectively), but in older animals (30-40 days post-hatching), the protein-carbonyl content was increased in *daf-2(e1370)* but not in *daf-2; pep-2* double mutant animals (5.2 ± 0.4; n = 3 and 3.2 ± 0.2; n = 5 respectively). These preliminary data suggest that the accumulation of oxidatively damaged proteins is delayed in the *pep-2(lg601)* mutant background.

4.3.3 Interaction between *pep-2* and the TOR signalling pathway

The TOR (Target of Rapamycin) proteins are members of the phosphatidylinositol 3-kinase (PI3K) superfamily and part of a signalling cascade that senses the cellular amino acid pool and regulates transcription, translation and protein degradation (Chou and Blenis 1995; Hara et al. 1998). Elimination of *C. elegans* TOR (encoded by *let-363*) results in developmental arrest at mid-to-late L3, accompanied by an increase in the gut lumen size and a compromise in the intestine's ability to digest and absorb nutrients (Long et al. 2002). To investigate a possible interaction between *pep-2(lg601)* and TOR-signalling, a *let-363/TOR(RNAi)* mutant was used for epistasis analysis with *pep-2(lg601)*. The recently developed technique of introducing RNAi into *C. elegans* through feeding worms bacteria expressing double-stranded RNA (dsRNA) (Kamath et al. 2001) provides a convenient way to examine the effect of disrupting a gene-of-interest on animal development and physiology. For the RNAi-through-feeding experiments in this work, the partial *let-363* cDNA from yk18c10 was cloned into the vector pPD129.36 (the plasmid was a gift from A. Gartner, Munich). The *let-363(RNAi)* mutant animals were analyzed in the *pep-2(lg601)* mutant background for development, fertility and intestinal phenotype.

4.3.3.1 Developmental arrest

To investigate the developmental arrest phenotype of *let-363* mutants, parent (P0) wild type and *pep-2(lg601)* animals were fed with *let-363* RNAi-producing bacteria starting in L3 larval stage and transferred to fresh plates every day for 3-5 days, depending on the strain. The F1 generation was observed and analysed for

developmental arrest for three to nine days, depending on the strain. In contrast to the *let-363(h111)* mutant allele (Long et al. 2002), *let-363(RNAi)* mutants developed to fertile adults like wild type animals (Fig. 4.29). However, the *let-363(RNAi); pep-2(lg601)* double mutant strain exhibited a strong developmental arrest at the L2 to L3 larva stage, which was neither observed for the *pep-2(lg601)* nor the let-363(RNAi) single mutants (Fig. 4.29). The *let-363(RNAi)* mutant strain did not arrest after feeding with *let-363* RNAi-producing bacteria for three generations.

Fig. 4.29 *pep-2* and *let-363*/TOR interact to cause developmental arrest. *let-363(RNAi)* animals develop like wild type (top) and reach the L4 larva stage 48 hours post egg-laying (PEL). *let-363(RNAi);pep-2(lg601)* double mutants arrested in larval development. 120 hours PEL the *pep-2(lg601)* single mutant animals developed to adults, the double mutants are still alive but arrested in L2 to L3 larval stage. Scale bars: 0.1mm

4.3.3.2 Brood size measurements

For brood size experiments, P0 wild type and *pep-2(lg601)* animals were placed on plates seeded with *let-363* RNAi-producing bacteria in adulthood and the brood size of F1 animals was counted. The progeny laid within 24 hours after transferring the P0 animals on RNAi-plates were used for brood size determinations to avoid developmental arrest in the *let-363(RNAi); pep-2(lg601)* double mutant animals. Since the let-363(RNAi) single mutants do not arrest, the brood size was measured for two generations on the RNAi-producing bacteria. The brood size of *let-363(RNAi)* mutant animals was reduced from 316.6 ± 21.5 (n = 54) in wild type to 263.9 ± 18.6 (n = 18, p = 0.18) and 251.6 ± 16.8 (n = 10, p = 0.21) in the first and second generation, respectively (Fig. 4.30). In addition, five of 15 analyzed F2 animals developed to sterile adults, thus excluding them from the brood size calculation. In

let-363(RNAi); pep-2(lg601) double mutants the number of progeny was significantly reduced form 109.3 ± 13.5 (n = 79) *in pep-2(lg601)* to 19.2 ± 4.0 (n = 20) in *let-363(RNAi); pep-2(lg601)* mutant animals (p = 0.01) (Fig. 4.30).

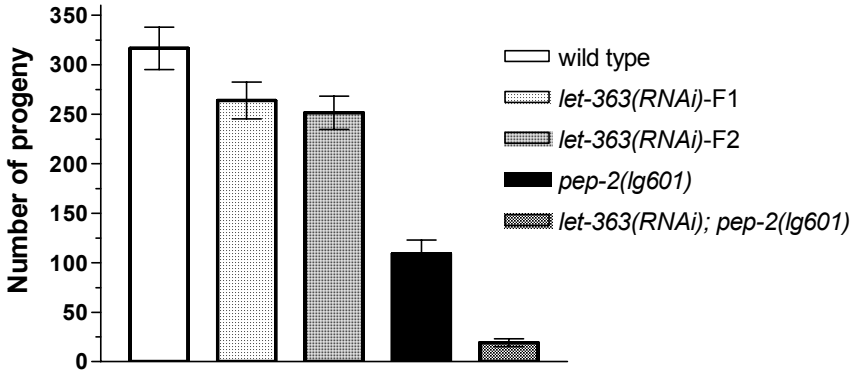

Fig. 4.30 *pep-2* and *let-363*/TOR interact to reduce fertility. Brood size of *pep-2*, *let-363(RNAi)* and double mutant animals at 20°C. F1: first generation feed on RNAi-producing bacteria; F2: second generation feed on RNAi-producing bacteria.

Thus, the brood size in the double mutant is decreased to 17.6 % compared to the *pep-2(lg601)* and to 7.3 % compared to the *let-363(RNAi)* single mutant.

4.3.3.3 Intestinal phenotype

let-363/TOR deficiency was shown to cause progressive intestinal atrophy and intestinal lumen enlargement in *C. elegans* (Long et al. 2002). The *let-363(RNAi)* mutant used in this work displayed just a weak enlargement of the intestinal lumen, but it is, like all aspects of the *let-363(RNAi)* phenotype, strongly enhanced in the *pep-2(lg601)* mutant background (Fig. 4.31).

In *let-363(RNAi)* mutant animals, the intestinal lumen was enlarged in L4 larva (48h PEL) compared to the wild type control (Fig. 4.31a). In adult animals (70h PEL), this phenotype nearly disappears, the intestinal lumen is scarcely wider than in the wild type control (Fig. 4.31a). This phenotype was also strongly enhanced in the *pep-2* mutant background. The intestinal lumen of *let-363(RNAi), pep-2(lg601)* double mutants enlarged progressively and by days 5-8 PEL, the intestinal cells are reduced to a thin layer around the wide lumen. In addition, clumps of half-digested or whole bacteria were visible in the lumen fluid of the double mutant animals (Fig. 4.31b).

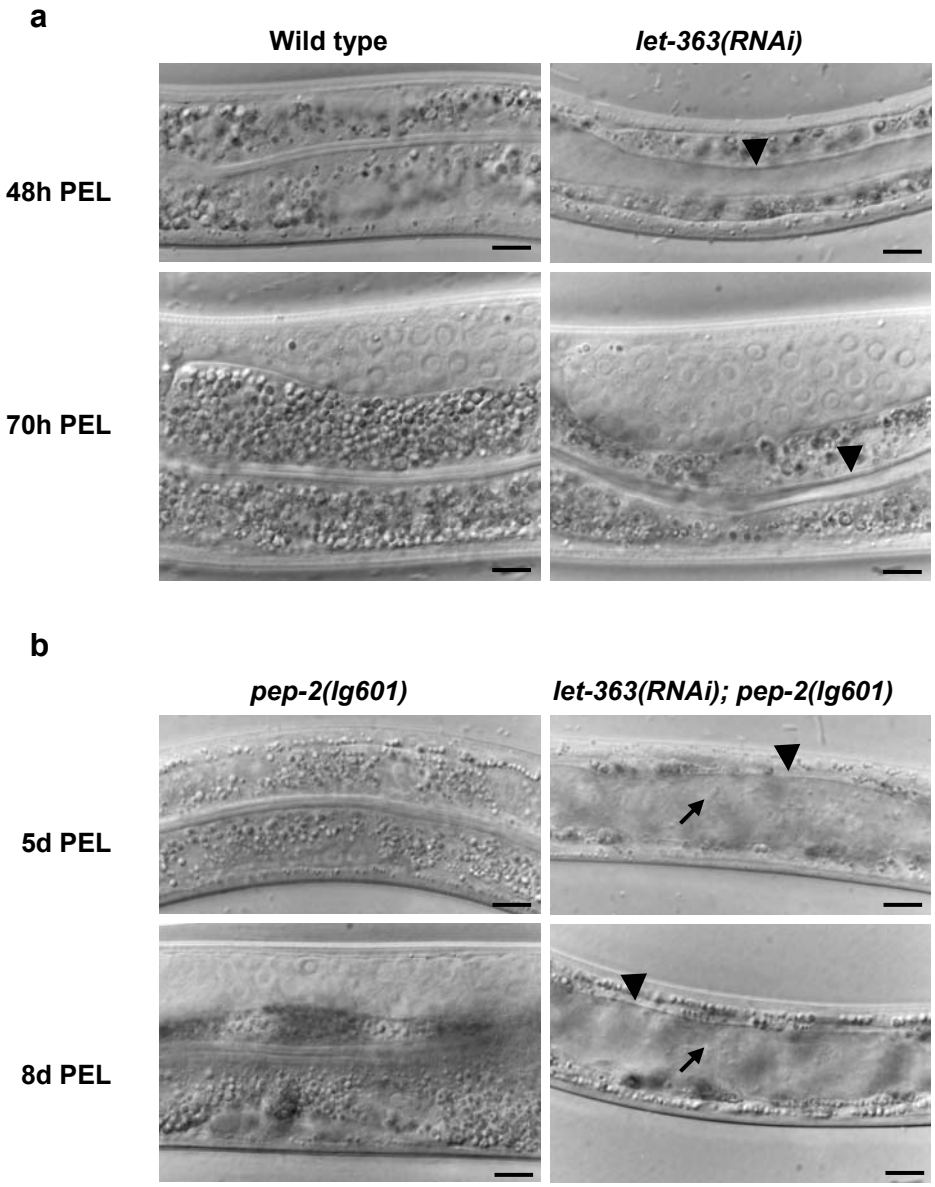

Fig. 4.31 Intestinal phenotype in *pep-2*, *let-363*/TOR and the double mutant animals. Pictures display a part of the intestine of individual animals. (a) *let-363RNAi* mutant animals 48 and 70 hours PEL display a more or less weak enlargement of the intestinal lumen (arrowhead). (b) *let-363(RNAi); pep-2(lg601)* double mutants exhibit a more severe intestinal phenotype at 5 and 8 days PEL. The intestinal lumen is extensively enlarged (arrowhead) and whole bacteria are visible in the lumen (arrows). PEL: post-egg laying. Scale bars: 20µm

5 Discussion

Although the function of mammalian peptide transporters has been studied extensively *in vitro*, less is known about the physiological importance of the proteins in living organisms. The intestinal isoform is thought to mediate uptake of di-and tripeptides from luminal protein break-down but its contribution to overall amino acid absorption in the gut and protein nutrition *in vivo* remained unknown. In 1998, two *C. elegans* oligopeptide transporters have been cloned and characterized by functional expression in *Xenopus leavis* oocytes and shown to resemble the two isoforms found in mammals (Fei et al. 1998).

In the present work, the model organism *C. elegans* was used to study the transporter proteins *in vivo* by analysing

- the expression pattern by generating transgenic animals that express reporter genes under the control of the specific promoters.
- the phenotype of deletion mutant strains for developmental and reproductive defects
- the interplay of transporter-dependent amino acid nutrition and signalling pathways involved in protein homeostasis employing animals with different genetic backgrounds.

5.1 Expression and function of proton-coupled peptide transporter in *C. elegans*

PEP-2, the functional homologue of the mammalian PEPT1, is expressed in intestinal cells throughout all developmental stages of *C. elegans*, starting in embryogenesis. This expression pattern is consistent with the proposed physiological function of PEP-2 in the intestinal uptake of di- and tripeptides derived from digestion of dietary proteins. An expression was also observed in the ASI sensory neurons in the head. This pair of neurons is known to be important for the transmission of environmental cues necessary for proper development of the animal, suggesting a possible link of *pep-2* to theses processes.

PEP-1 shows a much broader expression and tissue distribution, similar to its mammalian homologue PEPT2. The *pep-1* promoter-driven reporter construct is expressed in several tissues of *C. elegans* including vulva and nerve cord. A strong

neuronal expression is reminiscent of the tissue distribution of the mammalian PEPT2 protein, which has been identified in the rat peripheral and central nervous system. The function of this proton-coupled peptide transporters in neuronal cells remains to be determined.

To analyse the function of the *C. elegans* peptide transporter *in vivo*, deletion mutant strains for *pep-1* and *pep-2* were generated. The *pep-1(lg501)* mutant allele lacks 2.5 kb including 1269 bp of the promoter sequence, the translational start codon and the first six exons. The *pep-2(lg601)* mutant allele lacks 1.7 kb, including 257 bp of the promoter sequence, the translational start codon and the first six exons. Even if the proteins were produced in the mutants, they would lack most of the essential amino acid residues and domains known to be important for substrate binding and transport, mainly located in the N-terminal part. Thus, the reported mutant alleles most likely represent loss-of-function or null alleles.

To investigate the function of the *C. elegans* peptide transporter proteins in the intestine, both mutant strains were tested for the uptake of β-Ala-Lys-AMCA, a fluorescent model substrate, in feeding trials. Efficient uptake of the reporter molecule in *pep-1(lg501)* mutant animals was shown by a strong fluorescent staining in all intestinal cells as in wild type animals. In contrast, *pep-2(lg601)* mutant animals lacked this staining. The fluorescence was here detectable only in the intestinal lumen, indicating normal ingestion but lack of absorption of the reporter molecules. This experiment reveals that only a functional PEP-2 but not a PEP-1 protein is necessary for the uptake of β-Ala-Lys-AMCA and other di-/tripeptides across the apical membrane into the intestinal cells. Thus, PEP-1 seems not to be functional in the intestine and there is no redundancy of *pep-1* and *pep-2* with respect to the intestinal peptide transport in *C. elegans*.

Since the *pep-2(lg601)* mutant strain is viable, the intestinal peptide transport via PEP-2 is not essential for life, but the mutant animals show strong developmental and reproductive defects as analysed by various assays. The homozygote *pep-2(lg601)* mutant animals are smaller and display a prolonged postembryonic development compared to wild type animals. These data suggest that the intestinal peptide transporter PEP-2 represents a very important delivery system for peptide-bound amino acids during periods of a high demand for amino acids such as during growth and development. In addition, the *pep-2(lg601)* mutant strain exhibits strong defects in reproduction. It was shown that in *pep-2(-)* animals the number of eggs laid

per hour and the number of eggs in the uterus is decreased, and the egg-laying period is strongly extended. Since the oocytes in the mutant hermaphrodites accumulate as much yolk protein as the wild type control, it seems likely that the accumulation of yolk in the oocytes takes longer than in wild type animals. In addition, the total amount of progeny in *pep-2(lg601)* animals is reduced to 35% of that in wild type animals, indicative of a limited availability of amino acid nitrogen for the production of progeny. The limited fertility of *pep-2* males shown by a male mating test may be explained by a delayed or decreased production of sperm caused by amino acid deprivation. Since the feeding behaviour, measured by pharyngeal pumping and defecation cycle, is not affected in the mutant animals, the missing intestinal absorption of amino acid nitrogen via the peptide transporter seems to be the only limiting factor. Although the brush border transporters for free amino acids could provide sufficient essential amino acids for life, they obviously can not compensate for the lack of PEP-2 function to enable proper development, growth and reproduction.

To examine if a higher availability of free amino acids may prevent the phenotype in mutants lacking the intestinal peptide transporter, *pep-2(lg601)* animals were fed on amino acid supplemented agar plates. The supplemented mutant animals exhibited a partial rescue of the brood size and developmental delays. Therefore, when the dietary availability of free amino acids is increased, uptake via the different amino acid transporters can compensate partially the missing peptide transporter activity. Nevertheless, this is not sufficient to restore the wild type situation. This could be due to the insufficient activity of amino acid transporters in the intestinal membrane, or the insufficient ingestion of the supplemented amino acids into the gut. Interestingly, the rescue for the progeny production with an increase of 50% compared to *pep-2(lg601)* mutant animals without supplementation is much higher than the rescue achieved in the delayed postembryonic development. Even in the second generation of amino acid supplemented animals, the prolonged generation time in *pep-2(-)* animals was decreased only by about 10% compared to the non-supplemented animals. This might reflect an even higher demand for amino acid nitrogen during development as compared to reproduction.

To demonstrate finally that the deletion in the *pep-2* gene is responsible for the phenotype described, transgenic mutant animals carrying an extrachromosomal copy of the *pep-2* wild type gene were constructed. Two transgenic lines were analyzed for

β-Ala-Lys-AMCA staining and generation time and it was shown that a wild type copy of the *pep-2* gene is sufficient to rescue the *pep-2* mutant phenotype. Since an extrachromosomal array result in a mosaic expression in the animal, a complete reconstitution of the wild type situation is improbable.

There are several possible explanations for the observed phenotype of *pep-2(lg601)* mutant animals. The defects described above may be caused by a limited availability of amino acids as energy substrates resulting in caloric restriction, or a more specific effect by an insufficient supply of essential amino acids for protein synthesis. The consequences of a limited amino acid intake for the ageing process in an organism have not been addressed before, but it is known that restricting energy or glucose availability or perturbing the underlying signalling pathways prolongs lifespan in eukaryotes (Lin et al. 2000). The observation that a caloric restriction in rodents can modify the rate of ageing has been demonstrated repeatedly since the first report of McCay in 1935 (McCay et al. 1935) and the increase in life span was shown for a variety of animals (Weindruch and Walford 1988). About 25 years ago it was reported that life span of *C. elegans* can be extended by about 50% by growth in liquid cultures with relatively low concentrations of bacteria (Klass 1977). In addition, life span is known to be coupled to the rate of feeding in *C. elegans*. Some *eat* mutants that ingest bacteria less efficiently than wild type, which mimicks caloric restriction, live up to 50% longer (Lakowski and Hekimi 1998). Since *pep-2(lg601)* animals do not show a significant change in adult life span as compared to wild type or *eat-2(ad453)* as a positive control, the abolished intestinal peptide uptake does seemingly not cause a caloric restriction which is severe enough to lengthen life span.

The *pep-1(lg501)* mutant strain was analyzed for generation time and brood size. Neither in the single mutant strain nor in the *pep-1(lg501); pep-2(lg601)* double mutant, the deletion of *pep-1* did show any effect. These results lead to the conclusion that PEP-1, in contrast to PEP-2 is not necessary for proper growth, development and reproduction in *C. elegans*.

5.2 Function of *pep-2* in development, metabolism and aging

C. elegans has a dynamic ability to sense and respond to its environment, often integrating multiple sensory signals to generate one response. For example, dauer formation is induced by conditions of high dauer pheromone levels (crowding), starvation, and high temperature and is regulated by the activity of the chemosensory neurons ASI, ADF, and ASJ. DAF-7, a TGF-ß homologue, is constitutively expressed in the ASI neurons, to maintain normal reproductive growth. At high pheromone levels, the expression of *daf-7* is significantly downregulated and laser ablation of the ASI neurons was shown to cause dauer formation (Bargmann and Horvitz 1991). In addition, the metabolic dysregulation in *tph-1* mutants, which are defective in serotonin synthesis, is, in part, due to the downregulation of *daf-7* in the ASI neurons (Sze et al. 2000). Since *pep-2* was shown to be expressed in the ASI neurons it could be possible that the peptide transporter might function as a kind of food sensor and transmit signals via the DAF-7 pathway. For this reason, the expression of a *daf-7::GFP* reporter construct was analyzed in the *pep-2* mutant background. But unlike in *tph-1* mutants no reduction of *daf-7::GFP* expression was visible in the *pep-2* mutants. Thus, *pep-2* appears not to be directly involved in the regulation of the TGF-β homologue DAF-7 to control development and therefore the function of PEP-2 in the ASI neurons still remains unclear.

In *C. elegans*, a second pathway is known to regulate metabolism, the DAF-2 (Insulin/IGF-like signalling) pathway. The availability of dietary protein or amino acids is known to affect this pathway in Drosophila (Britton et al. 2002) and mammals (Takenaka et al. 2000; Gems and Partridge 2001) to regulate growth and development. On the other side, the mammalian peptide transporter PEPT1 is known to be regulated by insulin treatment (Thamotharan et al. 1999). In addition, *pep-2* was reported to be downregulated in a *daf-2(-)* mutant background (Murphy et al. 2003). To analyze a possible interaction of *pep-2* and *daf-2 in vivo*, a double mutant was generated in this work. If double mutants show a phenotype approximated by adding the strength of the single mutant phenotypes, the effect of the single mutations is additive. On the other hand, a much more intense phenotype in a double mutant defines a synergistic effect (Hekimi 2000). The analysis of the *daf-2(-); pep-2(-)* double mutant strain revealed additive and synergistic effects as compared to the single mutant phenotypes, depending on the phenotype observed.

In *daf-2(-)* animals, the brood size is reduced and generation time is enhanced at 20°C, like in the *pep-2(lg601)* mutant strain. In a *daf-2(-); pep-2(-)* double mutants, both phenotypes are significantly stronger compared to each of the single mutant strains, reflecting an additive effect. To further confirm this effect, *daf-2(-)*, *pep-2(-)* and the *daf-2(-); pep-2(-)* mutants were analyzed for suppression by *daf-16(-)*. All *daf-2(-)* mutant phenotypes are known to be dependent on a functional DAF-16 forkhead transcription factor, the main downstream target of the *daf-2* insulin-like signalling, resulting in a suppression of the *daf-2(-)* phenotype in a *daf-16(-); daf-2(-)* double mutant. This was also shown for brood size and postembryonic development in this work. In contrast, the decreased production of progeny and the increased generation time in the *pep-2(lg601)* mutant stain was not suppressed in the *daf-16(-)* background. Finally the *daf-16(-); daf-2(-); pep-2(-)* triple mutant behaves like the *pep-2* single mutant. Taken these data together leads to the conclusion that the impairments found in the *pep-2(lg601)* mutant phenotype are not directly transmitted via the *daf-2* insulin-like signalling pathway since they are not dependent on a functional DAF-16. Thus, *pep-2* is acting in parallel to *daf-2* to control development and reproduction (Fig. 5.1).

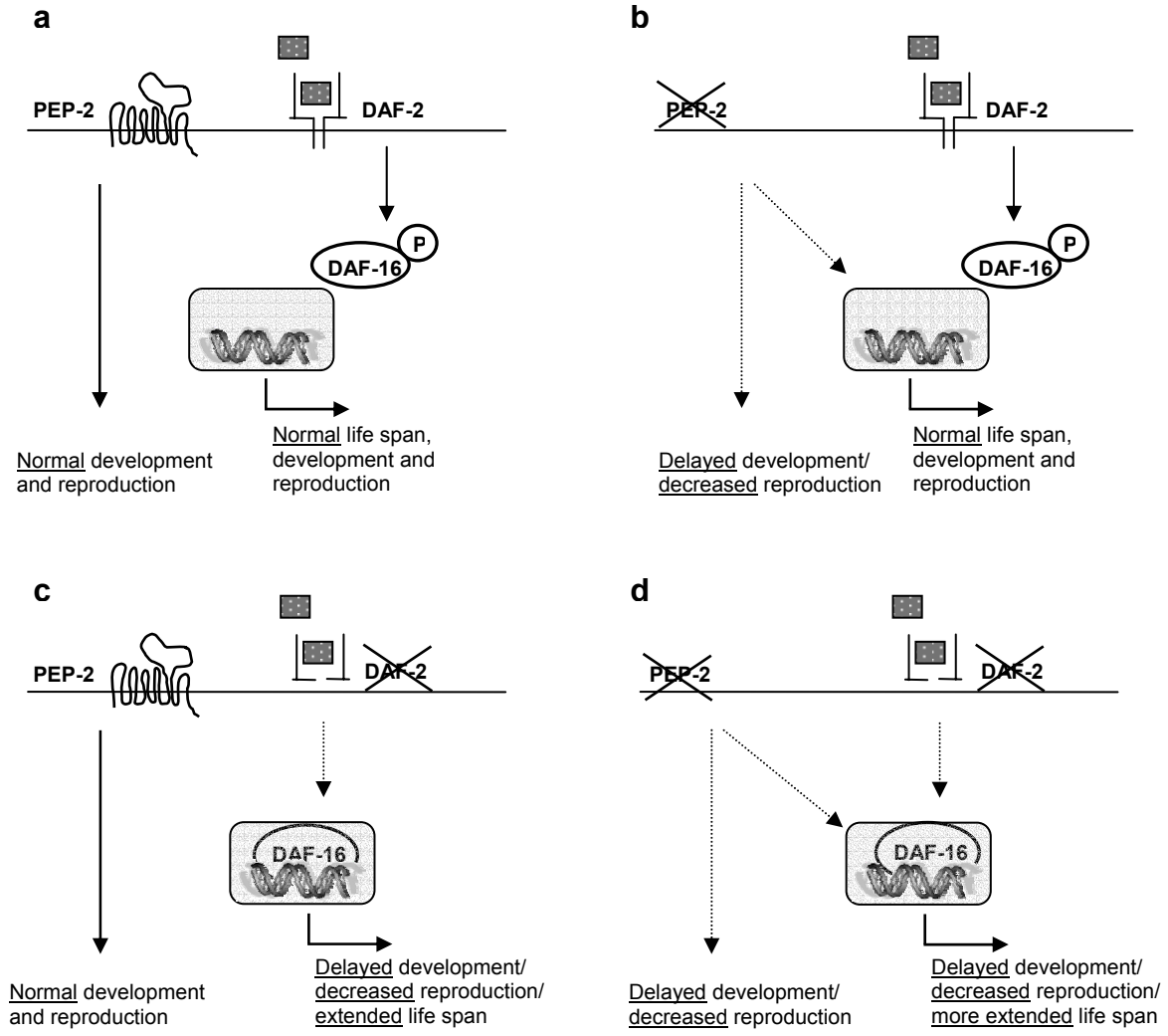

Fig. 5.1 Model for additive and synergistic effects of the *pep-2(lg601)* and *daf-2(e1370)* mutant phenotypes. (**a**) wild type situation: functional PEP-2 and DAF-2 proteins, DAF-16 is phosphorylated thus excluded from the nucleus; no effect on development, reproduction and life span. (**b**) *pep-2(-)* situation: loss of PEP-2 function results in delayed development and decreased reproduction, but normal life span. The phenotype is independent of DAF-16 function, which is still excluded from the nucleus. (**c**) *daf-2(-)* situation: DAF-16 can not be phosphorylated any more and enters the nucleus, resulting in delayed postembryonic development, decreased progeny production and an extended life span. This phenotype is dependent on a functional DAF-16 in the nucleus. (**d**) *daf-2(-);pep-2(-)* situation: development and reproduction are affected by both mutations in parallel thus the phenotypes of the single mutants add up in the double mutant. The life span is even more enhanced than in the *daf-2(-)* single mutant, representing a synergistic effect of *pep-2(-)* and *daf-2(-)*, which is dependent on a functional DAF-16.

In contrast, a synergistic effect was shown for the longevity phenotype of *daf-2(-); pep-2(-)* double mutants. Even in the absence of a significant life-span extension in *pep-2(-)* single mutants, the *pep-2* deletion causes a drastic increase in longevity in the *daf-2(e1370)* mutant background. Whereas the additive effect of *pep-2* was shown to be independent of DAF-16 function, the synergistic effect on the life span

could be completely suppressed by an additional mutation in the *daf-16* gene. Therefore, it seems likely that life span extension in *pep-2* mutant animals is only possible if there is a functional, nuclear localized DAF-16 transcription factor. In a *pep-2* single mutant, the *daf-2* signalling cascade is obviously able to phosphorylate DAF-16, resulting in inactivation by exclusion from nuclear translocation. Only in a *daf-2* mutant background the effects caused by the *pep-2* deletion can be transmitted via the active DAF-16 transcription factor in the nucleus to regulate longevity (Fig. 5.1).

To find an explanation for the life span effect seen in the *daf-2(-); pep-2(-)* double mutant strain, the animals were analysed for heat tolerance and oxidatively damaged proteins. Both parameters were shown to be directly correlated to ageing in *C. elegans*. Most of the long-lived mutant strains described before display greater resistance to one or more forms of environmental stress like oxidative stress (Vanfleteren 1993), heat (Lithgow et al. 1994) or UV light (Murakami and Johnson 1996). A positive correlation between stress resistance and life span and the role of oxidative damage and environmental stresses on aging are well studied subjects in *C. elegans* (Martin et al. 1996; Lithgow 2000; Johnson et al. 2001).

Lithgow et al. 1995 showed that mutants of the insulin-like signalling pathway (*daf-2* and *age-1*) display a higher resistance to thermal stress. To investigate a correlation between the extended life span in the *daf-2(-); pep-2(-)* double mutants and their thermoresistance, the heat tolerance for wild type, *pep-2(-), daf-2(-); pep-2(-)* and *daf-16(-); daf-2(-); pep-2(-)* has been determined in this work. This experiment revealed that the *daf-2(-); pep-2(-)* double mutants exhibited a higher thermotolerance as compared to each of the single mutants, corresponding to the life span phenotype observed for these mutant strains. Surprisingly, in *pep-2(-)* single mutants the tolerance is slightly increased even if their life span is not extended. The increased heat resistance measured in the *daf-2(-); pep-2(-)* double mutant seems to be additive rather than synergistic, in contrast to the synergistic effect shown for the ageing phenotype. Again, the *daf-16(-); daf-2(-); pep-2(-)* triple mutant strain behaves like the *pep-2(-)* single mutants. Thus, the weakly increased thermotolerance in *pep-2(lg601)* animals is not depended on a functional DAF-16 and is, therefore, independent of the *daf-2* signalling pathway. Taken these data together leads to the conclusion that an elevated thermotolerance appears to be important but not

sufficient for the pronounced life span extension caused by mutations in insulin/IGF-1 signalling pathway.

The amount of oxidatively damaged proteins is another parameter which is directly correlated with ageing in *C. elegans*. Mutants with a reduced activity of Cu/ZnSOD exhibit a higher sensitivity to the superoxide generator Paraquat and a reduced life span (Ishii et al. 1990; Ishii et al. 1994; Adachi et al. 1998). In addition it was shown that they accumulate age pigment and protein carbonyls at a faster rate than wild type, while longevity mutants do so at a slower rate (Hosokawa et al. 1994; Adachi et al. 1998; Yasuda et al. 1999). Protein carbonyls result from oxidative damage and are currently thought to be good markers for physiological age. For this reason, the amount of oxidatively damaged proteins was measured by the protein carbonyl content in wild type, *pep-2(lg601)*, *daf-2(e1370)* and *daf-2(e1370); pep-2(lg601)* strains. The *pep-2(-)* mutant animals show significant lower levels of protein carbonyl as compared to wild type at the days 16-17 after hatching, but in more aged animals (19-20 days after hatch) the amount of oxidative damaged protein reached the wild type level. This might be interpreted as a delayed accumulation of oxidative damaged proteins in *pep-2(-)* mutant animals, like the delay reported for all phenotypes demanding high protein production. Surprisingly, the protein carbonyl content in daf-2(-) single mutants at days 19-20 after hatching was as high as in wild type or *pep-2(-)* mutant animals, but decreased with further aging. This effect might be explained by the fact that there is an age-dependent increase of SOD and catalase (Larsen 1993) resulting in a higher stress response in aged animals. The *daf-2(-); pep-2(-)* double mutants exhibit a lower protein carbonyl content compared to the *daf-2(-)* single mutant in all ages, especially in the animals measured 30-40 days after hatching. The deletion of *pep-2* most likely affects the intrinsic amino acid availability and together with *daf-2* loss-of-function, this may result in reduced rates of protein synthesis, increased protein breakdown and/or an accelerated endogenous protein-turnover. One of the consequences of an accelerated protein-turnover would be a reduced accumulation of proteins damaged by radicals and other reactive oxygen intermediates.

Another pathway, which is known to be involved in cell growth and proliferation in response to nutritional cues, is the TOR (Target of Rapamycin) signalling pathway.

TOR is part of a signal transduction pathway that senses nutrients and regulates transcription, translation and protein degradation (Chou and Blenis 1995). TOR is a S/T kinase that belongs to the phospoinositide-3-kinase-related kinase (PIKK) family and is known to respond positively to the presence of amino acids by inducing an upregulation of translation through activation of ribosomal S6 kinase (p70S6K) (Dufner and Thomas 1999). S6K is another S/T kinase whose multistep activation involves TOR- and PDK1-dependent S/T phosphorylation (Chou and Blenis 1995; Dufner and Thomas 1999; Avruch et al. 2001). Since PDK-1 is regulated by insulin/IGF, both, hormonal and nutrient signals are prerequisite inputs necessary to fully activate S6K.

The phenotype of the *pep-2(lg601)* mutant strain described in this work is reminiscent of the phenotype observed in other organisms after mutating components of the TOR pathway. In Drosophila, a partial loss of TOR function was shown to reduce growth (Zhang et al. 2000) and flies deficient in the p70S6K gene are extremely delayed in development and smaller in size (Montagne et al. 1999). The elimination of TOR function in *C. elegans* (*let-363*) results in delayed development, developmental arrest at mid-to-late L3 larval stage accompanied by an increase in the gut lumen size and a compromise in the intestine's ability to digest and absorb nutrients (Long et al. 2002). To investigate possible effects of the *pep-2* deletion on the TOR signalling pathway that may be caused by a reduced availability of amino acids, a *let-363; pep-2* double mutant was analysed. Since the *let-363* null allele exhibit a severe phenotype with larval arrest and death (Long et al. 2002), a *let-363(RNAi)* mutant was used for epistasis analyses with *pep-2(lg601)*. *let-363(RNAi)* mutant animals develop into fertile adults without any delay and exhibit a very weak intestinal phenotype during larval development. In a *pep-2(lg601)* mutant background, all aspects of the *let-363(RNAi)* phenotype were strongly enhanced. First, the double mutants arrested at either L2 or L3 larval stage which is even earlier than observed for the *let-363(h111)* loss-of-function allele. In addition, they exhibited a substantially enlarged intestinal lumen that contained undigested bacteria as described for the *let-363* null mutant. This genetic approach leads to the conclusion that *pep-2* must be located in the same pathway like *let-363*. The physiological changes caused by the deletion of *pep-2* do not result in larval arrest, but they are able to enhance a weak *let-363(RNAi)* phenotype to a *let-363* loss-of-function phenotype. Thus, *pep-2* does not act in parallel to *let-363* and it seems likely that the

pep-2 mediated reduction in amino acid availability affects secondarily the TOR/S6K pathway to impair growth and development.

TOR has been implicated in the insulin/IGF network based on cell culture experiments (Scott et al. 1998; Raught et al. 2001) and in *Drosophila* it was shown that TOR is required for the growth-stimulating effect of the PI3K pathway (Zhang et al. 2000). However, this convergence is not yet evident in *C. elegans*, the data in this work suggest a connection between insulin signalling, amino acid availability and TOR signalling. A model showing the possible crosstalk between the pathways is provided in figure Fig. 5.2.

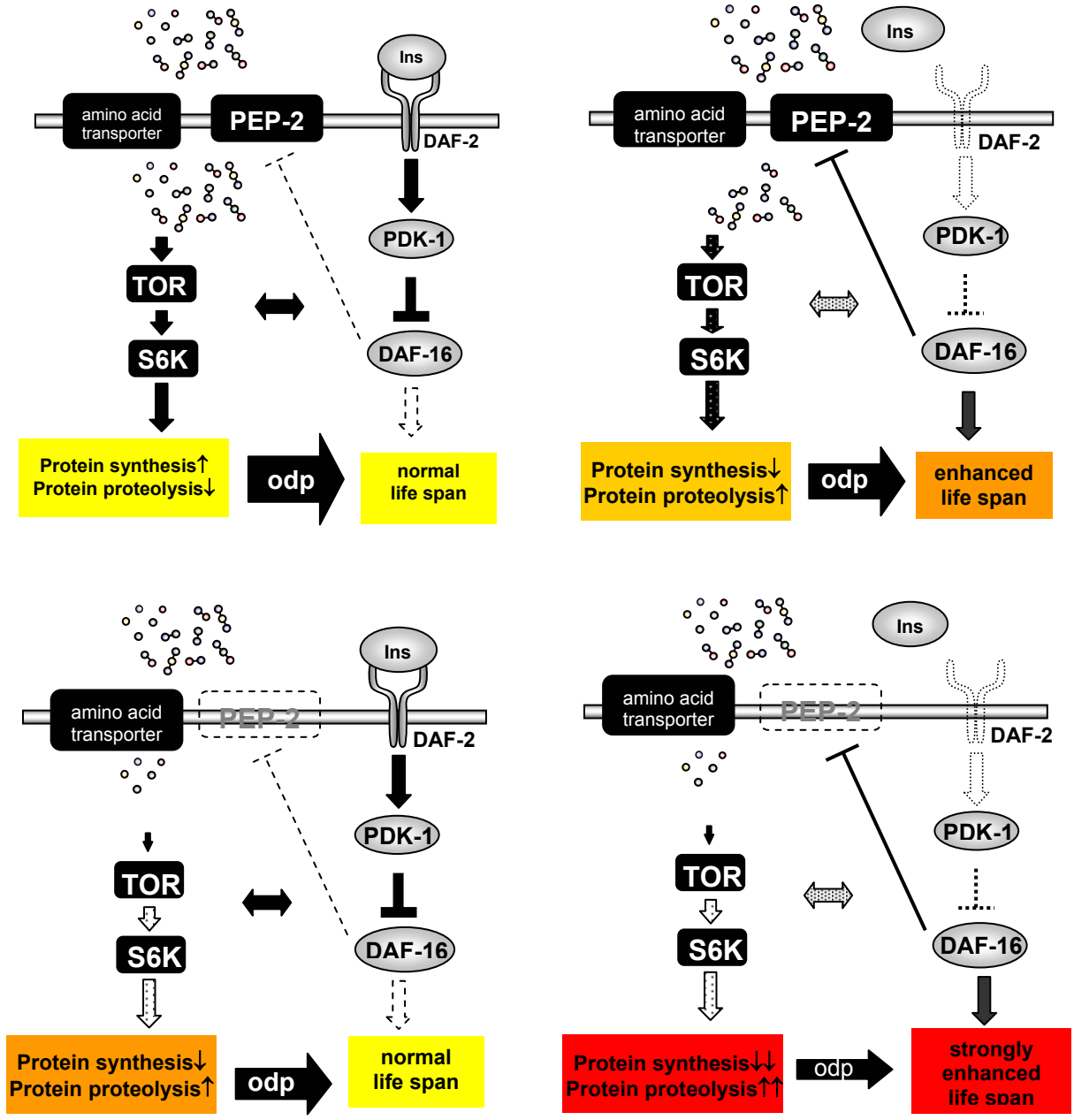

Fig. 5.2 **Crosstalk between amino acid metabolism, TOR and insulin-like signalling. Top left**: In wild type animals, amino acid and peptide transport, as well as active insulin signalling ensure normal growth, development, and reproduction. DAF-16 is inactive, resulting in normal life span. **Top right**: In *daf-2* mutant animals, the insulin signalling is perturbed, resulting in the activation of DAF-16, which regulates genes involved in development and life span. **Bottom left**: Elimination of di/tripeptide transport reduces intracellular amino acid levels, affects TOR function, and results in reduced growth, development and reproduction. Reduced amounts of oxidative damaged proteins are not sufficient to enhance life span since the genetic program is not activated (DAF-16 is inactive). **Bottom right**: In the *daf-2; pep-2* double mutant, DAF-16 is activated and executes the longevity program. *pep-2* mutant background further enhances longevity due to decreased amounts of oxidatively damaged proteins.

5.3 Outlook

Based on the present studies, the *pep-2(lg601)* mutant strain provides a very useful tool to study the phenotypical consequences in protein metabolism caused by restricted intestinal availability of amino acids. Of course, many questions including the interactions between amino acid absorption, insulin and TOR signalling, are still unanswered. Suppressor- and/or enhancer screens in a *pep-2* mutant background might reveal interesting new genes that will connect in TOR signalling and its dependency on the amino acid pool. A biochemical link between *pep-2* function and the TOR signalling pathway could be shown by measuring S6K activity (Oldham et al. 2000) in *pep-2* mutants, to verify that a decreased TOR signalling is due to the amino acid deprivation in the mutant animals.

To examine changes in protein metabolism, autophagy should be analysed in the different mutant backgrounds. Through autophagy, cells generate sufficient pools of amino acids to synthesize proteins that are essential for survival when the environmental food supply is limited. Since TOR is known to induce autophagy when the availability of amino acids is decreased, measurements of auophagy (Melendez et al. 2003) in *pep-2* mutant animals will be especially interesting.

In yeast it was shown that peptides control their membrane uptake by activating an ubiquitin-dependent proteolytic pathway (Turner et al. 2000). It therefore will be interesting to analyse the *C. elegans* orthologues involved in this pathway to assess whether an ubiquitin-dependent control of peptide uptake also exists in multicelluar organisms.

Pilot experiments for the uptake of radioactive labelled amino acids in different *C. elegans* mutant backgrounds have been performed within the scope of the present thesis. It was shown that the uptake of ^{3}H-Leucine is increased 5 to 8-fold in *pep-2(lg601)* mutant animals (Fig. 5.3a and b). In *daf-2(e1370)* mutant animals, the upregulation of ^{3}H-Leucine uptake was even higher than in *pep-2* mutants. In contrast, the uptake of ^{14}C-Lysine was decreased in both, *pep-2* and *daf-2* mutant animals as compared to the wild type control (Fig. 5.3c). These preliminary data, which should be reproduced at least some more times, deserve confirmation and interpretation. Nevertheless, these findings suggest that there could be a

compensation of the missing peptide transporter activity by upregulation of specific amino acid transporters (at least for neutral amino acids) in the *pep-2* mutant.

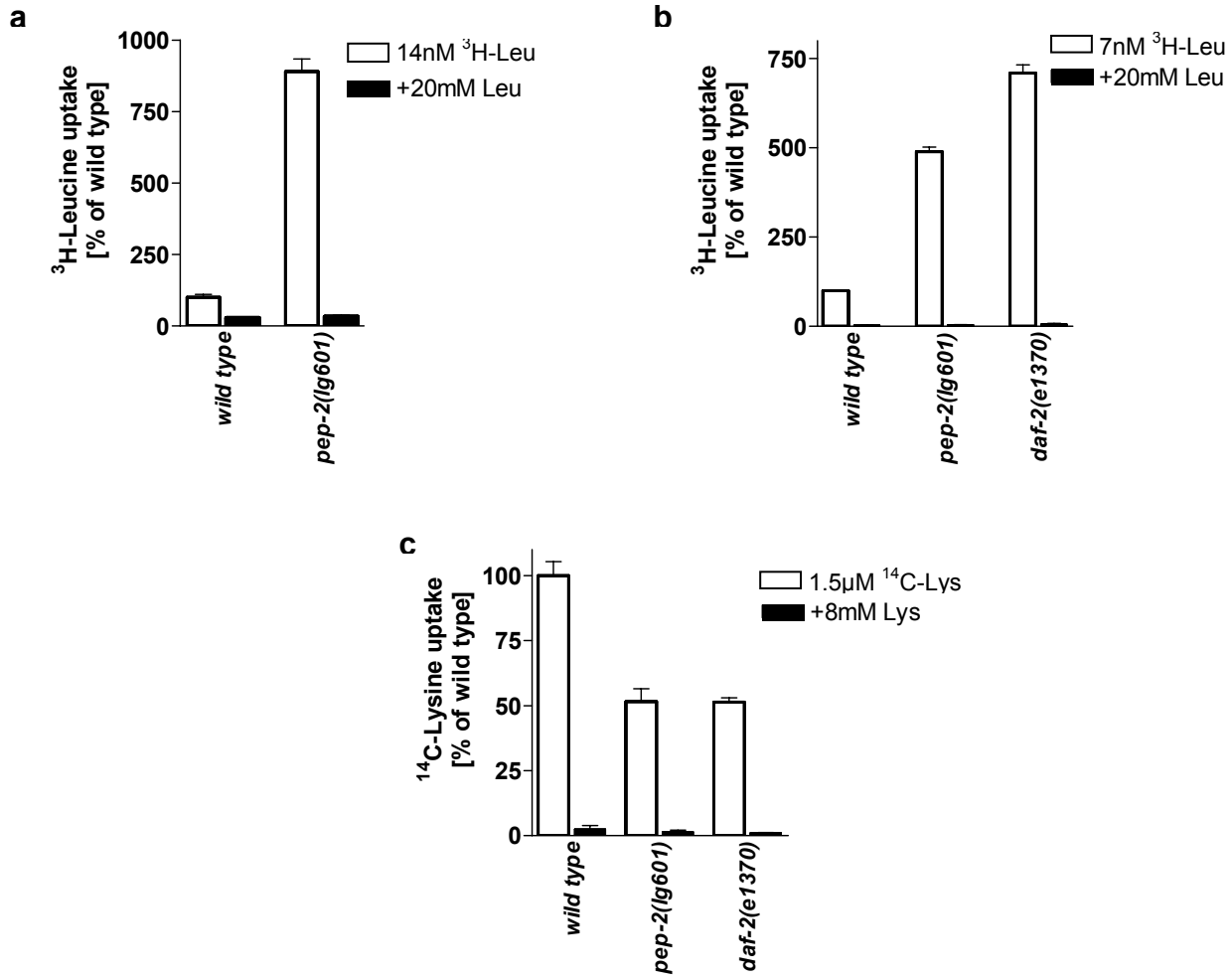

Fig. 5.3 Preliminary results of uptake studies with ^{3}H and ^{14}C labelled amino acids in wild type, *pep-2* and *daf-2* mutant animals. Data (cpm) see table 7.2 (Supplement).

For the interpretation of increased amino acid absorption rates in *pep-2* and *daf-2* it is interesting to note that AAT-1, a *C. elegans* homologue of the human large neutral amino acid transporter small subunit (hLAT1) (Fig. 5.4) encoded by F27C8.1, was shown to be a *daf-16* transcriptional target candidate (Lee et al. 2003). It could therefore be rewarding to investigate if the changes seen in the amino acid uptake can be suppressed in a *daf-16* mutant background. Finally, it might also be possible to determine the protein turnover in living animals by pulse-chase experiments with radiolabeled amino acids and to assess thereby whether the postulated changes in

protein degradation and synthesis in the transporter-deficient animals can be experimentally verified.

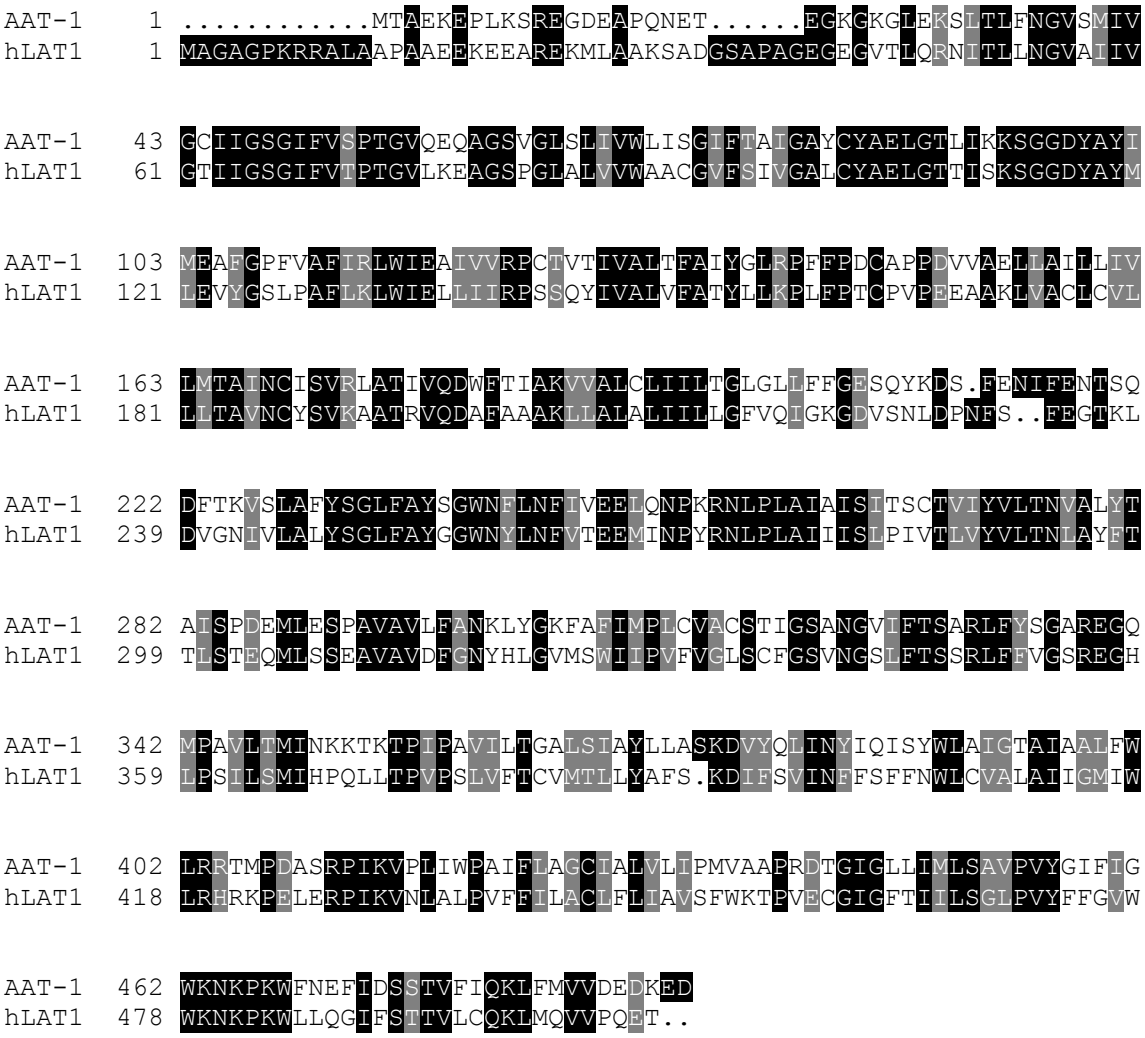

Fig. 5.4 Sequence alignment of the human large neutral amino acid transporter small subunit hLAT1 (SLC7A5) and the *C. elegans* AAT-1 protein. Identical amino acids are indicated by black boxes, similar amino acids are indicated by grey boxes. The proteins share a sequence identity of 45 % and a similarity of 62%.

Since nothing is known about the physiological function of the proton-coupled peptide transporters in neuronal cells, its role in the sensory neurons of *C. elegans* should be studied via genetic interaction with genes known to have important function in the ASI neurons. In addition, a characterization of the *pep-1* deletion mutant and/or an *opt-3* loss-of-function mutant could give some indications for the function of peptide transporters in neuronal cells.

6 Summary

The mammalian intestinal peptide transporter PEPT1 mediates the uptake of di- and tripeptides from the gut lumen into intestinal epithelial cells and acts in parallel to amino acid transporters. In this work, *C. elegans* was used to study the physiological role of the intestinal peptide transporter for overall amino acid absorption in the gut and for the delivery of protein nutrition *in vivo*. By expression pattern analysis it was shown that *pep-2*, the functional homologue of hPEPT1, is expressed in the intestinal cells and a pair of sensory neurons in the head of the animal. To study the physiological function and importance of the PEP-2 transporter *in vivo*, a *C. elegans pep-2* deletion mutant was constructed. Uptake studies with a fluorescent labelled model substrate have shown that the *pep-2(lg601)* mutant animals are incapable of taking up intact peptides from the gut lumen. This lack of intestinal peptide transport may result in a reduced availability of amino acids, which affects the TOR signalling pathway regulating protein synthesis. Indeed, the *pep-2* mutant phenotype characterized in this work is reminiscent of the phenotype observed in other organisms after mutating components of the TOR pathway. The consequences are severely retarded development, reduced body size and markedly decreased production of progeny in the *pep-2(lg601)* mutant animals. In addition it was shown that PEP-2 function directly affects the TOR signalling pathway, because the weak *let-363*/TOR (RNAi) phenotype in *C. elegans* was enhanced significantly in the *pep-2(lg601)* mutant background.

The developmental defects in *pep-2(-)* animals are not dependent on a functional DAF-16 forkhead transcription factor, the main downstream target of the DAF-2/insulin like signalling pathway. However, a crosstalk between both pathways was shown by a pronounced increase in adult life span of *daf-2(-); pep-2(-)* double mutants in the absence of an ageing phenotype in the *pep-2* single mutant. This suggests a link between the availability of dietary amino acids and the insulin/IGF signalling pathway in *C. elegans* and provides direct evidence for a predominant role of the intestinal peptide transporter not only in the delivery of amino acids for growth and development but also for signalling pathways that regulate metabolism and ageing.

7 Zusammenfassung

Der intestinale Peptidtransporter PEPT1 vermittelt die Aufnahme von Di- und Tripeptiden sowie Peptidomimetika über die apikale Membran der Darmepithelzellen in Säugetieren. Dieser Transport ist H^+-gekoppelt, sekundär aktiv und operiert parallel zu einer großen Anzahl von Aminosäuretransport-Systemen. Um die Bedeutung von PEPT1 für die Proteinversorgung eines Organismus und damit für Wachstum und Entwicklung *in vivo* zu bestimmen, wurde die Funktion des homologen Proteins PEP-2 in *C. elegans* untersucht. Mittels *pep-2* promotor-gesteuerter Reporterkonstrukte wurde gezeigt, dass *pep-2* in den Darmzellen und einem Paar sensorischer Neuronen im Kopf des Tieres exprimiert wird.

Um die physiologische Bedeutung des Transporters *in vivo* zu untersuchen, wurde ein Mutantenstamm mit einem Defekt im *pep-2* Genlocus generiert. Transportstudien mit einem fluoreszenzmarkierten Modellsubstrat konnten zeigen, dass die *pep-2* Mutante keine Dipeptide resorbieren kann. Dies könnte zu einer verringerten Verfügbarkeit von Aminosäuren im Organismus und somit zu einer veränderten Regulation der Proteinsynthese durch den TOR-Signaltransduktionsweg führen. Der Phänotyp der *pep-2* Mutante ist charakterisiert durch eine stark verzögerte postembryonale Entwicklungsphase, eine reduzierte Körperlänge und eine drastisch verminderte Nachkommenzahl, ähnlich der Phänotypen anderer Organismen mit Defekten im TOR Signalweg. Außerdem konnte gezeigt werden, dass die Funktion von PEP-2 einen direkten Einfluss auf den TOR Signalweg hat, da der sehr schwache Phänotyp einer TOR(RNAi) Mutante in *C. elegans* im *pep-2*-mutierten Hintergrund signifikant verstärkt wurde. Die Entwicklungsstörungen in der *pep-2* Mutante erwiesen sich als unabhängig von DAF-16, einem Transkriptionsfaktor der als zentrales Zielprotein des DAF-2/Insulin-/IGF-ähnlichen Signalwegs in *C. elegans* identifiziert wurde. Dennoch konnte mittels Experimenten zur Lebensdauer eine gegenseitige Beeinflussung beider Signalwege aufgezeigt werden. Die Mutation im *pep-2* Genlocus, welche per se keinen Effekt auf die Lebensdauer verursacht, führte in der *daf-2; pep-2* Doppelmutante zu einer signifikanten Verstärkung des *daf-2* Phänotyps.

Somit konnte belegt werden, dass der intestinale Peptidtansporter eine sehr wichtige Rolle bei der Bereitstellung von Aminosäuren für Wachstum und Entwicklung spielt. Weiterhin weisen die Befunde auf eine enge Verbindung des Aminosäurestoffwechsels mit dem Insulin-/IGF Signalweg und Alterungsprozessen hin.

8 Supplement

8.1 Crossing strategies

BR2513: *dpy-5(e61) daf-16(m26) unc-75(e950)*I ;*pep-2(lg601)*X
dpy-5(e61) daf-16(m26) unc-75(e950) I hermaphrodites were crossed with pep-2(lg601) males. The F2 generation was scored for Dpy, Unc and slow growing (the *pep-2* phenotype). The mutation in *pep-2* was additionally verified by PCR. The resulting strain *dpy-5(e61) daf-16(m26) unc-75(e950)*I ;*pep-2(lg601)*X was used for the generation of BR2689.

BR2751: *dpy-1(e1)*III; *pep-2(lg601)*X
*dpy-1(e1)*III hermaphrodites were crossed with *pep-2(lg601)X* males. The F2 generation was scored for Dpy and slow growing. The resulting strain *dpy-1(e1)*III; *pep-2(lg601)*X was used for the generation of BR2688.

BR2688: *daf-2(e1370)III; pep-2(lg601)*X
BR2751 hermaphrodites were crossed with *daf-2(e1370)III* males. The F2 generation was scored for non-Dpy, Daf-c and slow growing.

BR2689: *daf-16(m26)I; pep-2(lg601)*X
BR2513 hermaphrodites were crossed with *daf-16(m26)I* males. The F2 generation was scored for non-Dpy, non-Unc and slow growing.

BR3061: *daf-16(m26)I; daf-2(e1370)III; pep-2(lg601)X*
dpy-5(e61) unc-55(e1170)I; daf-2(e1370)III; pep-2(lg601)X was generated by crossing BR1472 (*dpy-5(e61) unc-55(e1170)I*) hermaphrodites with *daf-2(e1370)III; pep-2(lg601)X* males. *dpy-5(e61) unc-55(e1170)I; daf-2(e1370)III; pep-2(lg601)X* hermaphrodites were then crossed with *daf-16(m26)I; daf-2(e1370)III* males. The F2 generation was scored for non-Dpy, non-Unc and slow growing for *pep-2*. The mutation in *pep-2* was additionally verified by PCR.

BR2746: *bIs1[vit-2::gfp,pRF4,sqt-1(sc103)]; pep-2(lg601)*
bIs1[vit-2::gfp,pRF4,sqt-1(sc103)] hermaphrodites were crossed with *pep-2(lg601)X* males. The F2 generation was scored for slow-growing rollers (phenotype of the marker pRF4).

BR3059: *dbl-1(nk3)V; pep-2(lg601)X*
dbl-1(nk3) hermaphrodites were crossed with *pep-2(lg601)* males. The F2 generation was scored for slow-growing small, somewhat dumpy animals. The *pep-2* deletion was confirmed by SW-PCR.

BR3060: *ctIs40[dbl-1(+);sur-5::gfp]; pep-2(lg601)X*
ctIs40[dbl-1(+);sur-5::gfp] hermaphrodites were crossed wit *pep-2(lg601)* males. The F2 generation was scored for long, slow growing animals and the sur-5::gfp marker. The *pep-2* deletion was confirmed by SW-PCR.

BR3062: *mIs6[rol-6(su1006),daf-7p::GFP] ; pep-2(lg601)X*
mIs6[rol-6(su1006),daf-7p::GFP] hermaphrodites were crossed with *pep-2(lg601)* males. The F2 generation was scored for slow growing rollers.

8.2 Supplemental data

Tab. 8.1 Absolute data of the uptake measurements with radioactive labelled amino acids.

	Uptake of 14nM ^{3}H-Leucine per 200 animals		Uptake of 7nM ^{3}H-Leucine per 100 animals		Uptake of 1.5µM ^{14}C-Lysine per 100 animals	
	(cpm)	+ 20mM Leu (cpm)	(cpm)	+ 20mM Leu (cpm)	(cpm)	+ 8mM Lys (cpm)
wild type						
	12 001	3 644	4 608	165	4 035	153
	12 066	4 179	4 494	185	4 044	212
	16 644	4 592	4 451	114	4 183	188
	12 537	3 215	4 779	122	3 901	82
	10 895	4 140	4 901	117	4 148	40
	13 001		4 364	129	4 433	46
	22 337		4 274	72	4 900	39
					4 310	72
Mean ± SEM	**14 212 ± 3017**	**3 954 ± 420**	**4 553 ± 180**	**129 ± 26**	**4 244 ± 227**	**104 ± 60**
pep-2(lg601)						
	153 200	6 790	19 980	624	2 345	31
	133 096	3 062	23 703	147	1 788	54
	113 499	3 079	24 230	160	1 735	42
	104 719	5 033	23 524	151	2 418	42
	119 865		22 183	158	2 188	43
	130 103		21 451	249	2 449	50
	109 939		20 885	295	2 294	141
	147066				2 275	85
Mean ± SEM	**126 436 ± 13 124**	**4 491 ± 1 421**	**22 279 ± 1320**	**255 ± 117**	**2,187 ± 213**	**61 ± 26**
daf-2(e1370)						
	-	-	27 039	169	2 279	47
	-	-	36 454	123	2 297	42
	-	-	31 550	166	2 209	43
	-	-	32 995	220	2 175	40
	-	-	32 239	564	2 220	38
	-	-	32 719	555	1 971	54
	-	-	33 067		2 150	46
	-	-			2 143	
Mean ± SEM	**n.d.**	**n.d.**	**32 295 ± 1730**	**300 ± 173**	**2 181 ± 71**	**44 ± 4**

8.3 List of tables

8.4 List of figures

8.5 Abbreviations

°C	degree celcius
µg	microgram
µl	microlitre
µM	micromolar
ACE	angiotensin-converting-enzyme
Acetyl-coA	Acetyl-coenzymA
Age	ageing abnormal (*C. elegans* mutant phenotype)
C. elegans	*Caenorhabditis elegans*
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
Daf	abnormal dauer formation (*C. elegans* mutant phenotype)
DNA	deoxyribonucleic acid
DNPH	dinitrophenylhydrazine
Dpy	dumpy (*C. elegans* mutant phenotype, shorter than wild type)
Eat	eating abnormal (*C. elegans* mutant phenotype)
EDTA	ethylenediaminetetraacetic acid
GFP	green fluorescence protein
IGF	insulin-like growth factor
kb	kilobase
L(1-4)	*C. elegans* larval stages (1-4)
lacZ	β-galactosidase
Lon	long (*C. elegans* mutant phenotype)
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
NGM	nematode growth media
NHE3	sodium/hydrogen exchanger
OP50	*Escherichia coli* strain, food source for *C. elegans*
PCR	polymerase chain reaction
PDK	phosphtidylinositol-dependent kinase
PEP	peptidetransporter (protein in *C. elegans*)
PEPT	peptidetransporter (protein in mammals)

PI3K	phosphoinositide 3-kinase
PNS	peripheral nervous system
POT	proton-coupled oligopeptide transporter
RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
Sma	small (*C. elegans* mutant phenotype)
SOD	super oxide dismutase
TCA	tricarboxylic acid cycle
TMD	transmembrane domain
TOR	target of rapamycin
β-Ala-Lys-AMCA	β-Alanin-Lysine-N(epsilon)-7-amino-4-methylcoumarin-3-acetic acid.

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Lebenslauf

Name	Meissner
Vorname	Barbara
Geburtsort	Rosenheim
Geburtstag	23.03.1972

Schulbildung

1978-1982	Grundschule Vogtareuth
1982-1991	Finsterwalder-Gymnasium Rosenheim
Juli 1991	Abitur

Studium

04/1993 - 04/1995	Grundstudium der Haushalts- und Ernährungswissenschaften an der Justus-Liebig-Universität in Giessen
04/1995 – 10/1997	Hauptstudium mit dem Schwerpunkt Ernährungswissenschaft
10/1997 – 04/1999	Praktische Diplomarbeit in der Arbeitsgruppe von Prof. Dr. Hannelore Daniel am Institut für Ernährungswissenschaft in Giessen Thema der Arbeit: Funktionelle Charakterisierung der Arginin-Aufnahme in die humane Coloncarcinomzelllinie Caco-2
30.09.1999	Abschluss zur Diplom-Oecotrophologin
seit 02/2000	Anfertigung der Doktorarbeit am Lehrstuhl für Ernährunsphysiologie der Technischen Universität München in Kooperation mit dem Labor von Prof. Dr. Ralf Baumeister an der Ludwig-Maximilians-Universität in München
seit 11/2003	Wissenschaftliche Mitarbeiterin im Labor von Prof. Dr. Ralf Baumeister, Lehrstuhl für Stoffwechselbiochemie, an der Ludwig-Maximilians-Universität in München

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Vorträge:

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Barbara Meissner, Hannelore Daniel and Ralf Baumeister (2003) **Peptidtransporter-KO in *C. elegans* – influence in development, reproduction and life-span.** Colloquium at the WZW - Center of life science, Freising-Weihenstephan, Germany.

Barbara Meissner, Giuseppe Cassata, Michael Boll, Hannelore Daniel, and Ralf Baumeister (2003) **The intestinal peptide transporter controls development, reproduction and aging in *C. elegans.*** 14th International *C. elegans* Meeting, University of California, Los Angeles.

Posterbeiträge:

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Meissner B., Cassata G., Boll M., Daniel H. and Baumeister R. (2001) **The knock-out of the peptide transporter gene *pep-2* results in delayed development and extended life-span in *Caenorhabditis elegans.*** 13th International *C. elegans* Meeting, University of California, Los Angeles, USA.

B. Meissner, G. Cassata, M. Boll, H. Daniel, and R. Baumeister (2002) **The knock-out of the peptide transporter gene *pep-2* results in delayed development and reduced brood size in *Caenorhabditis elegans.*** Internationales Symposium on Membrane Transport and Transporters, Kloster Seeon, Germany.

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit

Phenotype analysis of *Caenorhabditis elegans* lacking the intestinal peptide transporter

selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen sind als solche kenntlich gemacht.

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München,

Barbara Meissner