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Immunolocalisation of the *D. melanogaster Nramp* homologue *Malvolio* to gut and Malpighian tubules provides evidence that *Malvolio* and *Nramp2* are orthologous

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Summary

***Nramp* (*Slc11a1*) genes in mammals are associated with the transport of iron and other divalent cations; *Nramp1* in macrophages involved in the innate immune response against intracellular pathogens, and *Nramp2* with duodenal iron uptake and the transferrin–transferrin-receptor pathway of iron assimilation. The *Drosophila melanogaster Nramp*-related gene is known as *Malvolio*. The localisation of Malvolio protein was inferred from the enhancer trap line initially used to isolate *Malvolio* in a screen for mutants with defects in taste perception. Here we describe the generation of a Malvolio-reactive polyclonal antibody and apply it to evaluate Malvolio localisation during stages of *D. melanogaster* development, and compare the results with the localisation of the enhancer trap line identified with beta-galactosidase. All immunolocalisation studies have been confirmed to be specific with Malvolio-blocking peptides. Our results demonstrated expression within Malpighian tubules, testis, brain, the amnioserosa of embryos, the larval and adult alimentary canal. Expression within the gut was of significant interest, as mammalian Nramp2 in the gut plays a primary role in the acquisition of dietary iron. We confirm expression within the central nervous system and in cells of the haematopoietic system. By immunohistochemistry we showed that expression within cells was either punctuate, diffuse cytoplasmic or plasma membrane associated, or both. The staining within the gut indicates a degree of conservation of components for iron acquisition between flies and mammals, suggesting that a comparable mechanism has been retained during evolution.**

Key words: Nramp, divalent cation transport, *Drosophila melanogaster*, innate immunity, brain, haemocyte, Malpighian tubule, gut cell.

Introduction

Nramp1/Slc11a1 (*natural resistance-associated macrophage protein 1/solute carrier family 11a member 1*), isolated by a positional cloning strategy (Vidal et al., 1993), is the prototypic member of a family of genes that are widely conserved (Cellier et al., 1995), and *Nramp1* controls natural resistance and susceptibility to infection within strains of mice. The *Nramp1* phenotype is associated with a non-conserved amino acid substitution *G169D* (Malo et al., 1994); allele *D169* is null (Vidal et al., 1995). *Nramp* genes encode highly hydrophobic polytopic integral membrane transporter proteins, and the mutation outlined above maps to the fourth of 12 putative membrane-spanning domains. Two paralogous genes have been identified in human and mouse (Vidal et al., 1993; Barton et al., 1994; Gruenheid et al., 1995), although in mouse a third gene fragment, more closely related to mouse *Nramp1* has been described, but not functionally characterised (Dosik et al., 1994). *Nramp1*, isolated for its role in controlling the growth of intracellular pathogens (Plant and Glynn, 1974; Bradley, 1974; Gros et al., 1981), is exclusively expressed in murine macrophages. The ubiquitously expressed *Nramp2/Slc11a2* gene is implicated in the proton-dependent divalent cation uptake into red blood cells, the uptake of cations by the duodenal enterocyte, and in the transferrin–transferrin-receptor-mediated pathway of cellular iron assimilation (Fleming et al., 1997; Gunshin et al., 1997). *Nramp2* is a carrier of a non-conserved mutation, *G185R*, in microcytic anaemia strains in both mouse (*mk*) (Fleming et al., 1997) and rat (Belgrade) (Fleming et al., 1998). The unequivocal role of *Nramp2* in divalent cation transport has lead to the suggestion that Nramp1 also transports divalent cations, and that susceptibility to infection is a manifestation of impaired macrophage cation transport. However, the precise role of Nramp1 in infection biology has been difficult to define. Consequently there is continuing controversy regarding the direction of cation transport and the mechanism of pathogen

growth-control (Kuhn et al., 1999; Goswami et al., 2001; Jabado et al., 2000; Gomes and Appelberg, 1998). This is proposed to be either by divalent cation starvation (Jabado et al., 2000; Gomes and Appelberg, 1998) or through a Fenton chemistry reactive-oxygen species killing process (Kuhn et al., 1999). What is clear is that Nramp1 plays a major role in regulating the inflammatory response within the macrophage; macrophages from *D169* or null strains exhibit a reduced ability to synthesize inflammatory mediators compared with their *G169* allele counterparts, and the action of *Nramp1* clearly constitutes a major component of the innate immune response (Blackwell and Searle, 1999). Nramp1 may also play a role in homoeostatic cation transport associated with the salvage and recycling of divalent cations within splenic macrophages and hepatic Kupffer cells from effete erythrocytes or other apoptotic cells, and also in cation transport in haemorrhagic disease (Atkinson and Barton, 1998; Biggs et al., 2001; Mulero et al., 2002). It is not clear if this role is a direct activity of *Nramp1* or a pleiotropic effect of $Nramp1^{G169}$ macrophages having a more hostile phagosomal microenvironment and greater ability to degrade ingested cells (Hackam et al., 1998).

The use of *Drosophila melanogaster* is increasing for the identification and study of the genes that play a role within the innate immune response, in view of the simplicity with which mutant strains can be generated and the similarities between flies and mammals in many aspects of these pathways (Hoffmann et al., 1999). Work by the Ezekowitz group (Ramet et al., 2002; Franc et al., 1999) has demonstrated the power of this organism in defining key genes associated with phagocytosis, a prerequisite process in the removal of adventitious pathogenic organisms, and dead or dying cells. The completion and on-going annotation of the *D. melanogaster* genome sequence has indicated that it is unique amongst many multicellular organisms analysed to date in that only a single copy of the *Nramp* gene family, known as *Mvl* (*Malvolio*), is found (Rodrigues et al., 1995), although there are multiple transcripts and two distinct polypeptide species differing at the C-terminal region. This finding begs the question as to whether *Mvl* represents an *Nramp1* or *Nramp2* orthologue, or whether *Mvl* is a bifunctional *Nramp* gene. If the latter scenario appears correct then *D. melanogaster* is a suitable organism to study the biology and function of a primitive *Nramp* gene that may have key properties from both mammalian paralogues. *Mvl* was cloned in a screen for genes that influence taste perception; a hypomorphic mutation in *Mvl,* results in decreased preference to sugar and an increased acceptance of salt (Rodrigues et al., 1995). This chemosensory phenotype is unusual for an *Nramp* gene based our knowledge from studies on mammalian *Nramps*. Electrophysiology in these *Mvl* mutants indicates no abnormal responses within the peripheral neurons suggesting that the mutation is likely to influence events downstream. Observations, using a β-galactosidase reporter gene assay, in the specific enhancer trap line used to isolate *Mvl*, indicate expression within the peripheral and central nervous system, as well as in some cells of the haematopoietic system, notably the macrophages of the embryo and the adult. However, studies were not directed to the tissue localisation of the Mvl protein or its sub-cellular localisation.

Recent complementation studies showed *Nramp2,* but not *Nramp1,* rescues the transport defect of yeast divalent cation transport mutants (Pinner et al., 1997; Tabuchi et al., 1999). Complementation studies in *D. melanogaster*, with an ectopically and ubiquitously expressed human NRAMP1 gene, show no behavioural or physiological effects in the normal animals, however the taste defect is rescued in the *Mvl* mutant strain by NRAMP1 (D'Sousa et al., 1999), and also by rearing of flies in the presence of millimole concentrations of divalent cations (Orgad et al., 1998), indicating that this phenotype is a direct consequence of sub-optimal cation concentrations. Unfortunately, results on the complementation by *Nramp2*/NRAMP2 were not reported. One conclusion from these data is that *Mvl* is orthologous with *Nramp1*/NRAMP1. However, the more widespread expression of *Mvl* than *Nramp1*, and the more restricted expression of *Mvl* compared with *Nramp2*, does not support this proposal. We, therefore, set out to investigate Mvl protein expression, using an in-house prepared anti-Mvl polyclonal antibody, and to compare it with the β-galactosidase reporter, using the *Mvl* enhancer trap line. Our results revealed expression in most tissues was either of a punctate nature, diffuse or both and was observed in macrophage-like cells, Malpighian tubules, testis, brain, the aminoserosa of embryos and the larva and adult alimentary canal.

Materials and methods

Preparation and characterisation of anti-Mvl polyclonal antibody

Rabbit anti-Mvl antibody was prepared against a recombinant GST-Mvl (glutathione S-transferase) fusion protein. Oligonucleotide primers to the Mvl cDNA were prepared (Eurogenetec, Seraing, Belgium), JF3/4 (ATG-GATCCATGTCTTCGAATGAGGCC, ATGAATCCTACT-TGCGAAAGCTGAA) and were used to prepare a DNA fragment from the Mvl cDNA (kindly provided by Veronica Rodrigues, Molecular Biology Unit, Bombay, India) encoding the N-terminal 76 codons by PCR, using standard procedures. This region is common to all known expressed Mvl polypeptides. Amplified DNA was purified and cloned into the pGex-2T expression plasmid between *Bam*HI and *Eco*RI restriction sites. Recombinant clones were screened for fusion protein expression by SDS-PAGE. Protein from a recombinant clone was prepared and used to immunise New Zealand white rabbits, using 500 μg in Freund's complete adjuvant, with a second and third immunisation 10 and 20 weeks later in Freund's incomplete adjuvant. Serum was prepared from whole blood and tested for immunoreactivity against the native protein and a second Mvl fusion protein constructed in pMal-p2. Immunoreactive serum was affinity purified against an agarose-immobilised MBP–Mvl fusion protein, as before

(Atkinson and Barton, 1999), and the eluted IgG was found to be reactive against the Mvl–GST fusion protein, but not native GST. Affinity-purified antibody was used in all histochemical applications. To confirm that the antibody was reacting against native Mvl sequences *in vivo* two overlapping oligopeptides were produced MSSNE AYAHEP GAGGD GPGGS SGASG GGSQR SNQLH HQQIL; HQQIL LNETT YLKPA AKQAY FSDEK VLIPD DDSTN VGFSF RK and used to block immunoreactivity. In control *in vitro* experiments the peptides blocked reactivity of the affinity-purified serum against the Mvl-GST, but not the crude serum against the native GST, as predicted, indicating that the blockade was specific. To further characterise the anti-Mvl antiserum the ORF from full-length Mvl cDNA was cloned, by PCR, into the pcDNA3.1 expression plasmid and transfected into Cos-1 cells, as before. Whole cell extracts from these cells were used in western blotting experiments, as described before, using ECL (GE Healthcare UK Ltd, Little Chalfont, UK).

Immunohistochemistry of Drosophila tissue

Oregon R *D. melanogaster* Meigen embryos were collected and prepared for immunohistochemistry using standard techniques. Tissues were dissected in Drosophila saline and fixed in 4% paraformaldehyde. Embryos and tissues were blocked in 3% normal horse serum (NHS; 3%), and incubated with anti-Mvl primary antibody (1:1000) overnight. Tissues were washed and incubated with biotinylated anti-rabbit IgG (1:200; Vector Laboratories, Peterborough, UK). After washing, tissues were incubated with avidin- and biotin-conjugated horseradish peroxidase, washed, and processed as recommended by the manufacturer. Embryos were covered in glycerol (70%) and mounted using a gelatin:glycerol mountant. Adult and larval brains and testis were treated in essentially the same way, with an acid wash after fixation to aid antibody penetration.

Results

Anti-Mvl antibody generation

We raised a polyclonal antiserum against the N-terminal sequence of the Mvl protein, residues 1–76. Our anti-Mvl antiserum demonstrated reactivity against the Mvl–GST chimera, and also against a Mvl–MBP protein, indicating that the antiserum was recognising the Mvl component of the chimeric protein (not shown). We opted to use a peptide-blocking strategy to demonstrate staining specificity, and two overlapping synthetic peptides, incorporating the immunogen, were generated. Their efficacy at blocking immunodetection was evaluated against recombinant proteins by western blotting. Extracts from Cos-1 cells transfected transiently with the full-length *Mvl* cDNA, mock transfectant cells and a sample of purified GST were probed with non-purified Mvl antiserum pre-incubated with peptide diluent (control), and a sample of antiserum incubated overnight with synthetic peptides. The control Mvl antiserum detected both the wild-type GST and a Mvl protein of 42–48 kDa expressed in transfectant Cos-1 cells, but not in mock transfectants (–ve) (Fig. 1A). Antiserum pre-incubated with synthetic peptides retained reactivity against the GST, indicating the peptides were not interfering with GST immunoreactivity, however, reactivity to the Cos-1 cell-expressed Mvl protein was lost (Fig. 1B). The bottom panel demonstrated comparable loading for the Cos-1 cell extracts on the two blots.

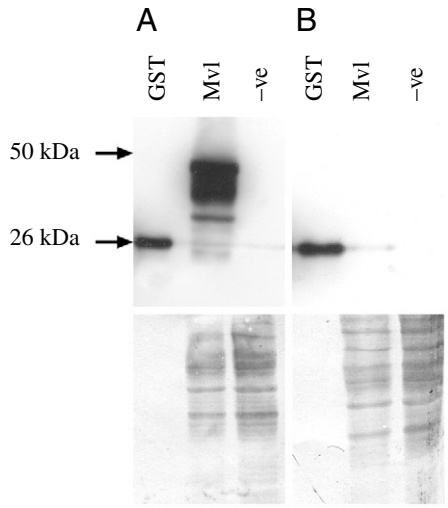


Fig. 1. Reactivity of anti-Mvl antiserum and demonstration of the specificity of blocking peptides. Western blots of purified wild-type GST, Mvl expressed in Cos-1 cells by transient transfection, and mock transfectants (–ve), were probed with anti-Mvl antiserum incubated overnight without peptide (A) or incubated with 25 μg of each peptide (B). Proteins were detected by ECL and size markers of the standard proteins. (Below) The amido black stain of the two membranes following immune detection.

Immunohistochemistry

Embryonic haemocytes

Mvl immunoreactivity was first detected in the syncitial blastoderm in a punctate pattern of expression (not shown), and later at stage 15 in the amnioserosa, as previously reported (Rodrigues et al., 1995). Mvl immunoreactivity was also detected at this stage, within a subset of cells that are scattered throughout the haemocoel of the embryo. These cells contain large phagosomes, are presumed to be phagocytic, and are possibly haemocytes. The Malvolio-positive staining within these macrophage-like cells was restricted to an unidentified subcellular compartment, which appeared to be in close association with the large phagosome (Fig. 2A,B). This subcellular staining was blocked with peptide pre-treatment (Fig. 2C). Haemocyte-like cells containing large phagosomes, and Mvl immunoreactivity were also seen throughout pupal stages (data not shown).

Larval brain and testis

In larvae, Mvl immunoreactivity revealed expression in a number of discrete tissues. In the CNS, Mvl expression was seen in a segmentally repeated pattern of neuronal cell bodies

(Fig. 3A). As with other cells, Mvl-positive staining appeared to be punctate within the cell bodies (Fig. 3B). Mvl immunoreactivity was also detected in two closely associated tissues: the gonad and the surrounding fat body (Fig. 3D–F). Within the fat body, punctate staining was observed associated with these large cells (Fig. 3D). Higher magnification revealed Mvl-positive staining associated with discrete structures within the cells of the gonad (Fig. 3E).

Gut

Immunohistochemistry, using the anti-Mvl antiserum, revealed striking expression within the alimentary canal of both the larvae and the adult imago, and the attached Malpighian tubules (Fig. 4). Staining appeared in the anterior midgut and the posterior midgut up to the midgut–hindgut boundary, although staining of these regions was not always consistent between samples. However, the appearance of staining within individual enterocytes was consistent. Mvl expression was revealed in enterocytes of the anterior midgut in both larvae (Fig. 4A–C) and adult (not shown). These enterocytes appeared to contain two types of staining. Firstly, a diffuse staining throughout the cell (Fig. 4A), and secondly, the familiar punctate subcellular pattern (Fig. 4B). In the posterior region of the midgut, Mvl immunoreactivity labelled two discrete cell types (Fig. 4D–F). Firstly, enterocytes of the midgut show diffuse staining, as well as some punctate staining (Fig. 4D). Secondly, small discrete cells that were found scattered amongst the enterocytes of the gut (Fig. 4E) showed strong Mvl immunoreactivity. In the Malpighian tubules two different cell types within distinct regions of the tubules were also positive for Mvl immunoreactivity. Firstly, diffuse staining was seen in patches of the large principal (type 1) cells (Dimitriadis and Kastritsis, 1983). The location of staining differed between samples, however, in all preparations examined staining was limited to within the region of the lower ureter to the main segment (Fig. 4F). The second, small discrete cell type (Fig. 4G), often had thin processes projecting into the haemocoel (not shown). These cells were limited to the upper ureter and lower tubule in all samples examined.

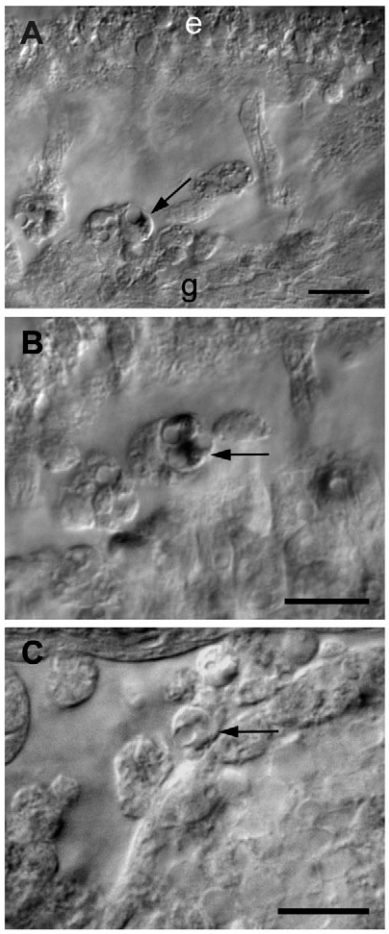


Fig. 2. Immunolocalisation of Mvl in the stage-15 embryo. Mvl-positive staining appears in a subset of cells scattered throughout the haemocoel between the developing gut (g) and the epidermis of the embryo (e). These scattered cells have large phagosomes (arrows). (A) The subcellular localisation of Mvl appears to be adjacent to the phagosome. (B) Mvl-positive staining in two discrete regions, both associated with the phagosome-like structures. (C) Mvl peptides compete out anti-Mvl staining. Scale bars, 100 μm.

Adult brain and gonad

Mvl immunoreactivity was observed within cells scattered throughout the adult brain. These cells were presumed to be neuronal, based on size and shape. Staining was again punctate and probably confined to a subcellular compartment (Fig. 5A). Staining was also found specifically within a sub-region of the gonad of the adult male fly known as the accessory gland. Cells at the apex of this gland had a diffuse sub-cellular stain that was blocked with peptides (Fig. 5C,D).

Western blotting

Western blot analysis of extracts from embryos, larvae, pupae and adult *Drosophila* revealed two distinct polypeptides of approximately 42 kDa and 50 kDa. The 50 kDa protein was detected in extracts from embryos and pupae, and the 42 kDa species was found in extracts from embryos larvae and adult, but not pupae (Fig. 6).

Discussion

This study has provided a definitive description for the temporal and spatial expression of Mvl encoded by *Mvl,* the *D. melanogaster Nramp* orthologue, by immunohistochemistry techniques, and *in vivo* immunolocalisation data were confirmed by immuno-blockade using synthetic Mvl peptides. Histochemical Mvl detection has not only enabled confirmation of many of the original observations (Rodrigues et al., 1995), but also allowed a much greater detailed analysis of *Mvl* expression. Study of Mvl is of particular interest, as it represents a prototypic member of the *Nramp* gene family, as genome sequencing has revealed only a single copy of this highly conserved gene family in *D. melanogaster* and it is unique amongst many other higher eukaryotes where at least

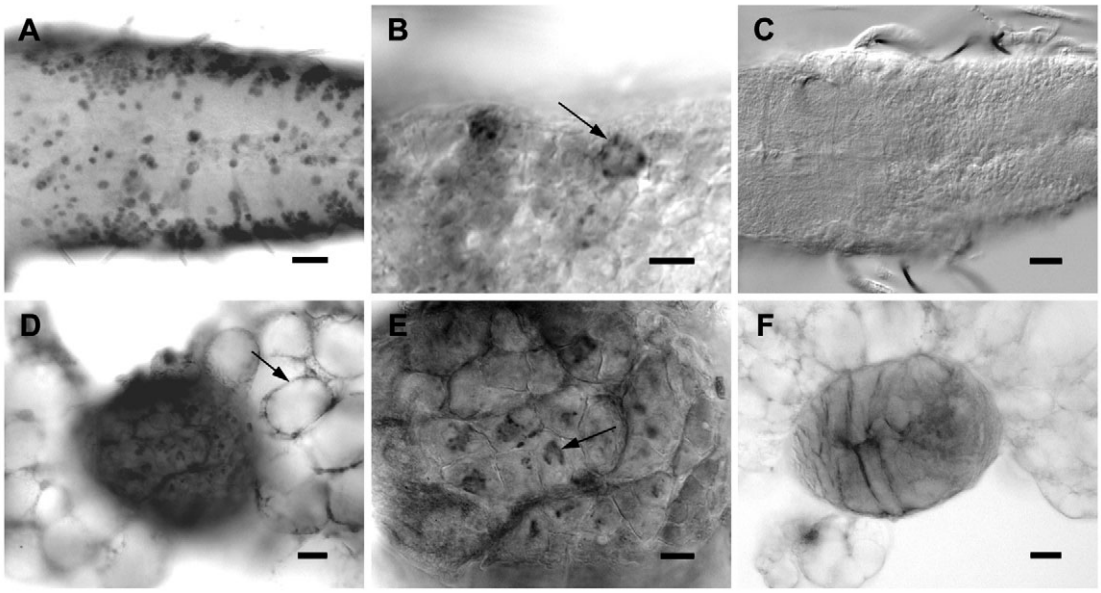


Fig. 3. Immunolocalisation of Mvl in the larval brain (A–C) and testis (D–F). (A) Reactivity in a subset of neurons within the larval brain. (B) Higher magnification of these neurons (arrows) shows staining associated with a sub-cellular compartment. (C) Immuno-blocking with Mvl peptides. (D) Reactivity within the larval gonad, and also staining associated with cells of the fat body (arrow). (E) Higher magnification show Mvl-positive staining associated with a subcellular structure within the cells of the testis (arrow). (F) Immuno-blocking with Mvl peptides. Scale bars, 200 μm (A,C,D,F); 100 μm (B,E).

two orthologues have been described. The *D. melanogaster* enhancer trap line, from which *Mvl* was identified, has an unusual phenotype, of taste perception, compared with that of the mammalian *Nramps*. The relationship between divalent cation transport and the function of the gustatory pathway is not obvious at present, however, in this study we were unable to detect expression of Malvolio within either the third antennal segment, neurons of the maxillary palps or labellar chemosensory neurons, which had been previously described by reporter gene. This difference could be due to low level or transient expression of Mvl in these tissues, making immunological detection difficult. Nramp-mediated cation transport is associated with both the acquisition of divalent cations from the duodenal lumen, the transport of divalent cations into cells through the transferrin–transferrin-receptor pathway of iron uptake and in the transport of cations associated with defence against intracellular macrophage pathogens. The transport of cations within macrophages by Nramp1 constitutes a branch of the innate immune response, but a role is also possible in homoeostasis and pathology associated with the salvage and recycling of cations from apoptotic cells, and effete red cells. The data from this current study support this concept of transport for iron salvage/recycling, as strong Mvl expression was demonstrated within haemocytes during embryonic and also pupal stages of the *D. melanogaster* life cycle, when considerable tissue remodelling occurs.

A significant and novel finding from this study was the observation that the Mvl protein was expressed within the *D. melanogaster* gut at both larval and adult stages. Expression within the midgut is consistently associated with two regions, the anterior midgut and the posterior midgut. The anterior region of the midgut, known as the cuprophilis region is an important site for nutrient absorption, especially dietary copper (Filshie et al., 1971; Dubreuil et al., 1998). The posterior region of the midgut is equally, if not more, important in nutrient absorption, and is populated by iron-accumulating cells (Dimitriadis and Kastritsis, 1983; Poulson and Bowen, 1952). A hallmark of Nramp2 is expression within enterocytes, where it has a pivotal role in the uptake of iron and other divalent cations from the duodenal lumen and where it is subjected to regulation by cation availability. It would be of significant interest to assess if *Mvl* expression was also sensitive to dietary iron. There is a precedent for this as iron loading can rescue the taste defect (Orgad et al., 1998). Although not the direct target of the pathogenic sequence alteration, there is an increased prevalence of NRAMP2 in patients with hereditary haemochromatosis, an iron overload disease, which is associated with increased uptake of divalent cations. It would be of interest to overexpress *Mvl* and examine whether enhanced iron acquisition also leads to tissue injury, as observed in untreated haemochromatosis. The decreased expression, or impaired targeting or function of Nramp2 also contributes to iron deficiency in microcytic anaemia. Since Mvl also was localised within gut cells, we conclude that it also participates in the acquisition of iron and other divalent cations for *Drosophila* growth and development. Conditional *Mvl* mutants could be of value for modelling this human disease. These data were supportive of an analogous mechanism for the acquisition of divalent cations from the gut

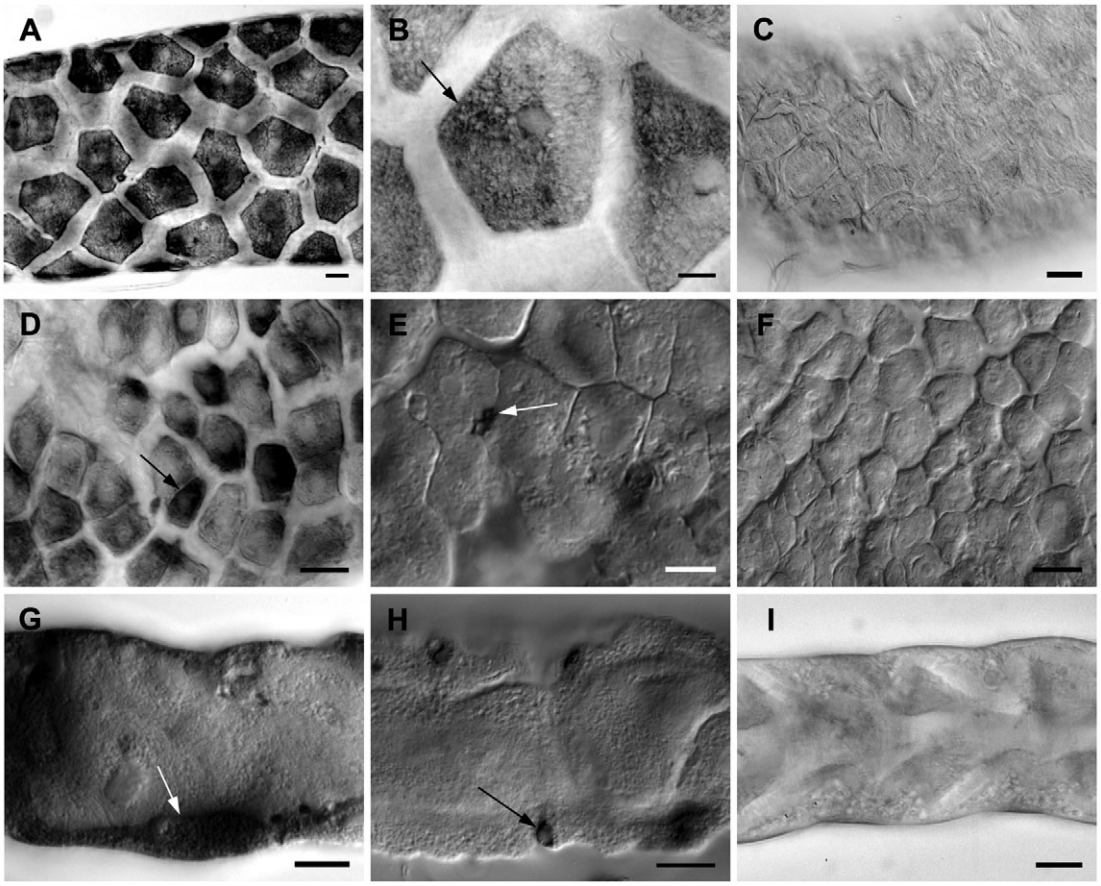
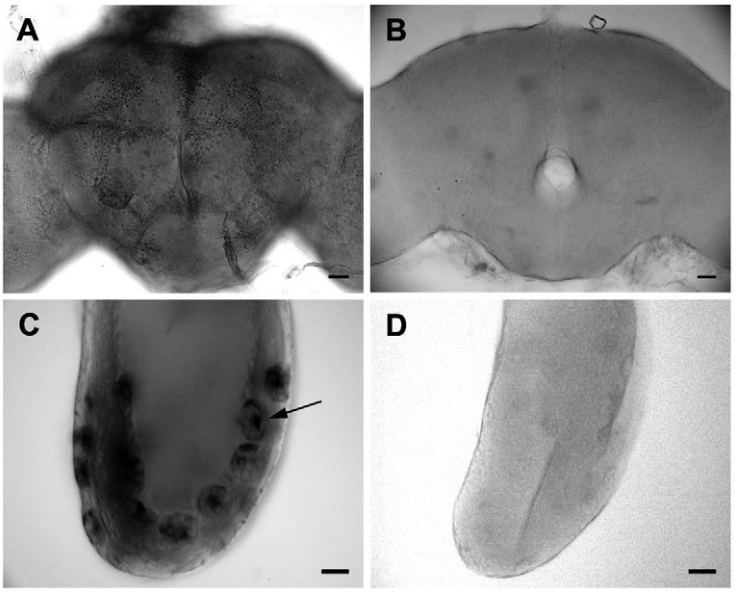


Fig. 4. Immunolocalisation of Mvl in both the larval and adult alimentary canal. (A–C) Larval anterior midgut. (A) Mvl staining within the larval anterior midgut. (B) Higher magnification reveals punctuate subcellular staining within enterocytes (arrow). (D–F) Mvl-positive staining within the adult posterior midgut. (D) Staining within these enterocytes appears to be diffuse (arrow), with some punctate staining. (E) Small scattered cells show strong Mvl staining (arrow). (G–I) Mvl localisation within the Malpighian tubules. (G) Diffuse staining within principal (type 1) cells (arrow). (H) Strong Mvl-positive staining within small neuroendocrine-like cells (arrow). (C,F,I) Mvl peptides confirm staining specificity in each cell type. Scale bars, 200 μm (A,C); 100 μm (B,D–I).



lumen for both flies and mammals. Interestingly, Mvl is also expressed in the *Drosophila* fat body, an organ that has been shown to play a role in iron uptake, storage and release back into the haemolymph in insects (Huebers et al., 1988). Since the release of the entire *D. melanogaster* genome sequence, it has become clear that whereas many genes involved in iron homeostasis are conserved between insects and vertebrates, many are not, indicating important differences in their metabolism of iron (Nichol et al., 2002). However, these data are clearly supportive of Mvl providing the conduit for iron acquisition for all stages of *D. melanogaster* development.

Patches of anti-Mvl staining were found along the length of the Malpighian tubules, within large cells that

Fig. 5. Immunolocalisation of Mvl in the adult brain and gonad. (A) Mvl-positive staining in neurons of the adult brain. (C) Mvl staining within a subset of cells in the accessory gland of the adult gonad (arrow). (B,D) Peptide block of staining. Scale bars, 20 μm.

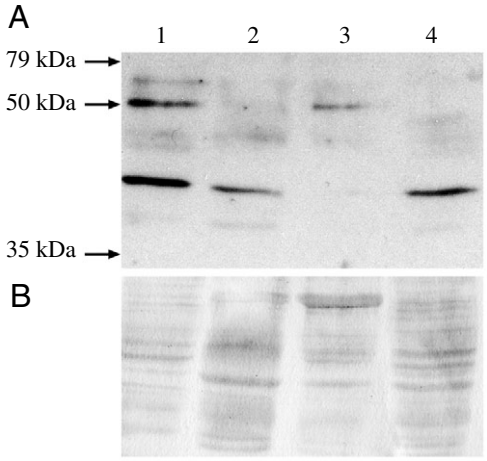


Fig. 6. Reactivity of anti-Mvl antiserum with *Drosophila* protein extracts. (A) Western blot of protein extracts from embryo (lane 1), larvae (lane 2), pupae (lane 3), adult (lane 4), probed with Mvl antiserum. Two strong bands appear of approximately 42 kDa, and 50 kDa, respectively. (B) Amido black-stained membranes show equal loading.

are similar in appearance to the principal (type 1) cells. In addition, a smaller cell type was also stained in a discrete area of the tubules. There were small cells of 3–4 μm diameter, with thin processes that, based on their size, appearance and location within the lower tubule, are likely to be the neuroendocrine cells described previously (Sozen et al., 1997). These cells demonstrated homogeneous staining throughout, which could either represent cytoplasmic or more probably surface staining. The patchy and inconsistent staining of the principal cells within the tubules is likely to reflect differences in the response to dietary iron load between specimens. Cells of the gut also displayed both punctate and diffuse staining that could also be surface staining. Given that Mvl shows punctate intracellular staining in many other cells, and that there are three variants of *Mvl*, it is possible that variant Mvl forms could be targeted to different subcellular structures (http://flybase.bio.indiana.edu/). It is also possible that Mvl reflects Nramp2 localisation, showing both plasma membrane and recycling endosome staining, where it is colocalised with transferrin. However, to address this issue would require the development of isotype-specific antibodies, but this is not without difficulty given the small differences between protein variants.

Expression of Mvl within the Malpighian tubules was novel and suggestive of this protein playing a role in divalent cation reabsorption. This is similar to the Nramp2 gene that is proposed to participate in a re-uptake system for divalent cation at the brush border of kidney proximal tubules (Canonne-Hergaux and Gros, 2002). Our studies also indicated that Mvl is expressed within the testis. A study of Nramp2 in phagocytic Sertoli cell lines of the testis shows Nramp2 association with early and late endosomes (Jabado et al., 2002). Following phagocytosis, Nramp2 becomes associated with the phagosome and is proposed to participate in active transport at the phagosomal membrane in these cells in providing essential iron for spermatogenesis. Animals that are hypotransferrinemic, have reduced levels of spermatogenesis indicating the important role of iron in this and other processes. The localisation of Mvl within the testis may support an analogous function in providing iron for the maturing sperm cells.

Western blot analysis of homogenates from embryos, larvae, pupae and adult *Drosophila*, revealed two distinct bands of approximately 42 kDa and 50 kDa. This compares with the predicted molecular masses of 53.3 kDa and 65.5 kDa. However, hydrophobic proteins often run anomalously in SDS-PAGE and post-translational modification of Nramp1 also influences apparent size. The 50 kDa band appeared in extracts from embryos and pupae. The 42 kDa band appeared in extracts from embryos, larvae and adult, but not pupae. We have shown that at these stages Mvl is expressed in phagocytic haemocytes, and it is possible that it is the 50 kDa Mvl that plays a role in divalent cation transport, during the extensive tissue remodelling at these stages. As the 42 kDa band appeared in extracts from embryos, larvae and adult, but not pupae it is possible that the 42 kDa Mvl is involved in divalent metal uptake, and distribution at these stages, but not transport associated with the phagosome.

These studies have revealed novel sites for Mvl expression that clearly supports its orthology with Nramp2, however, expression within cells displaying a phagocytic morphology also supports its orthology with Nramp1. We conclude that Mvl is a bifunctional divalent cation transporter protein, based on cell distribution and is likely to have functional characteristics of both proteins, although it is not clear how the antiport–synport controversy of Nramp1 (Goswami et al., 2001; Jabado et al., 2000) will relate to Mvl function. Furthermore, this study does not reveal how divalent cation transport is linked to the gustatory circuit.

Future studies might be directed at examining the DNA regulatory sequences controlling Mvl expression within particular cellular structures and the identification of motifs within the protein that target it to particular sub-cellular regions. Furthermore, given that iron regulatory protein (IRP) proteins are found in *Drosophila* (Muckenthaler et al., 1998) and that *Drosophila* ferritin carries an iron response element (Georgieva et al., 1999) it is a distinct possibility that other genes, possibly including *Mvl*, may carry a functional iron response element to modulate function.

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