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# The Drosophila Ortholog of the Endolysosomal Membrane Protein, Endolyn, Regulates Cell Proliferation

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**Abstract** Endolyn (CD164) is a sialomucin that regulates the proliferation, adhesion, and migration of human haematopoietic stem and progenitor cells. This molecule is predominately localized in endocytotic compartments, where it may contribute to endolysosomal biogenesis and trafficking. In order to more closely define the function of endolyn from an evolutionary view-point, we first analyzed endolyn orthologs in species ranging from insects, fish, and birds to mammals. The predicted molecular structures of the endolyn orthologs from these species are well conserved, particularly with respect to significant O-linked glycosylation of the extracellular domain, and the high degree of amino acid similarities within their transmembrane and cytoplasmic domains, with the latter possessing the lysosomal target signal, YXXϕ. Focusing on Drosophila, our studies showed that the subcellular distribution of endolyn in non-polarized Drosophila S2 cells resembles that of its human counterpart in hematopoietic cells, with its predominant localization being within intracellular vesicles, while a small fraction occurs on the cell surface. Both Y → A and L → A mutations in the YHTL motif perturbed the normal subcellular distribution of Drosophila endolyn. Interestingly, embryonic and early larval development was often arrested in endolyn-deficient Drosophila mutants. This may partly be due to the role of endolyn in regulating cell proliferation, since knock-down of endolyn expression in S2 cells resulted in up to 50% inhibition of cell growth, with a proportion of cells undergoing apoptosis. Taken together, these results demonstrate that endolyn is an evolutionarily conserved sialomucin fundamentally involved in cell proliferation in both the human and Drosophila melanogaster. J. Cell. Biochem. 99: 1380–1396, 2006. 

**Key words:** endolyn; CD164; Drosophila melanogaster; endosome; lysosome; cell growth; apoptosis

Endosomes and lysosomes are dynamic membrane-sealed acidic intracellular organelles, within which vesicle trafficking, membrane fusion and digestion of endocytosed macromolecules take place. They contribute to maintaining cell homeostasis. Membrane constituents play vital roles in the functions and biogenesis of these organelles. These include a set of highly glycosylated proteins contained in the limiting membrane, including lysosomal associated membrane protein 1–2 (LAMP1-2), lysosomal integrated membrane protein I–II (LIMP I–II), macrosialin (CD68), lysosomal acid phosphatase (LAP) and many others [Eskelinen et al., 2003]. Many of these proteins are ubiquitously expressed, while others are restricted to specific cell types. For example, CD208 occurs in dendritic cells and type II pneumocytes [Salaun et al., 2004], CD68 in monocytes and macrophages [Holness and Simmons, 1993], and the ocular albinism type 1 gene product, OA1, resides exclusively in the retina and in melanocytes [Bassi et al., 2001].

Intracellular trafficking and sorting of endolysosomal membrane proteins have been shown to be mediated by their interaction with distinct

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hetero tetrameric adaptor complexes, AP-1, -2, -3, and -4, which recognize the YXXΦ (where X represents any amino acid and Φ a hydrophobic amino acid) or the di-leucine-based sorting motifs located within the cytoplasmic tail of sorted molecules [Hunziker and Geuze, 1996; Bonifacino and Traub, 2003]. Although various functions have been suggested for these molecules, including sequestration of the active lysosomal enzymes, transport of degradation products from the lysosomal lumen to the cytoplasm, and the regulation of fusion and fission events between endolysosomes and related organelles, their specific roles are only now being elucidated. Accordingly, certain diseases can be attributed to the functional alterations in some of these proteins [Eskelinen et al., 2003].

Recent studies demonstrate that endolyn (CD164) is highly conserved in the human and rodents [Zannettino et al., 1998; Kurosawa et al., 1999; Chan et al., 2001; Ihrke et al., 2001]. In these species, genomic sequences are organized into 6 exons, with the genes being located on the syntenic regions of human and mouse chromosomes (6q21 and 10B1-B2, respectively) [Chan et al., 2001]. The mature endolyn proteins in both the human and rodents are predicted to contain two mucin domains (I and II) linked by a cysteine-rich domain [Watt et al., 1998; Zannettino et al., 1998; Doyonnas et al., 2000; Ihrke et al., 2000; Chan et al., 2001]. They possess an identical transmembrane region and a short cytoplasmic domain, in which the lysosomal sorting signal, YHTL, is located. Endolyn is expressed in many tissues in these species, predominately being distributed in intracellular organelles, but also occurring on the cell surface [Zannettino et al., 1998; Doyonnas et al., 2000; Ihrke et al., 2000, 2001; Watt et al., 2000; Chan et al., 2001]. Newly synthesized endolyn protein is delivered to cell surface in an N-linked glycan-dependent manner, with its internalization to endolysosomes being mediated via its YHTL sorting signal [Ihrke et al., 2001, 2004; Potter et al., 2004]. In the human, the amino terminal domain I of endolyn has been shown to be differentially glycosylated in different cell types and at different stages of cell lineage development [Watt et al., 2000; Kuci et al., 2003; McGuckin et al., 2003; Hennersdorf et al., 2005; Jorgensen-Tye et al., 2005; Vodyanik et al., 2005]. This differential glycosylation regulates its functional interaction with other receptors involved in the adhesion, migration and proliferation of hematopoietic stem/progenitor cells [Zannettino et al., 1998; Chan and Watt, 2001; unpublished data]. Endolyn also plays a role in the in vitro differentiation of mouse myoblasts [Lee et al., 2001]. Yet, the specific in vivo function of endolyn remains to be elucidated.

Comparative sequencing of the whole genomes from various species has emerged as a powerful tool for identifying functionally conserved genomic elements that play important roles in various cell physiological processes. Proteins with significant similarity to human LAMPs and/or CD68 have been identified in lower species, such as Caenorhabditis elegans, which provides further insights into their functions [Kostich et al., 2000]. Identification and characterization of endolyn orthologs in a genetically less complex species may also facilitate the study of in vivo function of endolyn. The general molecular machinery for membrane trafficking and endocytosis is conserved in eukaryotic cells [Stenmark and Zerial, 2001] and AP-1, -2 and -3 homologs have been shown to exist in lower species [Burd et al., 1998]. However, the involvement of YXXΦ or di-leucine motifs in the delivery of proteins to endolysosmes has not been demonstrated experimentally in insect cells [Sun et al., 2004].

Therefore, we have in this study compared endolyn in Drosophila melanogaster with its orthologs in the human and other species, with respect to primary structures, predicted oligosaccharide modifications and subcellular distribution. Furthermore, we demonstrate that endolyn is involved in the embryonic and larval development of Drosophila and provide evidence for its role in regulating the growth of Drosophila cells in vitro.

## MATERIALS AND METHODS

### Antibodies and Cell Culture

Human endolyn specific monoclonal antibodies (Mabs) N6B6 and 105A5 have been described previously [Watt et al., 1998, 2000]. The Rab11 Mab (Clone 47) was purchased from BD Pharmingen, CA, and the anti-GFP Mab (clone GFP20) from Sigma Chemical Co., MO. Alexa Fluor488- and 546-conjugated secondary antibodies were obtained from Molecular Probes (OR). Drosophila S2 cells were cultured

in serum-free DES expression medium (Invitrogen Ltd, Parsley, UK). The human haematopoietic cell line KG1a was cultured as described [Watt et al., 2000].

### Fly Strains

Three fly strains with a P-insertion at different sites within or adjacent to the Drosophila endolyn gene were included in this study. These were $vsg^{BG00708}$ (P{GT1}$vsg^{BG00708}$) (GT-VSG) with the P-element inserted just downstream of the endolyn transcription start site, $vsg^{EY05552}$ ({EPgy2}vsg) (EP-VSG) with the P-element inserted in the first intron (both mutants were obtained from the Bloomington Drosophila Stock Center, Indiana University, USA), and P{lacZ}$vsg^{1}$ (E4203), with the P-element just prior to the transcription initiation site, provided by Dr. Josh Dubnau at Cold Spring Harbour Laboratories, USA. The strain, $w^{1118}$ (isocJ1), provided by Dr. Dubnau, served as the control in the growth rate observations. In all mutants, the position of the P-element insertion has been verified by the providers using the inverse PCR techniques.

### Bioinformatics Analysis of Endolyn Orthologs in Multiple Species

The Genetic Computer Group (GCG) software package Version 10 was used for all comparative analyses. Sequence information for endolyn orthologs from all species was retrieved from the NCBI database. The deduced amino acid sequences were compared using the Pileup program for progressive and pairwise alignment. The deduced amino acid sequences were submitted to online NetOGlyc 3.1, etNGlyc 1.0, and YinOYang 1.2 servers (http://www.humgen.nl/programs.html) for glycosylation analysis. The software SignalP 3.0 and PSORTII (http://psort.nibb.ac.jp) programs were used for the prediction of the leader peptide and transmembrane domains, respectively.

### RNA Extraction and RT-PCR

Total RNA was extracted from cultured S2 cells or larvae at different stages using the Trizol reagent (Invitrogen). Reverse-transcription was carried out using reverse transcriptase and the Platinum PCR SuperMix High Fidelity kit (Invitrogen) with the primers 5′-ACCACAATGAATCGGCAGGCG-3′ and 5′-CTCAAAGGGTGTGGTAGTTGCGC-3′ flanking the full coding region of predicted Drosophila endolyn gene. To examine RNA levels of Drosophila endolyn after transfection with double stranded RNA (dsRNA), the primer sequences 5′-ACTCAGCGTTTAACGACCAC-3′ and 5′-TGCCGCATTGAAGGCCGAC-3′, respectively, were used to allow the amplification products from indigenous endolyn mRNA, but with no amplification from the transduced endolyn dsRNA. The PCR products were visualized by electrophoresis in 1% (w/v) agarose gels and stained with ethidium bromide, excised from the gel and cloned into the pGEM-T easy plasmid vector (Promega Inc., WI).

### Constructs and Overexpression of GFP-Drosophila Endolyn Fusion Protein (GFP-Endolyn)

The DNA sequence coding for green fluorescent protein (GFP) was amplified by PCR with the nucleotide oligos 5′-GGTCGCCACCATGGTG-3′ and 5′-CTTGTACAGCTCGTCC-3′ as PCR primers and pEGFP-1 plasmid (BD Biosciences, Oxford, England, UK) as templates. The amplified DNA fragment was linked to the 5′ end of a Drosophila endolyn cDNA. The recombinant sequence was inserted into the Drosophila expression vector pMT/V5-His (Invitrogen). Mutations of the YHTL motif were generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene, CA) and the recommended procedures. The primers for creating the AHTL mutation were 5′-ACGAGCGCAACGCCCACACCCTTTGAG-3′ and CTCAAAGGGTGTGGGCGTTGCGCTCGT-3′. To mutate the YHTL motif into the YHTA, the 5′-CGCAACTACCACACCGCTTGAGCCGCCACCGC-3′ and 5′-GCGGTGGCGGCTCAAGCGGTGTGGTAGTTGCG-3′ primers were used. The resultant expression constructs, together with the pCoHygro vector (Invitrogen) for hygromycin resistance, were co-transfected into Drosophila S2 cells using the Lipofectamine 2000 reagent (Invitrogen) and transfected cells were selected against 300 μg/ml hygromycin. After stable cell clones were established, GFP-endolyn expression was induced by addition of 500 μM of copper sulfate to the culture medium. pMT/V5-His containing the GFP sequence alone was used as the control.

### Immunofluorescent Staining and Confocal Microscopy

Cells were suspended in PBS containing 0.5% (w/v) BSA, labeled with primary Mabs or isotype

matched negative control Mabs, followed by fluorescein isothiocyanate (FITC)- or R-phycoerytherin (RPE)-conjugated anti-mouse isotype specific antibodies (Southern Biotechnology Associates Inc., AL) and analyzed by flow cytometry as previously described [Chan et al., 2001]. Alternatively, cells were allowed to adhere to Superfrost glass slides (BDH, Poole, UK) for 30 min at 4°C and incubated with 10 μg/ml of specific or the relevant isotype matched control Mabs, followed by 10 μg/ml anti-mouse isotype-matched secondary antibody, prior to fixation with 1% (w/v) paraformaldehyde for 10 min, and then washed and mounted with DAKO fluorescent mounting medium (DAKO A/S, Glostrup, Denmark). For labeling intracellular proteins, cells were fixed with paraformaldehyde for 5–10 min prior to incubation with antibodies. Cells on slides were assessed using the Nikon ECLIPSE600 fluorescence microscope or the Zeiss 510 automated confocal microscope, which was fitted with HeNe 543, HeNe 633, and argon 488 lasers.

### Immuno-Electron Microscopy (ImmunoEM)

Pellets of $2 \times 10^6$ cells were fixed in 5% (v/v) glutaraldehyde in 0.1 M phosphate buffer, followed by post-fixation in osmium tetroxide, dehydration and embedding in Spurr's epoxy resin. Identical cell samples were also processed for immuno-electron microscopy, which involved fixation in 2% (w/v) paraformaldehyde in phosphate buffer, and dehydration and embedding in LR White acrylic resin. For immunostaining, thin sections mounted on nickel grids were floated on drops of 1% (w/v) bovine serum albumin in Tris/HCl buffer (pH 7.2), to reduce non-specific staining, followed by the specific primary antibody (anti-GFP or anti-human endolyn) diluted in Tris buffer. After washing, the grids were floated on secondary antibody conjugated to either 5 or 10 nm colloidal gold particles. Sections were stained with uranyl acetate prior to the examination under the electron microscope.

### Carbohydrate Analysis and Immunoblotting

Lysates of GFP-endolyn expressing Drosophila S2 and human KG1a cells were subjected to various carbohydrate cleavage analyses. To cleave the N-glycans, 50 μg cell lysate protein in sodium phosphate buffer (60 mM sodium phosphate, 150 mM EDTA, 2% (w/v) SDS, 2 μl β-mercaptoethanol, pH 7.5) was boiled for 2 min prior to the addition of 15 units of N-glycosidase F (Roche Diagnostics, East Sussex, UK), followed by incubation at 37°C for 16 h. To cleave the terminal α2–3 and α2–6 linked sialic acid residues from both N- and O-glycans on this molecule, cell lysates were incubated with the Arthrobacter ureafaciens sialidase (50 U/ml) in 50 mM sodium phosphate buffer, pH 6.0 for 16 h at 37°C. For O-sialoglycoprotease treatment, 15 μg of O-sialoglycoprotease (Cedarlane, Canada) was added to cell lysates in 25 mM sodium phosphate buffer, pH7.4 at 37°C for 4 h. Lectin binding of GFP-Endolyn proteins was analyzed using the DIG glycoprotein detection kit (Roche) and included Galanthus nivalis agglutinin (GNA), Datura stramonium agglutinin (DSA), Sambucus nigra agglutinin (SNA), Maackia amurensis agglutinin (MAA), and peanut agglutinin (PNA) [Jorgensen-Tye et al., 2005]. Anti-GFP or anti-human endolyn antibodies at a concentration of 100 ng/ml were coated onto Immulon 2HB plates (Dynex Technologies, VA) at 4°C overnight and plates were blocked for 1 h with PBS containing 0.2% (w/v) BSA before the addition of cell lysates at a concentration of 200 μg/ml, at 4°C for 1 h. Following washing with PBS, 100 μl of each lectin (1 μg/ml) was added to triplicate wells and allowed to bind for 2 h at room temperatures. The bound lectins were labeled for 2 h with 100 μl, 1 μg/ml anti-DIG-AP antibody. After washing with water, 200 μl pNPP (Sigma) was added and the absorbance was read using a Bio Rad Model 450 microplate reader (Hemel Hempstead, UK) at 405 nm.

### RNA Interference (RNAi) Experiments

To further explore the role of enodolyn at the cellular and molecular levels, the T7 promoter sequence was incorporated into PCR primers and the resultant PCR products were used as templates for in vitro RNA transcription using the MEGAscript transcription kit (Ambion Ltd., Huntingdon, UK). The bacterial lacZ sequence was used as an irrelevant control. The sequences of PCR primers used for amplifying Drosophila endolyn were: 5′-TTAATACGACTCACTATAGGGACAATGAATCGGCAGGCG-3′, and 5′-TTAATACGACTCACTATAGGGACTCAAAGGGTGTTGGTAGTTG-3′, and those for amplifying the lacZ fragment were 5′-TTAATACGACTCACTATAGGGAGGCGTAATCATGGTCATAGC-3′ and 5′-TTAATACGACTCACTATAGGGAGTAAGCGGCAGGGTCGG-3′. In

vitro transcription, the annealing of dsRNAs and the recovery of dsRNAs were performed using MEGAscript kit (Ambion).

### Cell Proliferation Assays

Cell counts at indicated time points were recorded directly using a hemocytometer or measured using the MTT assay. For the latter assay, cells cultured in 96-well plates were incubated with 20 μl of MTT (5 mg/ml) in PBS for 5 h in a $CO_2$ incubator at 37°C, prior to the addition of 200 μl of DMSO at 37°C for 5 min. The reaction was read at 550 nm using the Bio Rad plate reader.

### Apoptosis Assays

Apoptosis was determined by three independent methods, using the APO-BrdU kit (BD Pharmingen) for measuring DNA breaks, the CaspACE assay system (Promega) for caspase 3 activities, and the Annexin V-PE probe (BD Pharmingen) for detecting cell surface phosphatidylserine, typically 48 h after transfection with dsRNA or siRNA constructs, according to the manufacturers' procedures.

### Statistical Analysis

Values for all results are shown as mean ± SEM. Specific differences between treatments were examined using Student's $t$-test, and $P \leq 0.05$ was accepted as being statistically significant.

## RESULTS

### Structural Conservation of Endolyn in Multiple Species

Homologous and orthologous genes across various species can now be readily assigned using the available genome sequence information. Among a few mucin-like molecule-encoding genes identified in the fruit fly, CG16707/GH15083 has been identified as a mammalian endolyn-like gene (http://flybase.bio.indiana. edu). This Drosophila endolyn ortholog spans 3.4 kb of DNA in chromosome 3L and comprises 2 exons separated by an intron of 1.12 kb. All transcripts so far deposited in the Drosophila database, although with slightly different transcription initiation sites, are approximately 2.4 kb in length and encode a polypeptide of 182 amino acid residues. Expression of Drosophila endolyn was found to be broad and over 50 deposited expressed sequence tags (ESTs) were found in diverse cell and tissue types, including the embryo, larva, pupa, adult head, testes, ovary, and cultured Schneider L2 cell lines.

More extensive searches of the NCBI database (http://www.ncbi.nlm.nih.gov), including the EST and protein databases, revealed that endolyn-related sequences also exist in many other species ranging from insects (e.g., Drosophila, mosquito and bee), vertebrates (e.g. xenopus, birds, and fish), to mammals (e.g., mouse, rat, pig, dog, cat, horse, and calf) and primates (monkeys and human). The deduced amino acid sequences in species that occupy the distinctive positions in evolution were comparatively aligned (Fig. 1A). These included human (NM_020404), chimpanzee (XM_518677), mouse (NM_054042), rat (BV166072), chicken (AJ292037), zebra fish (CAH68969), and Drosophila (NM_168363). As previously shown [Zannettino et al., 1998; Kurosawa et al., 1999; Doyonnas et al., 2000; Chan et al., 2001], the amino acid sequences for human and rodent endolyns are highly conserved, with the overall similarities of 68–85%. Here we show that chimpanzee and human endolyn molecules are almost identical. Even though the amino acid sequences of the endolyn orthologs of insects, fish and birds appear to be more divergent (~37% overall similarity) than those in mammals, the extensive O-linked glycosylation sites and the short C-terminal tail containing the YXXΦ motif are all maintained (Fig. 1). O-linked carbohydrates appeared to be the dominant form of glycosylation for the Drosophila endolyn ortholog as there are only two potential N-linked glycosylation sites predicted. The amino acid sequence similarities for the C-terminal region of all orthologs compared was found to be in the range from 80 to 100%. The other overt divergence is that, in the lower vertebrates (birds and fish), the cysteine-rich region is located at the N-terminus of the mature polypeptides, resulting in a single O-glycosylated mucin domain, while the extracellular region of mammalian endolyns is divided into two mucin domains separated by a short cysteine-rich region. The Drosophila endolyn ortholog lacked such a cysteine rich region (Fig. 1).

### Characterization of Drosophila Endolyn

To further characterize Drosophila endolyn, we overexpressed Drosophila endolyn tagged with GFP at its N-terminal (GFP-Endolyn) in

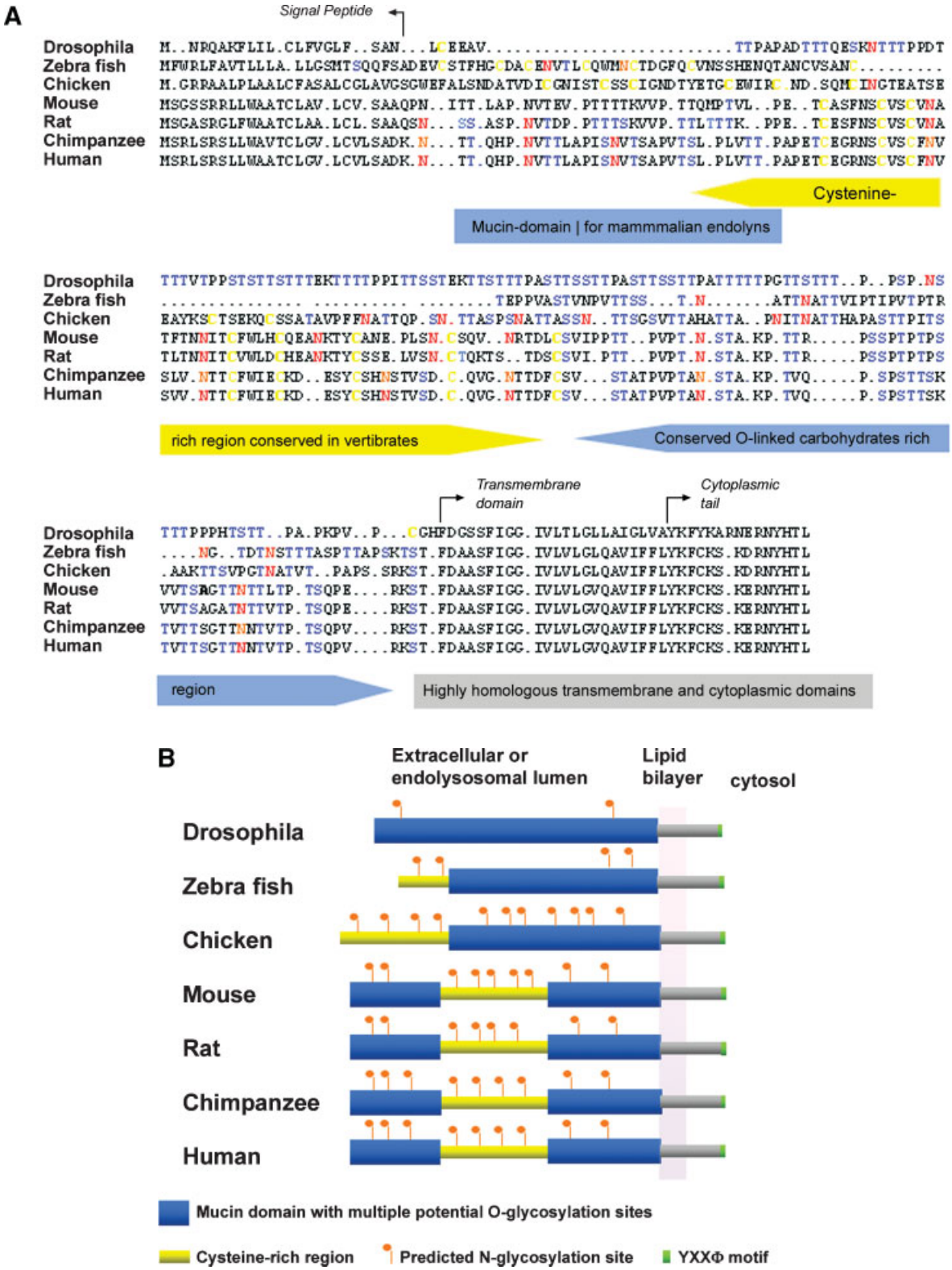


**Fig. 1.** Conserved structural features of endolyn orthologs in various species. A comparative alignment of amino acid sequences of endolyn orthologs (**A**). Potential sites for N-glycosylation are in red; potential sites for O-glycosylation are in blue; cysteine residues are in yellow. A schematic display of domain organizations and major structural features of endolyn orthologs (**B**).

Drosophila S2 cells, a macrophage-like nonpolarized cell line. Using immunoblotting analysis with a GFP-specific antibody, we were able to estimate the relative molecular weight of Drosophila endolyn. The fusion protein was estimated to have a molecular weight of ~45 kDa when unglycosylated, with 26.6 kDa contributed by GFP and about 18 kDa by Drosophila endolyn. As shown in Figure 2A, the fusion protein detected in S2 cell lysates was approximately 68 kDa in size. The mature Drosophila endolyn could therefore be estimated to carry ~56% carbohydrates. Very few carbohydrates on Drosophila endolyn appeared to be N-linked, as neither tunicamycin treatment of cultured cells nor N-glycosidase F treatment of cell lysates altered the apparent molecular mass of the recombinant protein (data not shown). As with human endolyn (H endolyn, Fig. 2B, Jorgensen-Tye et al., 2005), the Drosophila endolyn (F endolyn) contained α2-6 linked sialic acid residues (Fig. 2B), which were resistant to N-glycosidase F but sensitive to sialidase treatments. The SNA binding activity of GFP-Endolyn was diminished with sialidase treatment. Treatment with O-sialoglycoprotease, which specifically cleaves mucin-type glycoproteins containing closely spaced sialylated O-linked oligosaccharides [Norgard et al., 1993], almost completely abolished the binding of both human and Drosophila endolyn to all tested lectins (Fig. 2C). Interestingly, GFP-Endolyn could also be detected in the culture media of S2 cells, with the apparent molecular mass being slightly smaller than that from cell lysates (Fig. 2A), suggesting the existence of a secretory form of Drosophila endolyn protein.

### Subcellular Distribution of the Drosophila Endolyn Resembles That of its Human Counterpart

In contrast to GFP alone, which appeared to be highly soluble and homogeneously distributed in the cytosol with no visible aggregations (data not shown), GFP-Endolyn in S2 cells was distributed in the cytoplasm and appeared to be well compartmentalized (Fig. 3A). GFP-Endolyn

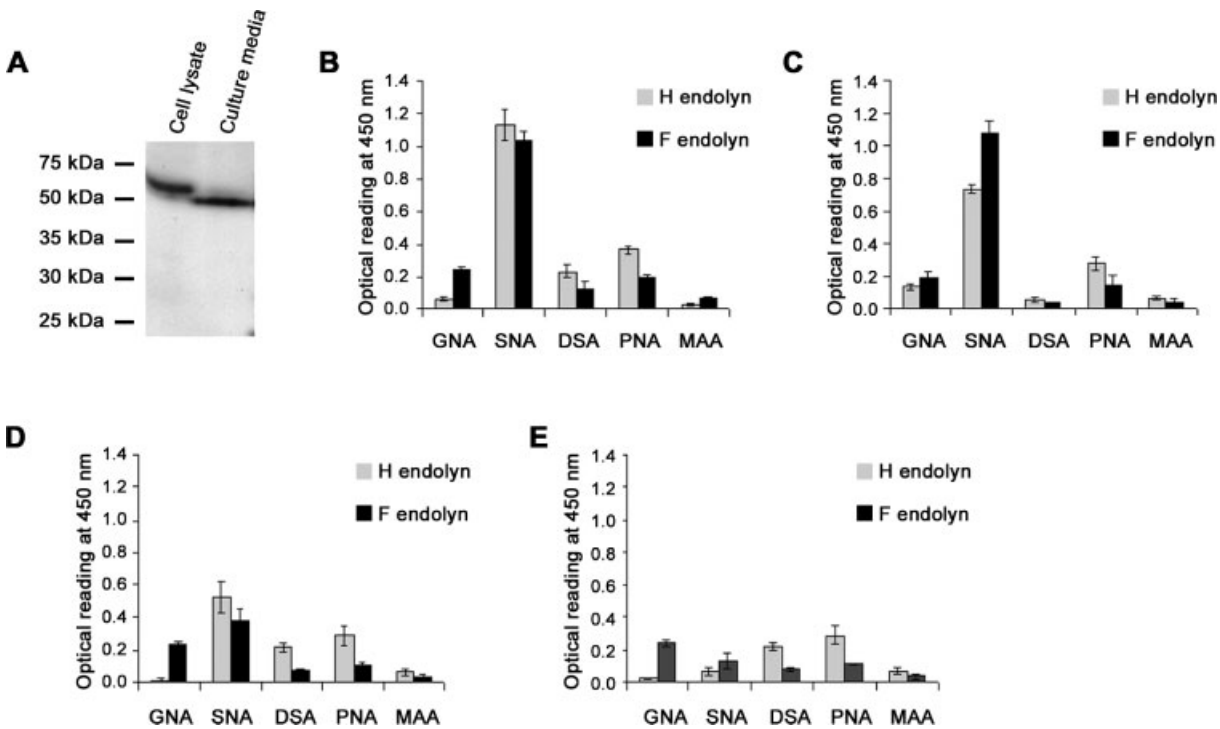


**Fig. 2.** Western blot and glycosylation analysis of recombinant GFP-Endolyn. Anti-GFP immunoblotting of S2 cell lysates and of culture medium from GFP-Endolyn expressing Drosophila S2 cells (**A**). Lectin binding of Drosophila (F endolyn) and human endolyn (H endolyn) from S2 and KG1a cells, respectively, before (**B**) and after treatment with N-glycosidase F (**C**), sialidase (**D**) and O-sialoglycoprotease (**E**). The specificity for GNA, N-linked high mannose; for SNA, sialic acid α(2–6) linked to N- or O-glycan structures; for DSA, galactose present in N-glycan structures; for PNA, galactose forming the core unit of O-glycans; and for MAA, sialic acid α(2–3) linked to N- or O-glycan structures. The data included in the analysis are means ± SEM from at least three experiments.

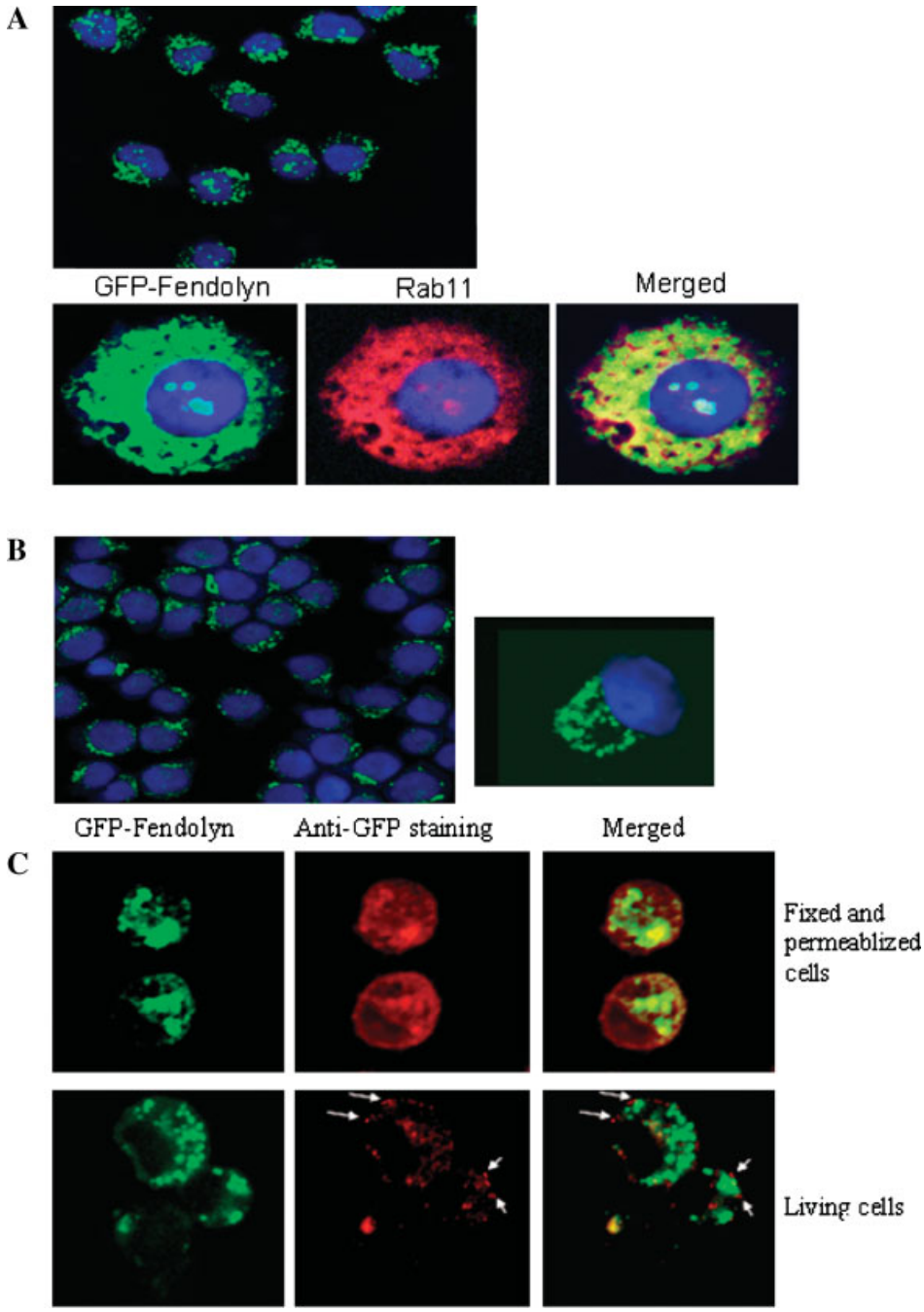


**Fig. 3.** Subcellular distribution of endolyn orthologs. GFP-Endolyn expression in Drosophila S2 cells and co-localization with Rab11 (**A**). Rab11 expression was detected by immunocytochemistry using an anti-Rab11 antibody followed by RPE-conjugated anti-mouse antibodies. GFP-Endolyn in S2 cells was detected by confocal microscopy. Nuclei are stained with TOTO-3 iodide (Invitrogen). Human endolyn in KG1a cells (**B**). KG1a cells were stained with the anti-human endolyn antibody, 105A5, followed by the secondary anti-mouse FITC-conjugated antibody. Comparison of GFP-endolyn in permeablized and live S2 Drosophila cells (**C**). The upper panel shows that anti-GFP labeling (red) and GFP fluorescence from GFP-Endolyn (green) were co-localized using a confocal fluorescent microscope when cells were fixed and permeablized; the lower panel shows that on living cells, anti-GFP labeling (red) was detectable on the cell surface with the GFP fluorescence from GFP-Endolyn also seen in the cytoplasmic vacuoles. Note weak staining with anti-GFP antibody on the surface of and not in the cytoplasm of living cells. Flow cytometry analysis of cells labeled in the same manner as C (**D**).

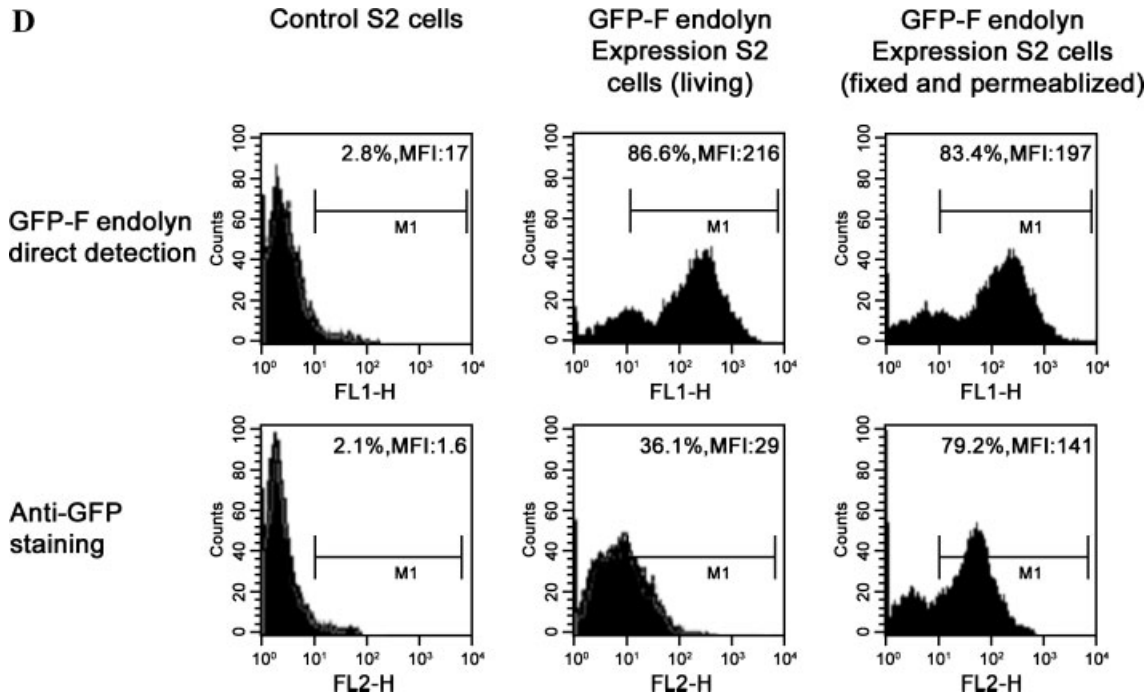


**Fig. 3.** (*Continued*)

was found to mainly co-distribute with Rab11, a small GTPase implicated in intracellular membrane fusion reactions and a known marker for protein trafficking, sorting, and recycling in the endosomal pathways [Prekeris, 2003]. The compartmentalization pattern of GFP-Endolyn was similar to that of human endolyn in non-polarized haematopoietic cells including cord blood $CD34^{+}$ cells, KG1a and Jurkat cells (Fig. 3B, Chan et al., 2001). While the majority of expressed GFP-Endolyn was present in intracellular compartments, a small amount of the fluorescent staining of living cells also revealed expression on the cell surface (Fig. 3C), as for its human counterpart [Chan et al., 2001]. The surface expression was also demonstrated by FACS analysis (Fig. 3D).

To further reveal the detailed structures where endolyn was localized, we conducted immunoEM studies of endolyn in KG1a cells and Drosophila S2 cells. In haematopoietic KG1a cells, examined for routine morphology, a number of small mitochondria and two types of vacuolar structures, consisting of vacuoles with electron dense contents (lysosomes) and lucent vacuoles containing variable numbers of vesicles (multi-vesicular bodies), were observed in the cytoplasm (Fig. 4A). When identical cells were examined by immunoEM after staining with the specific anti-endolyn antibody 105A5, it was observed that the positive staining was predominately associated with the intracellular vacuolar structures (Fig. 4B), although a few gold particles were also observed on the plasmalemma (Fig. 4B). The multi-vesicular vacuoles showed strong labeling with numerous gold particles on the limiting membrane and also the contents of the vacuoles (Fig. 4B and C). The specificity of the labeling was confirmed by the absence of gold particles when the primary antibody was omitted (Fig. 4D). In addition, certain cells contained larger vacuoles with membranous contents (secondary lysosomes) and these were also labeled with anti-endolyn (Fig. 4E). In the case of S2 cells, positive labeling using anti-GFP antibody was found associated with large intracellular structures (Fig. 5A) although a few gold particle were also observed on the plasmalemma (Fig. 5C). There were some variations in the sub-structure of the labeled vacuoles. Certain vacuoles had labeled electron dense contents (Fig. 5B) while, in others with electron lucent contents, the labeling was limited to the periphery (Fig. 5C). There were also vacuoles with myelin-like membranes with the staining limited to the membranous region (Fig. 5D). Occasionally, anti-GFP staining was also seen in some loose extracellular membrane

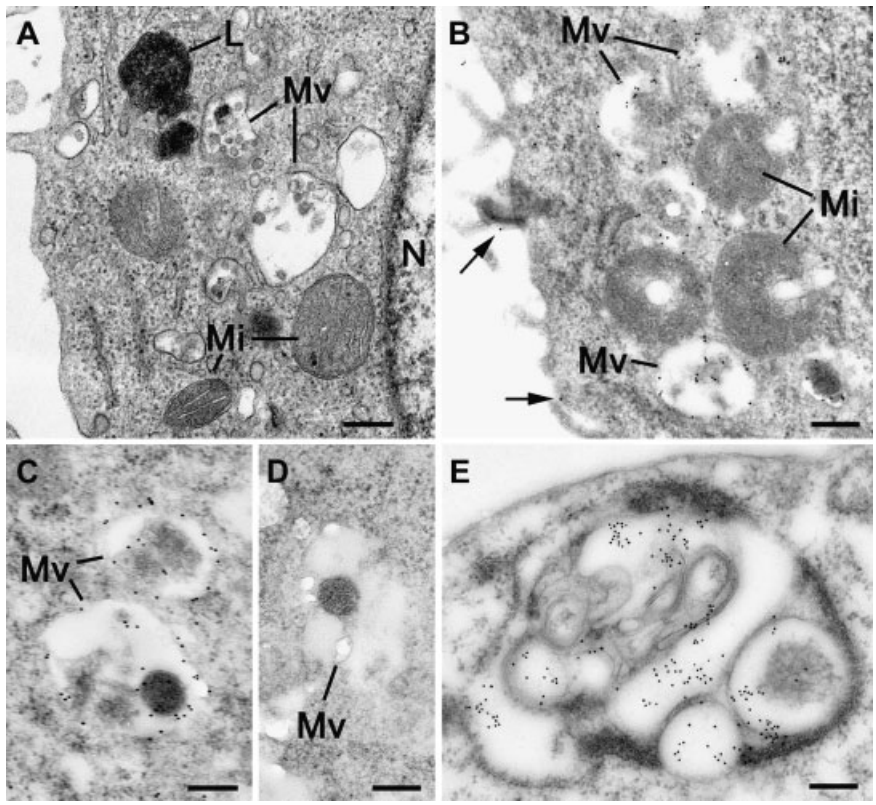


**Fig. 4.** Immuno-electron microscopic analyses of human endolyn in KG1a cells. Endolyn was labeled with anti-human endolyn Mab, 105A5, and visualized using rabbit anti-mouse IgG antibodies conjugated with 10 nm colloidal gold. Bars represent 200 nm segments. **A**: Part of a routinely processed KG1a cell showing the cytoplasm containing a nucleus (N), mitochondria (Mi) plus lysosome (L) and vacuole containing vesicular structures (Mv). **B**: Part of a KG1a cell processed for immuno-EM and stained with anti-endolyn Mab, 105A5, showing numerous gold particles associated with the vacuolar structures (Mv) but not the mitochondria (Mi). Note the few gold particles associated with the plasmalemma (arrows). **C**: Detail of the multi-vesicular vacuoles (Mv) in KG1a cells showing the gold particles located along the limiting membrane and also on the electron dense contents. **D**: Negative control in which the anti-endolyn Mab was omitted showing the absence of any gold label associated with the multi-vesicular vacuole (Mv). **E**: Detail of a larger vacuole with membranous contents that are strongly labeled with the anti-endolyn Mab, 105A5.

structures (not shown) that might be exosomes secreted from cells.

### The YXXΦ Motif is Required for the Correct Compartmentalization of Drosophila Endolyn

To examine whether or not the cellular and molecular machinery uses the YXXΦ motif in Drosophila cells, we mutated the YHTL sequence of the GFP-endolyn fusion protein to AHTL or YHTA. These mutations altered the intracellular distribution of the fusion protein, with most of mutated proteins being packed into large vacuoles close to the plasma membrane (Fig. 6). This was in contrast to the wild-type endolyn that appeared in small vesicles that were evenly distributed in the whole cytosol. The distribution of Rab11 was not altered by overexpressed GFP-endolyn in S2 cells (data not shown).

### Developmental Defects of Drosophila Mutants With a Disrupted Endolyn Gene

Endolyn mRNA expression was assessed using RT-PCR for the L1-to L3 larvae of all Drosophila strains and was found deficient in the GT-VSG and E4203 strains (Fig. 7A). All Drosophila endolyn mutants were homozygous viable, indicating that the gene product is dispensable for survival and fertility. Fecundity was not affected in any of the mutant lines examined, since the number of embryos produced from the same number of virgin female and male flies for each mutant was not significantly different (data not shown). Neither was longevity impaired, since mutant flies that survived into adulthood exhibited life spans comparable to endolyn wild-type flies. However, it was found that development of a proportion

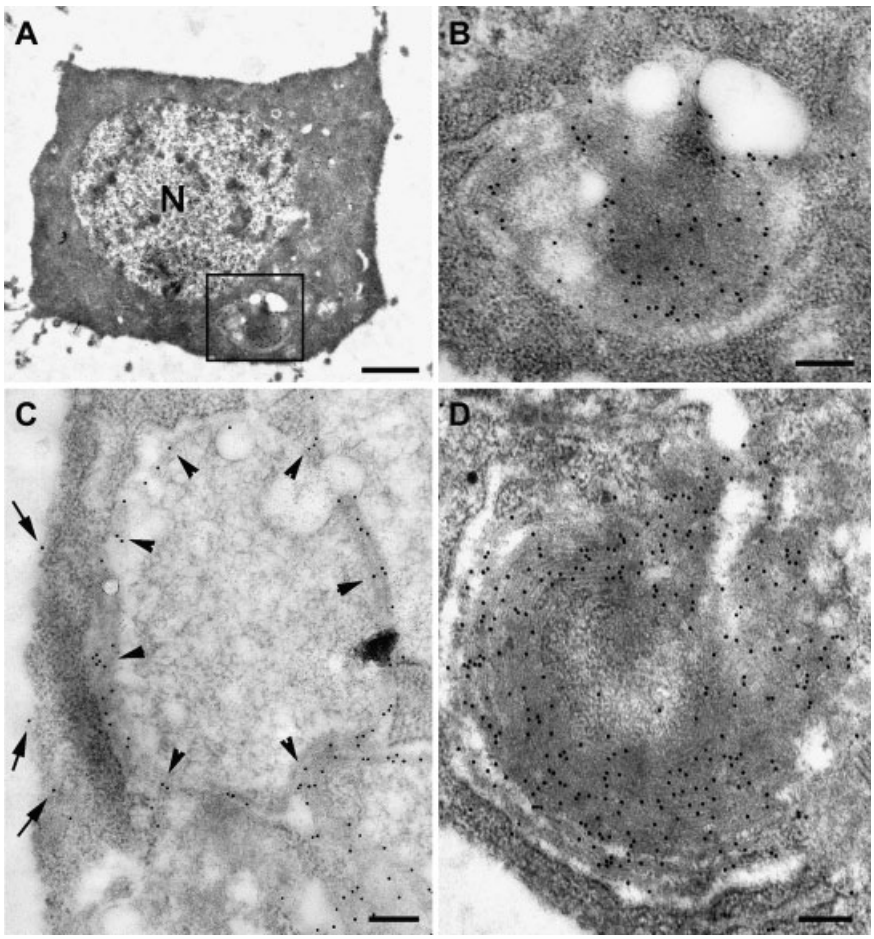


**Fig. 5.** Immuno-electron microscopic analyses of GFP-Endolyn expressed in Drosophila S2 cells. Drosophila endolyn was labeled using an anti-GFP antibody and visualized using rabbit anti-mouse IgG conjugated with 10 nm colloidal gold particles. **A**: Low power of an S2 cell showing the centrally located nucleus (N). Bar is 1 μm. **B**: Enlargement of the enclosed area showing a vacuole with electron dense content labeled with gold particles. Bar is 200 nm. **C**: Detail from the periphery of a cell showing a large vacuole with electron lucent contents in which the labeling is limited to the periphery (arrowheads). Note the few gold particles associated with the plasmalemma (arrows). Bar is 200 nm in length. **D**: Detail of a vacuole in a S2 cell showing the gold particles located along the multilayer membrane that encircle the vacuole. Bar is 200 nm.

of the embryos and larvae into adults was impaired, particularly with P-element insertion close to the transcription start site (Fig. 7B). Less than 50% of GT-VSG and E4203 embryos were able to develop into adults, compared to the wild-type. The development of first instar larvae (L1) was also impaired for the GT-VSG mutant, with only about 50% being able to

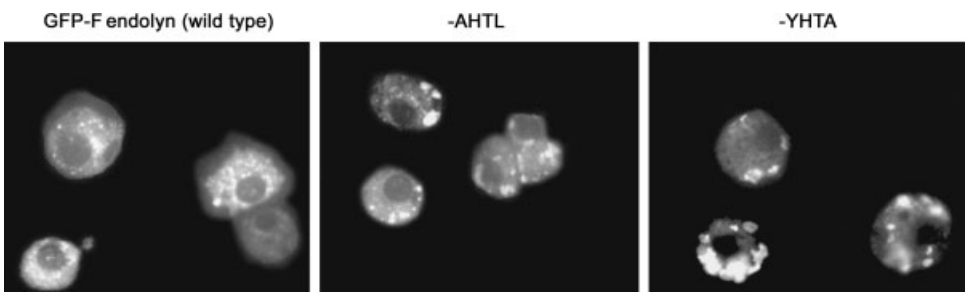


**Fig. 6.** Altered cellular distribution of GFP-Endolyn in Drosophila S2 cells after mutation of the YXXΦ motif. Both AHTL and YHTA mutations resulted in the accumulation of recombinant protein in large vesicles around the plasma membrane, while the wild-type GFP-Endolyn appeared as small vesicles evenly distributed within the cytosol.

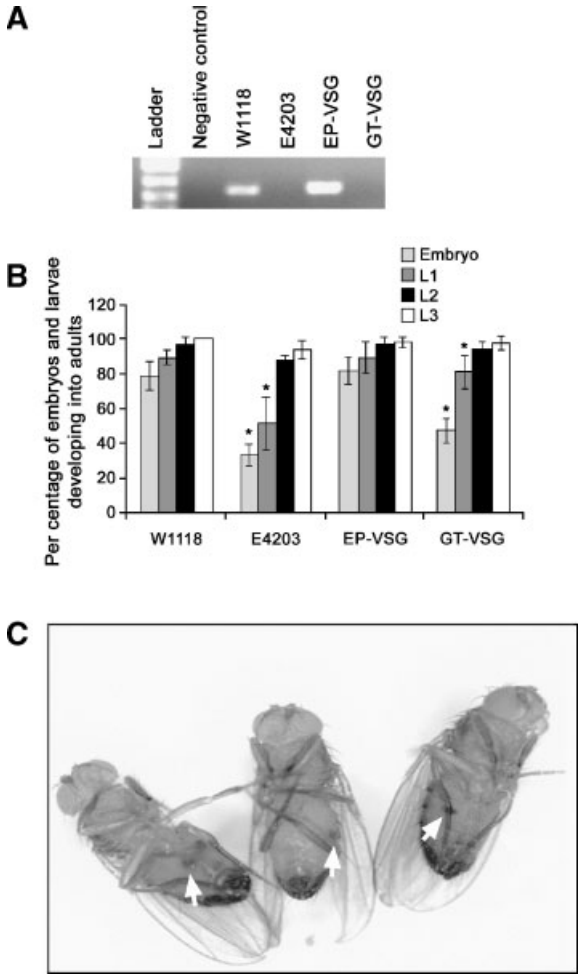


**Fig. 7.** Growth characteristics of Drosophila mutants with impaired endolyn expression. Endolyn mRNA expression in Drosophila mutants as determined with RT-PCR with total RNA extracted from larvae of different fly strains (**A**). Successful development into adults from embryos and larvae of wild type and edolyn-deficient mutants (**B**). One hundred embryos and larvae at three developmental stages (L1, L2, and L3) from the different strains described in the Materials and Methods and as indicated were separately cultured and the number of adult flies counted at 12 days post-egg laying. Results represent means ± SEM from three observations $^*P < 0.05$. Appearance of melanotic capsules (arrowed) occurring within fat bodies in GT-VSG mutant flies (**C**).

develop into the adult stage. Once the larvae had developed beyond the L1 stage, no obvious impairment of development into adulthood was observed (L2 and L3 histograms in Fig. 7B). In 20–30% of the third instar larval and adult flies of the GT-VSG strain, melanotic capsules, which were embedded in the fat bodies, were observed (Fig. 7C). The capsules had high density and did not contain identifiable cellular structures when observed under light microscopy following their dissection from the fat body.

## Ablation of Endolyn Inhibits Cell Proliferation

To shed light on the cellular mechanisms by which Drosophila endolyn is involved in the development, we transfected a fragment of dsRNA with the sequences derived from the coding region of Drosophila endolyn into S2 cells, in order to knock-down endolyn expression. The level of endogenous endolyn expression in endolyn dsRNA-transfected S2 cells was clearly lower than in untransfected cells or cells transfected with unrelated dsRNA (derived from the bacteria lacZ gene), as shown by RT-PCR (Fig. 8A) and by visibly decreased GFP-endolyn expression when transfected into GFP-endolyn expressing-cells (Fig. 8B). The decrease in GFP-endolyn expression level was quantified using flow cytometry and the median fluorescence intensity for GFP-endolyn expressing cells was decreased approximately 5-fold from 197 to just 40 after endolyn dsRNA transfection (data not shown). The specificity of endolyn knock-down was verified by the observation that the expression of an unrelated gene product, Rab11, was not altered. The introduction of LacZ dsRNA did not affect the expression levels of either endolyn or Rab11 (Fig. 8B). Together with the decreased endolyn expression, we observed that the growth rate of S2 cells was inhibited by 40–50% with endolyn knock-down relative to untreated cells or cells treated with LacZ dsRNA (Fig. 8C), particularly within the first week post-transfection. Figure 8D shows the significant reduction in cell number ($P < 0.05$) 48 h post-transfection of dsRNA, compared with the control cells described above. This growth inhibition was, at least in part, due to programmed cell death, since a proportion of cells were shown to be undergoing apoptosis. This is illustrated in Figure 8E for the 48-h time point by the increased caspase three activity. Furthermore, at this time point, approximately 15% of endolyn dsRNA-transfected S2 cells were positive for fluorescent-annexin V staining, an indicator of intermediate stages of apoptosis (Fig. 8F), while the untreated and LacZ dsRNA-transfected S2 cells showed lower and similar background levels of annexin V (2–4%). Similarly, about 20% of BrdU positive cells were detected in endolyn dsRNA-transfected cells, with low background levels being found in the untreated and LacZ dsRNA-transfected cells (Fig. 8G).

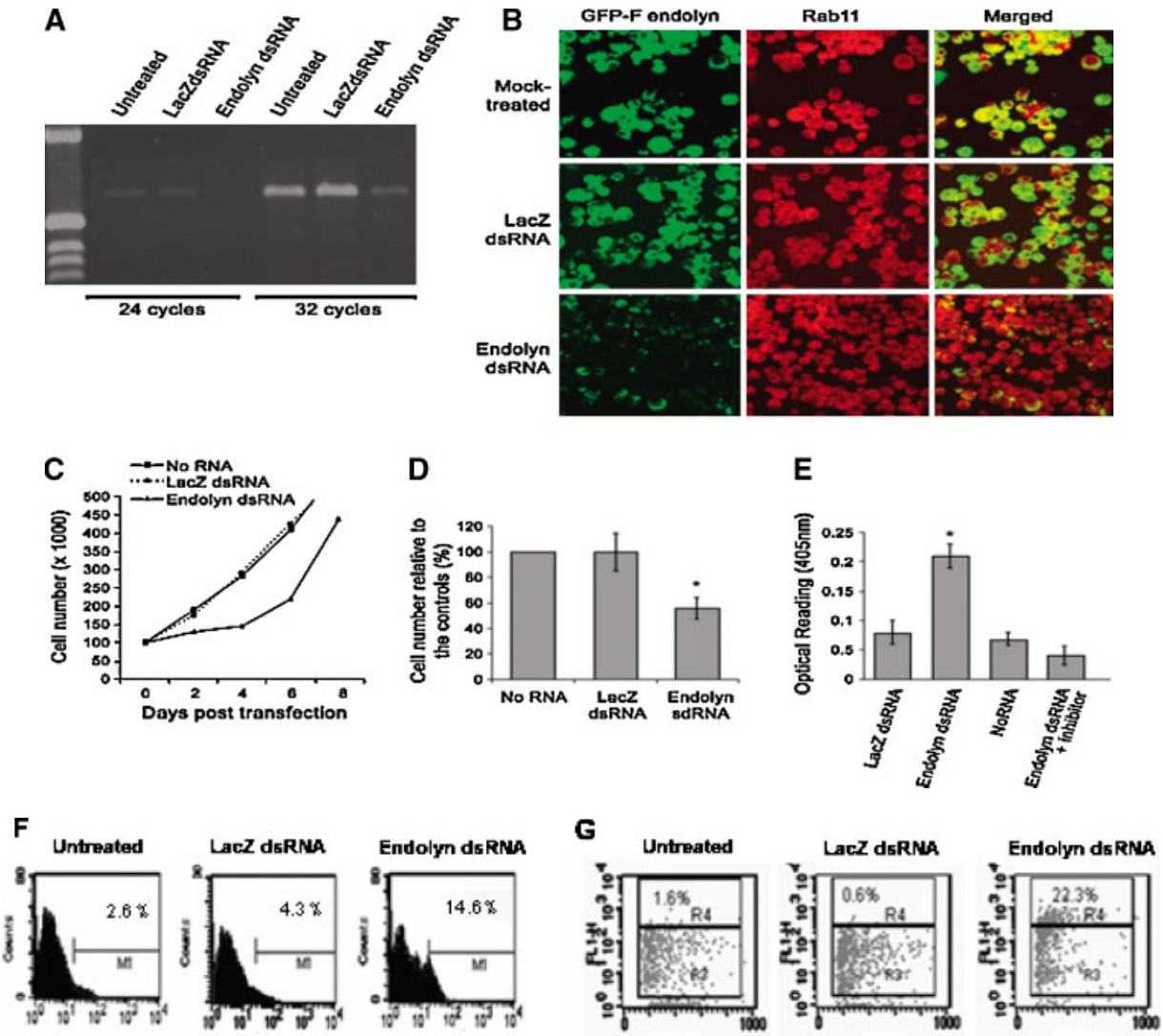


**Fig. 8.** Knocking-down endolyn inhibits Drosophila S2 cell growth. Endogenous endolyn mRNA expression was knocked down by introduction of endolyn dsRNA into S2 Drosophila cells. Endolyn mRNA was measured by RT-PCR at both the 24th and 32nd cycle of amplification (**A**). Expression of GFP-Endolyn, as a reporter in this case, was knocked down by endolyn dsRNA, while the expression of Rab11, an endosomal membrane protein, was not changed. LacZ dsRNA did not alter endolyn or Rab11 expression (**B**). A representative experiment showing the effect of endolyn dsRNA on the cell growth over 6 days, after which cells started to proliferate. The experiments were repeated 3 times (**C**). The growth of S2 cells was inhibited with endolyn knock-down (**D**). Caspase three activity was increased in endolyn dsRNA-transfected S2 cells, while caspase three activities in untreated or Lac Z dsRNA-transfected S2 were similar to the control, to which a caspase inhibitor (Z-VAD-FMK) was added (**E**). Data are means ± SEM with $*P < 0.05$. **F**: Approximately 15% of endolyn dsRNA-transfected cells were positive for the fluorescent annexin V staining. **G**: BrdU-fluorescein positive cells were detected in endolyn dsRNA-transfected S2 cells but not in untreated or Lac Z dsRNA-transfected cells. Separate boxes were drawn around cells that stain positive (upper box) and negative (lower box) with the fluorescein-BrdU mAb. Before detection, cells had been incubated with Br-dUTP in the presence of TdT enzyme in order to incorporate Br-dUTP into exposed 3′-OH DNA ends. Br-dUTP sites were then detected with a fluorescein-labeled anti-BrdU mAb. At least three experiments were performed to obtain data for cell growth and apoptosis assays. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

## DISCUSSION

In this study, we first compared the putative structures of endolyn orthologs in multiple species. We demonstrated that endolyn orthologs in Drosophila and the human are well conserved in several respects, namely the presence of extensive mucin domains and the localization in endocytotic organelles. We have also observed the developmental defects

in Drosophila mutants deficient in endolyn expression, and more interestingly, have extended this to reveal the function of endolyn in regulating cell proliferation.

Mammalian endolyn orthologs are highly conserved, containing identical transmembrane and cytoplasmic domains and showing the overall similarity (68–85%) higher than that (61–78%) shown by other known mammalian lysosomal membrane proteins including LAMPs and LIMPs. The amino acid sequence of endolyn orthologs in a broad range of species analyzed, in particular the transmembrane and cytoplasmic domains containing an unchanged lysosome-sorting signal YHTL, are also well conserved. This seems to be a unique feature of endolyn, since the putative orthologs of other mammalian endolysosomal proteins, such as mammalian LAMP-1/2, in Drosophila (CG3305), show much lower similarities (<30%). While the extracellular domains of all endolyn orthologs retain their mucin properties, with O-linked carbohydrates predominating in lower species, the presence of N-linked carbohydrates together with the occurrence of the cysteine-rich region in vertebrates most likely reflects the evolutionary tendency of endolyn and relates to its function on cells from higher species. The cysteine-rich domain in human endolyn has been shown to be necessary for the formation of dimers or oligomers [Watt et al., 1998]. This was not observed for Drosophila endolyn. In addition, Drosophila endolyn, like its mammalian counterparts [Matsui et al., 2000], can be detected in culture supernatants. This suggests that Drosophila endolyn may also exist as a soluble form. The mechanism for producing soluble endolyn in both the human and insects is unclear, but it may result from proteolytic processing of the membrane-bound form either at the cell surface or within the lysosome, followed by exocytosis from the cell, as has been speculated for human LAMP-1 [Meikle et al., 1999], or be generated by differential gene splicing [Kurosawa et al., 1999; Matsui et al., 2000]. While the soluble form of LAMP-1 has been shown to interact with transthyretin in the circulation [Chang et al., 2004], the functional significance of soluble endolyn is unclear, although over-expressed soluble endolyn modulates myocyte differentiation in the mouse [Lee et al., 2001].

In mammals, endolyn is delivered along an indirect route through the cell surface, re-internalized, and transported from early to late endosomes [Ihrke et al., 2001, 2004]. It has been previously demonstrated that the majority of endolyn is localized intracellularly, with only a small fraction of endolyn molecules reaching the surface of polarized epithelial or non-polarized hematopoietic cells [Chan et al., 2001; Ihrke et al., 2001]. Varying amounts of intracellular endolyn localize in early endosomes, recycling enodosomes and lysosomes in mammalian cells, with a large portion overlapping other lysosomal membrane proteins such as LAMP-1 [Doyonnas et al., 2000; Chan et al., 2001]. We have shown here, as well, that Drosophila endolyn distributes mainly in intracellular vesicles, with a minor amount detectable on the cell surface. Some endolysosomal proteins such as LIMP-II can also be detected on the surface of white blood cells and platelets that are activated [Nieuwenhuis et al., 1987; Mahmudi-Azer et al., 2002], or of cytotoxic T-lymphocytes that exocytose granules during specific interaction with target cells [Pfistershammer et al., 2004]. Hennersdorf et al. [2005] have further shown that cell surface expression of human endolyn may also serve as a basophil-activation marker. The presence of endolyn on the cell surface reaches maximum levels after 5–15 min of stimulation with tetradecanoylphorbol-13-acetate, whereas the maximum upregulation of LIMP-I (CD63) and LAMP-1 (CD107a) is detected only after 20–40 min and these reach maximum levels after 60 min. Due to limited information available for Drosophila lysosomal proteins and the lack of detection reagents, we were unable to compare endolyn with endolysosomal membrane proteins other than Rab11 for their cellular trafficking and localization in the current study. In addition, a large number of glycan moieties contained in endolyn and in the other major lysosomal membrane proteins, LAMPs and LIMPs, may be important for them to maintain their function under the high hydrolytic and low pH environment of the lysosome [Silva and Gordon, 1999; Tomlinson et al., 2000].

The conservation of the cytoplasmic motif, YXXΦ, across all the species analyzed is of particular interest. In lower species, the role of this cytoplamic motif has only been demonstrated in one yeast protein very recently [Sun et al., 2004]. We have now provided the experimental evidence that the YXXΦ motif contained in Drosophila endolyn functions in mediating

its appropriate intracellular trafficking and localization, further suggesting that both the cellular organelles and the molecular machinery for the general endocytotic pathways, including intracellular trafficking of endolyn, are conserved in insects. The asparagine (N) residue prior to the YXXΦ motif in the mammalian endolyn cytoplasmic tail determines the specificity of recognition by AP-3 [Ihrke et al., 2004]. This residue was also found to be conserved in all known endolyn orthologs. N-glycosylation at certain positions has been suggested to be mandatory for mammalian endolyn delivery to plasma membrane [Potter et al., 2004]. Since there is little N-glycosylation present in Drosophila endolyn, the process of delivering Drosophila endolyn to the plasma membrane may therefore be mediated by a mechanism distinct from that in mammalian cells.

Gene expression profiling has shown that, at the all stages of Drosophila embryo development, endolyn expression levels remain constant in wild type Drosophila (http://www.fruitfly.org/cgi-bin/ex/insitu.pl). In situ hybridization studies further show that after the maternal-contributed signal declines, endolyn expression occurs at later stages of embryogenesis in the dorsal ectoderm primordium (stages 9–0) and the dorsal epidermis primordium (stages 11–12) (http://www.fruitfly.org/cgi-bin/ex/insitu.pl). The strongest expression is observed in embryonic/larval garland cells (stages 13–16), which are thought to function as nephrocytes and to have a rapid rate of fluid-phase endocytosis [Kosaka and Ikeda, 1983]. Although similar expression studies were not performed for the mutants included in this work, expression of endolyn mRNA was not detectable in larvae of the two fly strains, GT-VSG and E4203, and developmental arrest of embryos and early larvae with endolyn-deficient expression was often observed. Taken together, this information suggests that endolyn may indeed play an essential role at the early stages of Drosophila development, although a memory defect in the adult E4203 line has also been reported [Dubnau et al., 2003]. The presence of melanotic capsules in the GT-VSG but not E4203 line, which both lack endolyn mRNA, may reflect a difference in genetic backgrounds of the two strains, with E4203 being W1118 (cisocj1); Ecol\LacZVSG-1 and GT-VSG W1118; P{W + mGT = GT1} VSGBG00708, or a difference in the positions of the P-element insertions. Whichever is the case, the significance of the melanotic capsules in GT-VSG and their relevance to endolyn deficiency requires further study.

As an initial step in deciphering the possible cellular and molecular mechanisms underlying the developmental defects seen in the Drosophila mutants, we knocked down endolyn expression in Drosophila S2 cells in vitro and showed both apoptosis and inhibition of their proliferation. The results are in line with our previous studies that reveal a function for endolyn in regulating the proliferation of human $CD34^{+}CD38^{-}$ hematopoietic stem/progenitor cells [Watt et al., 1998; Chan et al., 2001].

Endolyn is not unique amongst the endolysosomal-related proteins that have been shown to be important for the cell growth control. Hsp70, for instance, has been demonstrated to promote cell survival by inhibiting lysosomal membrane permeabilization, while the depletion of Hsp70 triggers the death of certain cell types [Nylandsted et al., 2004]. Accumulation of autophagic vacuoles with myopathy has been observed in LAMP-2- and LAMP-1/2 double-knock out mice, which also display elevated mortality and reduced weight compared with their wild-type littermates [Tanaka et al., 2002; Eskelinen et al., 2002]. The results described here show that endolyn contributes to both cell proliferation and embryonic development. In support of this cell proliferation related role, our preliminary results show that human endolyn is upregulated in many malignant tumors, which display dysregulated growth.

Further understanding of the molecular mechanism underlying the inhibition of proliferation remains to be investigated. It would be possible that the altered expression of endolyn modulates the endocytic process at a general mechanistic level or in respect to specific receptors. Using electron microscopy, we could not, however, observe any apparent morphological differences in the hemocytes dissected from the wild-type and endolyn-deficient fly strains. The bulk endocytosis rate of FITC-dextran by these cells appeared not to be changed either. The binding of a ligand to cellular receptors often initiates a series of events including the activation of receptor and endocytosis of the activated ligand-receptor

complex to the endolysosomal system, from where they may be returned to the plasma membrane and participate in further signaling, or delivered to lysosomes and degraded, resulting in signal attenuation. Whether endolyn plays a role in these processes has not been determined for specific receptors, although our recent unpublished results show that human endolyn interacts with and modulates the function of the chemokine receptor, CXCR4, in human hematopoietic precursor cells. Our studies on Drosophila endolyn will hopefully contribute to our further understanding of the functional significance of endolyn and to the identification of its interacting partners.

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