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Research Article

The *Drosophila* reticulon, Rtnl-1, has multiple differentially expressed isoforms that are associated with a sub-compartment of the endoplasmic reticulum

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Abstract. The reticulons are a recently discovered family of proteins that have a predominant localisation to the membrane of the endoplasmic reticulum. The precise function of the reticulons is unclear despite their presence in a wide variety of eukaryotic organisms. Here we describe the characterisation of the *Drosophila* reticulon, reticulon-like1 (Rtnl1), which is the only functional reticulon in *Drosophila*. The *Rtnl1* locus produces seven predicted mRNA transcripts encoding five different protein isoforms. The different transcripts have tissue-specific expression patterns remarkably similar to their mammalian counterparts. Rtnl1 protein is associated with organelles of the secretory pathway including the endoplasmic reticulum and the Golgi apparatus. Rtnl1 function appears to be non-essential or redundant since loss of function *Rtnl1* mutants are viable. However, a significant reduction in life expectancy was seen in *Rtnl1* mutant flies. This may point towards a possible protective role for reticulons against conditions of environmental stress.

Keywords. Reticulon, *Drosophila,* endoplasmic reticulum, expression, isoforms, mutation.

Introduction

The reticulons are a recently discovered family of proteins that derive their name from their predominant localisation to the membrane of the endoplasmic reticulum (ER) [1]. The reticulons were originally identified as markers for carcinomas with neuro-endocrine characteristics [2]; subsequently reticulons have been identified in all eukaryotic organisms studied to date including yeast, plants and fungi, suggesting an evolutionary conserved role for these proteins in the eukaryotic cell [3].

All reticulons possess a highly conserved ~200 amino acid reticulon homology domain (RHD), generally located at the C terminus of the protein. This RHD is characterised by the presence of two large (~35 amino acid) putative transmembrane hydrophobic stretches separated by a 66-amino acid hydrophilic loop [4]. In contrast, the N-terminal regions of reticulons are highly divergent, and share no similarity to known domains or sequence motifs [3].

In the chordates four major reticulon paralogues can be identified: *RTN1*, *RTN2*, *RTN3* and *RTN4*/*Nogo* [5]. Each paralogue encodes several different mRNA transcripts that arise through alternative splicing and/or differential promoter usage and consequently, several isoforms are produced [3, 6]. These isoforms always share the same C-terminal RHD but differ at the N terminus. The C-terminal RHD of mammalian paralogues share 70% identity to each other, but in contrast, no similarity exists between paralogous N-terminal regions [5], suggesting that a conserved reticulon function most likely resides in the RHD [3]. It is possible RHD function may be modified through

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specific properties provided by the N terminal regions and this notion is supported by the different tissue-specific expression patterns seen for different isoforms. For example RTN2-B is localised to the nervous system, while RTN2-C is highly expressed in skeletal muscle [7], and RTN4/Nogo-B has ubiquitous expression, while RTN4/Nogo-A and RTN4/Nogo-C are enriched in nervous and skeletal tissues respectively [8].

The precise function of the reticulons has been unclear but roles for these molecules have begun to emerge. In particular, identification of RTN4/Nogo-A as a potent myelin-derived inhibitor of neurite outgrowth has led to intensive research into its potential role in prevention of axonal regeneration following spinal injury [9, 10]. RTN4/Nogo-A was identified through its recognition by the monoclonal antibody IN-1 [5, 11, 12], which improved axon outgrowth and functional recovery when infused into the site of a neural lesion in certain animal models [13–15]. In addition, reticulons have been identified as binding partners to several proteins linked to neurodegenerative diseases (reviewed in [16]), including β-amyloid converting enzyme (BACE) that processes amyloid precursor protein (APP) in Alzheimer's disease [17, 18], and most recently Spastin that is linked to hereditary spastic paraplegia [19]. Analysis of *RTN4*/*Nogo-B* knockout mice suggests *RTN4*/*Nogo-B* may regulate vascular remodelling following injury [20]. Reticulons have also been implicated in apoptosis through interactions with members of the anti-apoptotic Bcl-2 family [21] and the ability of RTN4/Nogo-B to induce apoptosis in certain carcinoma cell lines [22]; however, this pro-apoptotic effect may be a consequence of protein accumulation in the ER [23]. Clues to the cellular function of reticulons have also come from their interaction with several constituents of endocytic and secretory pathways including *C. elegans* Rme-1 [24], SNAREs [25] and *S. cerevisiae* Yip3p [26], although the functional significance of these interactions remains largely obscure.

Reticulons are predominantly localised to the ER [1, 21], although there is evidence that they are additionally localised to the Golgi apparatus [3, 17, 27]. Uniquely amongst the reticulons, a small proportion of the RTN4/Nogo protein pool has been found at low levels on the cell surface; however, this appears to be cell type specific [5, 28, 29]. Within the ER the reticulons may not be uniformly distributed, but may instead localise to a sub-compartment. RTN1-C has been found to co-localise with the ER marker SERCA2, but not with another ER marker, calreticulin [25]. Recently, it has been demonstrated that RTN4/NogoA is associated with the tubular ER and is excluded from the nuclear envelope and the sheet ER [30]. Voeltz et al. [30] also presented compelling evidence that the major function of RTN4/Nogo is in the formation of the tubular ER.

Here we describe the characterisation of the *Drosophila* reticulon *reticulon-like 1* (*Rtnl1*). We describe the molecular organisation of the *Rtnl1* locus and demonstrate that in common with their chordate relatives the locus encodes several isoforms that display differential patterns of expression in the developing embryo. Using GFP-tagged versions of Rtnl1 and antibodies to the protein, we revealed that Rtnl1 is localised to a sub-compartment of the ER. We also showed that removal of *Rtnl1* from the *Drosophila* genome decreases the lifespan of the organism, suggesting an important but non-essential role for this gene.

Materials and methods

Drosophila strains. The G9 insertion in *Rtnl1* and G198 insertion in *Pdi* were from a protein trap screen [31]. The NP7026 insertion was obtained from the Kyoto *Drosophila* Genetic Resource Center (http://www.dgrc.kit.ac.jp/en/index.html). The $Rtnl1^{1}$ mutation was generated using a combination of male recombination and P-element excision. To put the $Rtnl1^{1}$ mutation in an isogenised background, the chromosome carrying the mutation was backcrossed for two generations to the second chromosome carrying the original P-insertion (NP7026). A chromosome isogenic to this one was then derived by crossing together two other Chr 2 insertions from the NP collection and recombining off both P-element insertions. Isogenised Chr X and Chr 3 were provided by crossing to the isogenised stocks available from the Bloomington Stock Center (BL-5905, BL-5906, and BL-5907). Oregon R flies were used as the wild-type strain. All strains were raised at 25 °C on standard cornmeal agar medium.

Live GFP imaging. Embryos were dechorionated for 5 min in 50% sodium hypochlorite, rinsed in distilled water, mounted in Voltalef halocarbon oil (Atofina) and visualised using a Zeiss LSM510 confocal microscope.

Immunohistochemistry. Embryos were staged according to Campos-Ortega and Hartenstein [32]. Embryos were fixed for 20 min with 4% paraformaldehyde and stained according to standard protocols as described by Patel [33]. Following dissection, third instar eye discs and brains were processed in the same way. Third instar muscle dissections were carried out as described by Lin et al. [34]. Primary antibodies were used at the following dilutions: rabbit anti-GFP (Molecular Probes) 1 : 1000; Cy3-anti-HRP (Jackson ImmunoResearch) 1 : 500; 22C10 (Developmental Studies Hybridoma Bank, [35]) 1 : 5; rat polyclonal anti-Rtnl1 raised against Rtnl1-PB/PE isoform (gift of W. Chia, National University of Singapore), 1 : 100. Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories and used at a dilution of 1 : 300 (FITC) to 1 : 600 (Cy3). Embryos and larval tissue were cleared in Vectorshield (Vector Laboratories) and mounted on slides for viewing.

***In situ* hybridisation.** *In situ* hybridisation was performed as described by Tear et al. [36]. To create probes specific to the unique 5′ exons of each transcript precise regions were amplified from genomic DNA (*Rtnl1-RB*/*RE* and *Rtnl1-RA*), cDNA extracted from whole embryos (*Rtnl1-RD*/*RG*), or expressed sequence tags (ESTs) (*Rtnl1-RF* using EST RH01247 from the Berkeley *Drosophila* Genome Project). These regions were either cloned into the dual RNA transcription vector pCRII-Topo (Invitrogen) for the production of sense and anti-sense riboprobes or amplified with a 3′ primer that included a T7 promoter site (underlined) and a 5′ primer that included a T3 promoter site (dashed underline) and used directly as a template for RNA transcription of anti-sense and sense riboprobes, respectively. Probes were purified using Ambion Megaclear columns. The *Rtnl1-RA* probe consists of a direct repeat of two copies of the first exon of *Rtnl1-RA* (forward primers: TTCAGACGGTCGGCCGAAT and TAGCTAGCGAGTTTTCCCAATCGTCCCATTCGTGTG; reverse primers: GGGAAAACTCGCTAGCTAAAGCGGCTGAAAATTGCC and TGGTCCGCGTTCTGGAAG). The *Rtnl1-RD*/*RG* probe consists of the first two exons of *Rtnl1-RD* (forward primer: CACGCTAGCCAACGTCACATTGCCCCAG; reverse: CAACGACTCGCGGTTCAG). The *Rtnl1-RB*/*RE* probe consists of the first exon of *Rtnl1-RE* (forward primer: AATTAACCCTCACTAAAGGGGGAACGTCAAACGTAGGAG; reverse primer: GTTAATACGACTCACTATAGGTGGGATCGAGTATGGAGC). The *Rtnl1-RF* probe consists of a 1.1-kb sequence from the open reading frame (ORF) of *Rtnl1-RF* (forward primer: ACGAGGACATCTTCAAGCAG; reverse primer: AGAAGATCTCCTCGACGGAC). The common *Rtnl1-RHD* probe hybridises to the ORF within the four RHD-encoding exons (forward primer: AATTAACCCTCACTAAAGGGTACTTGTCGCTCCTAACCC; reverse primer: TAATACGACTCACTATAGGGTTACTTGTCCTTCTCAGACTC).

S2 cell immunocytochemistry. S2 cells suspended in PBS were incubated in Nunc Lab-Tek II chamber slides and allowed to adhere for 10 min to the coated surface before fixing in 2% paraformaldehyde for 15 min at room temperature. Cells were washed three times for 5 min in PBS, and incubated with primary antibodies in PBS 0.1% Tween-20, 5% normal goat serum for 1 h at room temperature. After three 5-min washes in PBS, cells were incubated in secondary antibody in PBS for 1 h and washed again three times for 5 min in PBS before mounting in Vectorshield. Primary antibody dilutions were as follows: anti-Rtnl1, 1 : 200; mouse anti-KDEL (Stressgen) 1 : 50; guinea pig anti-Boca (gift of R. Mann [37]), 1 : 200; mouse anti-Golgi (Calbiochem, Stanley et al. [38]), 1 : 50. Secondary antibodies were as described above.

Rtnl1 gene model and Clustal W analysis. The gene model shown is based on the prediction available from Flybase (http://flybase.org/). Sequences corresponding to the RHD of each protein were aligned using Clustal W [39]. The resulting phylogenic tree was plotted using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). GenBank accessions for the sequences used in the alignments are as follows: CAE48546, AAF52196, AAF54008, NP_996734, NP_996784, NP_958832, NP_008939, NP_001007597, NP_001020535, AAR98631, NP_077188, AY316183, AY316191, AY495963, AY495964, BK004058, BK004007, BK004057, BK004012, AAT78355, AAT64106, AAT64112, AAT64119, AAT64120, AAT64133, AAT64105, CAB37626, NP_010519, NP_010077.

Lifespan studies. Flies were reared at standard larval density and adults collected over a period of 24 h. Lifespan was scored using males as standard [40] (10 flies per vial) on regular food medium at 29 °C. Increased oxidative stress at this elevated temperature reduced the length of the assay but gave qualitatively the same outcome as flies raised at room temperature. Flies were scored every 3–4 days and transferred to fresh vials. The Log-Rank test was performed using the R statistical software package.

Results

We identified a functional *Drosophila* reticulon in a screen to identify proteins enriched in axons of the developing *Drosophila* embryonic nervous system. This screen made use of a library of *Drosophila* lines expressing GFP-fusion proteins generated by Morin et al. [31] in which an exon encoding GFP is inserted within introns of genes at random. Each line expresses a GFP-fusion protein from their endogenous locus. One line, G9, identified a GFP-fusion protein that was expressed throughout the embryo but whose expression becomes upregulated in the axons of late stage embryos (Fig. 1a, b). This line was selected for further study and the insertion was identified by inverse PCR to be within a *Drosophila* reticulon gene, *reticulon-like 1* (*Rtnl1*, Fig. 2). We performed a Clustal analysis, which confirmed that *Drosophila Rtnl1* bears no closer resemblance to RTN1, RTN3 or RTN4/Nogo, but is more similar to this group than it is to RTN2 (Fig. 2a). RTN2 has previously been identified as a particularly divergent member of the chordate reticulon family [3]. This suggests the cellular function of *Rtnl1* may be common to mammalian reticulons, but it is unlikely *Rtnl1* will share with RTN4/Nogo the more specialised function of neurite outgrowth inhibition. An additional reticulon-like sequence is also present in the *Drosophila* genome, *Rtnl2,* which is more similar to RTN2; however, no expression of this gene was detected

in the embryo (data not shown) and no ESTs exist in public databases from wild-type flies maintained under standard laboratory conditions.

The expression of Rtnl1-GFP extends into post-embryonic stages where it has a similar pattern to that seen in the embryo. In third instar G9 larvae, expression of Rtnl1-GFP is also found in all cells of the animal with high levels obvious within neural tissue. At the neuromuscular junction Rtnl1-GFP is expressed within axons and within the synaptic boutons (Fig. 1d–g) and Rtnl1-GFP is highly expressed within photoreceptor axons that project from the developing eye disc to the optic lobes within the brain (Fig. 1h–j). The Rtnl1-GFP has a punctate pattern in photoreceptor axons, while in muscles Rtnl1-GFP shows a striated expression pattern (Fig. 1d).

Closer examination of Rtnl1-GFP expression in epithelial cells of gastrula stage embryos, revealed a feathery pattern in the cytoplasm (Fig. 1c) consistent with a localisation to the ER as reported for other members of the reticulon family [1, 21].

Genomic organisation of the *Drosophila Rtnl-1* locus. Annotation of the sequenced *Drosophila* genome had previously identified the *Drosophila Rtnl1* locus (see http://www.flybase.org). This annotation together with information from over 100 ESTs has revealed that

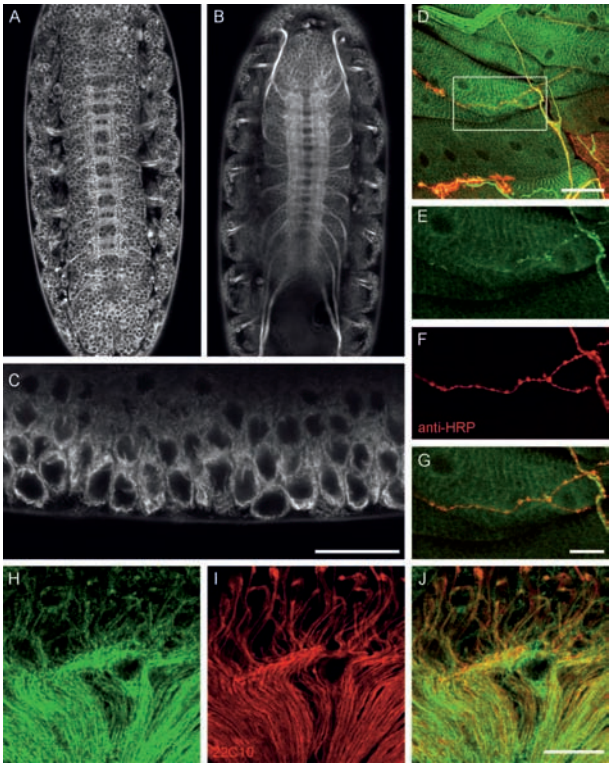

Figure 1. *Rtnl1* is ubiquitously expressed and up-regulated in the axons of late stage *Drosophila* embryos. (*a–c*) Rtnl1-GFP expression in live G9 embryos. (*a*) Rtnl1-GFP is expressed ubiquitously within the embryo. Towards the end of embryogenesis at stage 17 (*b*), an up-regulation of Rtnl1-GFP expression in axons, particularly motor axons, is observed. At the earlier gastrula stage (*c*), the localisation of Rtnl1-GFP in the invaginating epithelial cells of the embryo has a feather-like appearance, suggesting localisation to the ER. (*d–j*), Rtnl1-GFP expression in third instar larvae (fixed tissue). (*d–g*) Rtnl1-GFP (green) is expressed in a striated pattern in the muscles (*d*) and localises to the boutons of the NMJ (*e–g*), which are visualised using anti-HRP (red) that recognises an epitope present on all *Drosophila* neuronal membranes. The region highlighted by the box in (*d*) is shown in (*e–g*). (*h–j*) Axons projecting from the photoreceptors towards the optic lobes in the third instar eye disc visualized using mAb 22C10 that recognizes the microtubule-associated protein, Futsch (red). (*h*) A punctate pattern of Rtnl1-GFP immunoreactivity (green) can be seen within the photoreceptor axons. Scale: for *c*, *g* and *j*, bar represents 20 µm; for *d* bar represents 50 µm. *a*, *b* and *h–j*: anterior is top. *c*: dorsal is top and anterior to the left.

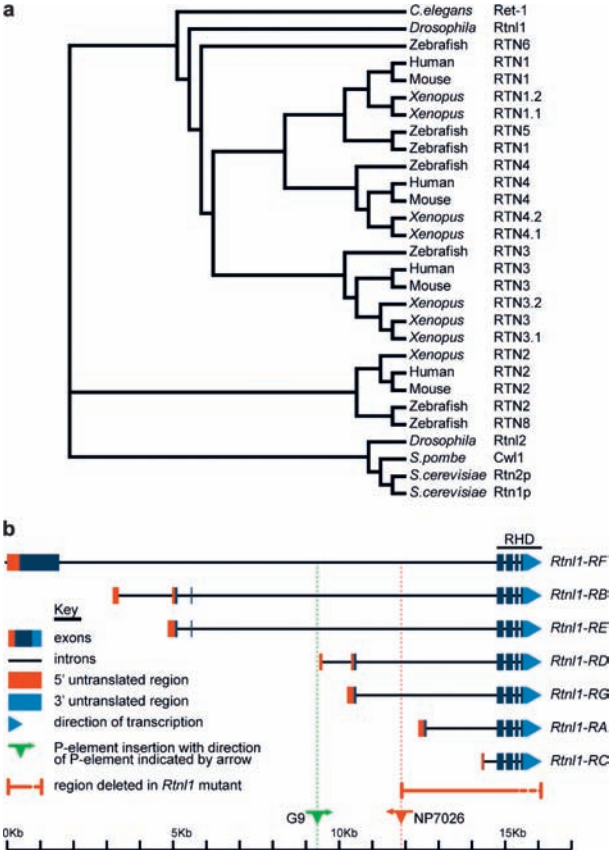

Figure 2. The *Rtnl1* locus and the relationship between Rtnl1 and other reticulons. (*a*) Cladogram obtained by performing a Clustal W alignment of the RHD encoded by reticulon genes identified in major model organisms. Both *Drosophila* Rtnl1 and *C. elegans* Ret-1 align closely with vertebrate reticulons, whereas yeast reticulons and Rtnl2 are more distantly related. (*b*) Scale diagram of the *Rtnl1* gene locus with the organization of the transcripts predicted by Flybase and confirmed by EST data. The common RHD is encoded by four exons at the 3′ of the gene. The transcripts *Rtnl1-RF*, *-RA* and *-RC* have unique 5′ exons, while *Rtnl1-RB* and *-RE* share exons and encode the same isoform. Similarly, *Rtnl1-RD* and *-RG* share exons and encode the same isoform. The locations of P-element insertions used in this study are indicated by the coloured triangles. The mutant produced in this study contains a deletion of the region indicated by the red bar. The precise endpoint has not been determined but lies within the 3′UTR of *Rtnl1* (dashed red line).

the gene is transcribed from seven promoters producing seven transcripts, *Rtnl1-RA* to *Rtnl1-RF,* which encode five different polypeptides (Fig. 2b). Each of these transcripts include at their 3′ end the four C-terminal exons that encode the RHD. This production of multiple transcripts from the reticulon locus is a common feature found for the reticulons. The unique N-terminal regions of the Rtnl1 isoforms are not homologous to N-terminal regions of reticulons from other species, except for the closely related species *Drosophila pseudoobscura*. Nevertheless, the N-terminal regions of Rtnl1 do share some characteristics common to the mammalian reticulons in that these N-terminal regions are generally acidic and contain a large number of proline residues. This organisation conforms to the model that a basic function is provided by the RHD that is modified by the appendage of unique N-terminal sequences.

The GFP-protein trap insertion in line G9 is located to the 5′ side of the start of the *Rtnl1-RD* transcript, so that it can only be spliced into three mRNA transcripts: *Rtnl1-RF*, *-RB* and *-RE*; these encode two protein isoforms: Rtnl1-PF and Rtnl1-PB/E (Fig. 2b). Thus, the Rtnl1-GFP expression pattern represents a combination of the expression patterns of these proteins and does not give information on the expression of individual *Rtnl1* transcripts.

***Rtnl1* mRNA transcripts are expressed in tissue-specific, developmentally regulated patterns.** Previous reports describing the expression patterns of the different reticulon isoforms encoded by each vertebrate reticulon gene have revealed that they are generally expressed in tissue-specific and developmentally regulated patterns (for example [6, 8, 41, 42]). To determine whether this is also the case for *Drosophila Rtnl1*, we designed *in situ* riboprobes recognising the short, unique 5′ exons of several *Rtnl1* transcripts and hybridised them to wild-type embryos (Fig. 3). Due to the short length of the unique *Rtnl1-RB* and *-RE* 5′ exons (Fig. 2b), we de-

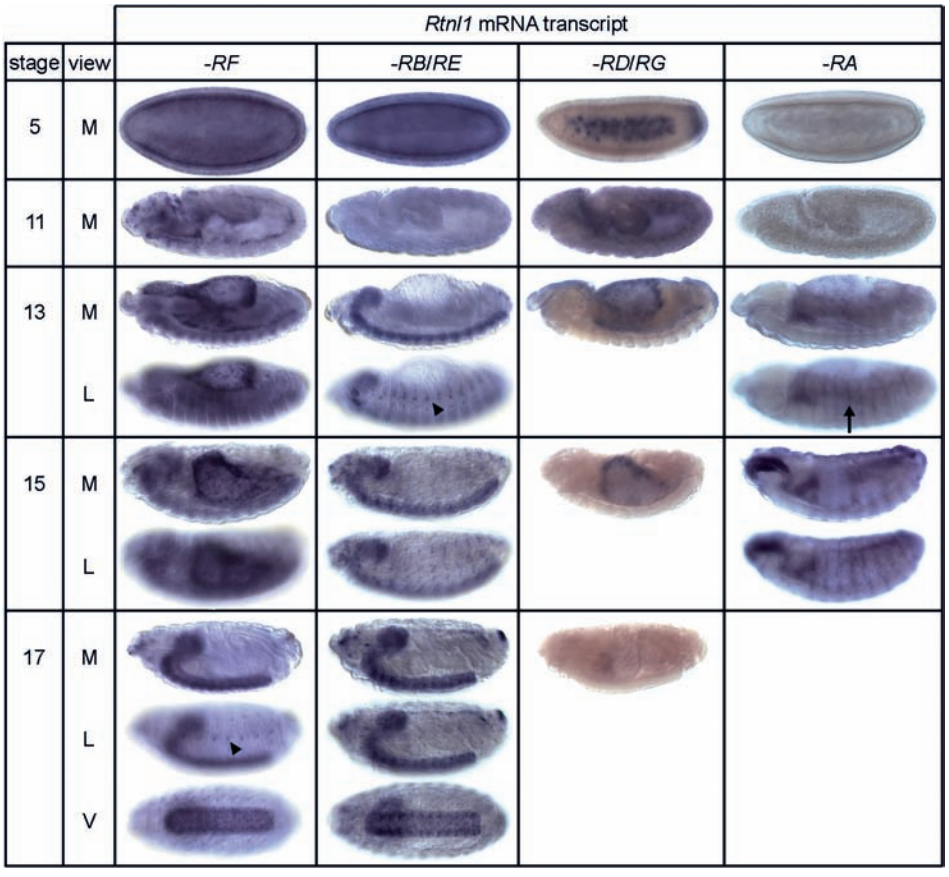

Figure 3. Individual *Rtnl1* transcripts are expressed in tissue-specific and developmentally regulated patterns. The expression of *Rtnl1* mRNA transcripts as revealed by whole mount *in situ* hybridisation using *Rtnl1* isoform-specific probes. M, medial view, a saggital optical section focused at the midline of the embryo. L, lateral view focused at the lateral epidermal surface. V, ventral view focused at the level of the ventral nerve cord. In all images, anterior is to the left. In views M and L dorsal is top, while in view V ventral is uppermost. The *Rtnl1-RF* and *Rtnl1-RA* transcripts were visualised uniquely while the limited amount of unique sequence separating *Rtnl1-RB* from *Rtnl1-RE*, or *Rtnl1-RD* from *-RG* necessitated probes that recognise both *Rtnl1-RB* and *-RE* or both *Rtnl1-RD* and *-RG* transcripts. However, in both cases, the two transcripts encode the same Rtnl1 isoform. Arrowheads indicate expression of *Rtnl1-RB*/*RE* and *Rtnl1-RF* in the PNS and the arrow indicates expression of *Rtnl1-RA* in the epidermis in a segmentally repeated stripe close to the segmental boundary. No specific staining above background levels was observed when control sense probes corresponding to the same regions of *Rtnl1* were hybridised to wild-type embryos (data not shown).

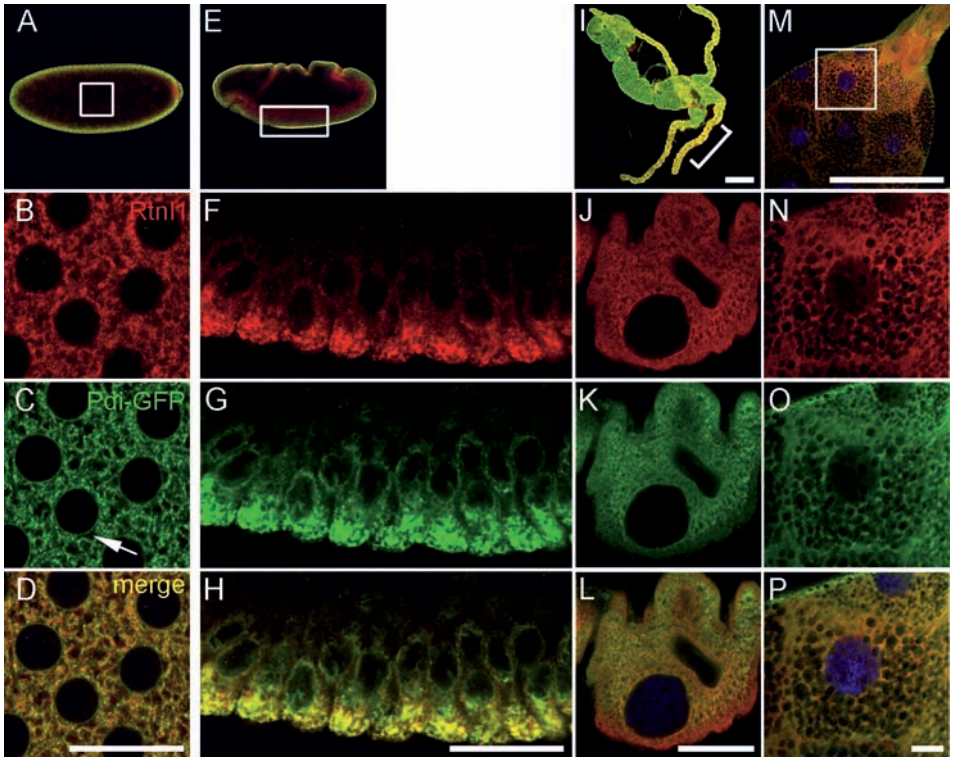

Figure 4. Subcellular localisation of Rtnl1 in embryos and third instar larvae. Rtnl1 expression *in vivo* was compared with that of an ER resident protein, Pdi. Rtnl1 was visualised with anti-Rtnl1 (red) within G198 embryos and third instar larvae that contain a GFP-tagged Pdi fusion protein, visualised here with anti-GFP (green). (*a–d*) Pdi and Rtnl1 expression in a stage 4 embryo. The region highlighted by the box in (*a*) is shown in (*b–d*). (*b–d*) Within the syncytial blastoderm Pdi-GFP localises to the nuclear envelope (*c*, arrow) but Rtnl1 is excluded. (*e–h*) Stage 7 embryo. The region highlighted by the box in (*e*) is shown in (*f–h*). (*f–h*) In the cellular blastoderm Pdi continues to be visible in the nuclear membrane, while Rtnl1 is excluded from this membrane. Rtnl1 is predominantly localised to the peripheral endomembrane within the cell. (*i–p*) Pdi and Rtnl1 expression in secretory tissues within the third instar G198 larvae, (*i*) dissected third instar midgut showing the four gastric caecae and (*m*) salivary gland. (*j–k*) Confocal section through one of the midgut luminal cells. (*n–p*) Salivary gland cell highlighted in the box in (*m*). Rtnl1 shows extensive overlap with Pdi-GFP in the gastric caeca (*i–l*) and salivary gland (*m–p*). In (*l*) and (*p*), DNA is stained with ToPro3 (blue). Scale: *i* and *m*, bar represents 200 µm; *d*, *h*, *l* and *p*, bar represents 20 µm.

signed a *Rtnl1-RB*/*RE* probe that recognises both transcripts. However, both transcripts encode the same Rtnl1 isoform. The same is also true of the *Rtnl1-RD* and *-RG* transcripts, and the *Rtnl1-RD*/*RG* probe identifies transcripts encoding the Rtnl1-PD/PG isoform.

The first three transcripts (*Rtnl1-RF* and *-RB*/*RE*) are present at high levels in the syncitial blastoderm, before major zygotic transcription is initiated at mid-blastula transition, suggesting they are largely of maternal origin; the remaining transcripts that were studied (*Rtnl1-RD*/*RG* and *-RA*) do not appear until later (stages 5 and 12, respectively), indicating they are not maternally supplied (Fig. 3 and data not shown).

Maternal *Rtnl1-RF* mRNA is steadily degraded after stage 5. The first zygotic *Rtnl1-RF* expression appears in the nuclei of the yolk sac (vitellophages) at around stage 11, and increases until enclosure of the yolk sac in the midgut at stage 15, when *Rtnl1-RF* mRNA in the yolk sac is rapidly lost. In addition to expression in the yolk nuclei, *Rtnl1-RF* mRNA expression is initiated in the ventral nerve cord (VNC) from stage 12 onwards. From around stage 14, expression can also be seen in the peripheral nervous system (PNS), including the lateral chordotonal organs (Fig. 3, stage 17, arrowheads). By stage 17, expression in other tissues has diminished so that *Rtnl1-RF* mRNA is restricted solely to the nervous system. The *Rtnl1-RF* mRNA is present in cell bodies and cannot be seen in axons.

Rtnl1-RB/*RE* expression is very similar to *Rtnl1-RF*, with the exception that yolk sac expression is absent. As a consequence, lower expression levels in surrounding tissue allow expression in the PNS chordotonals to be seen as early as stage 13 (arrowheads). At stage 17, the ventral view of the VNC (Fig. 3, stage 17) reveals differences between *Rtnl1-RF* and *Rtnl1-RB*/*RE*: *Rtnl1-RB*/*RE* appears to be upregulated in a subset of cells in the VNC, with higher expression in these cells in the thoracic segments. No such variations within the VNC are seen for *Rtnl1-RF*.

Rtnl1-RD/*RG* expression is initiated in the yolk nuclei and at the posterior pole of the embryo at stage 5. During stages 10–11 *Rtnl1-RD*/*RG* is transiently expressed ubi-

quitously, after which this expression ceases and *Rtnl1-RD/RG* transcripts only continue to be expressed in the yolk nuclei until their enclosure in the gut at stage 15.

There is no early expression of *Rtnl1-RA* transcripts in the embryo. Weak expression begins in the yolk sac from stage 12. From stage 13, *Rtnl1-RA* is expressed in the epidermis and gut. In the epidermis, expression is strongest in a series of segmentally repeated stripes lying close to the segmental boundaries (Fig. 3, arrow). Expression in the gut is strongest in the pharynx and proventriculus, but can also be seen in the hindgut. Like *Rtnl1-RD/RG* there is no expression of *Rtnl1-RA* in the nervous system.

Due to the short length (83 bp) of the unique 5′ exon of *Rtnl1-RC*, we were not able to identify the specific pattern of expression of *Rtnl1-RC*. However, use of a probe to the common RHD exons revealed ubiquitous expression of *Rtnl1* transcription (data not shown), suggesting that *Rtnl1-RC* may have widespread expression that includes regions of the embryo where expression is not seen for the alternative transcripts described above.

Subcellular localisation of Rtnl1. The reticulons have predominantly been described as components of the ER [1, 3, 21], although they additionally localise to other regions of the secretory pathway such as the Golgi [3, 27]. The position of lysines present at the C terminus of Rtnl1 (positions -13,-12, -3 and -1 from the C terminus) differ somewhat from the ER retention motif consensus sequences KKXX or KXKXX [43], found at the C terminus of most reticulons. To confirm that Rtnl1 nevertheless shares the same subcellular localisation described for other reticulon family members, we examined the subcellular distribution of Rtnl1 both *in vivo* and *in vitro*.

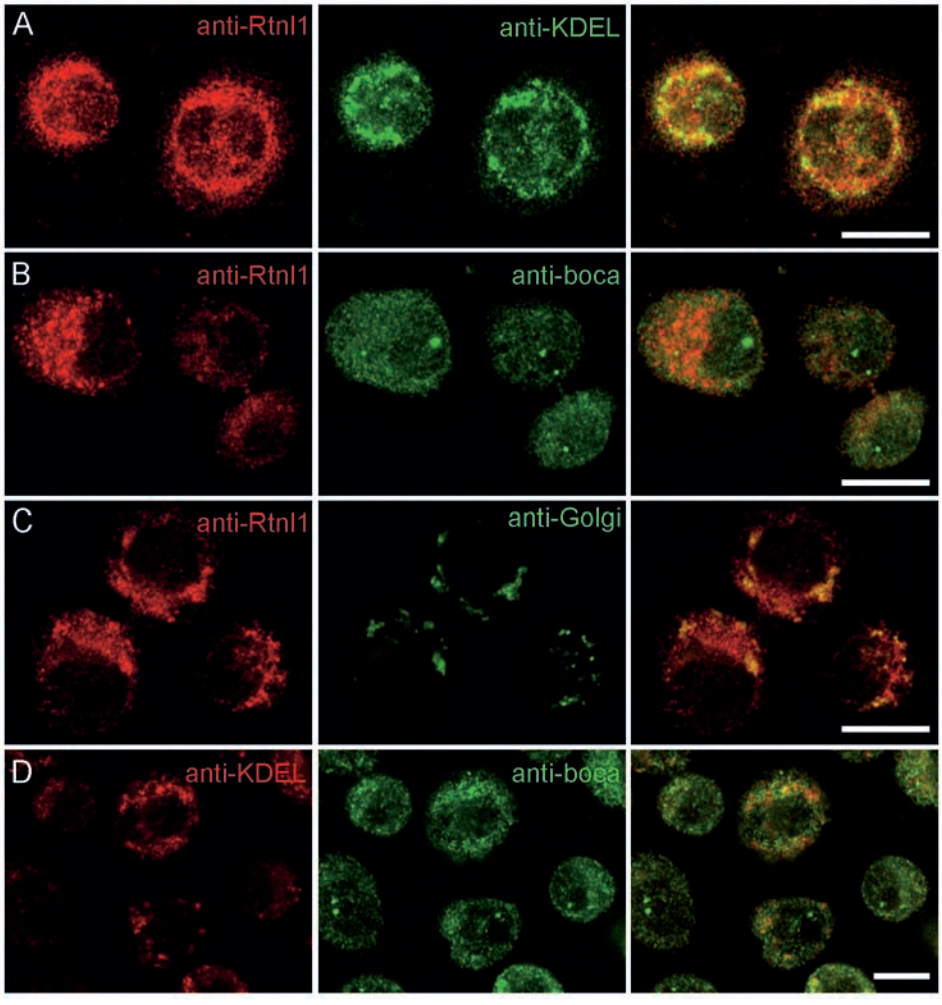

Figure 5. Subcellular localisation of Rtnl1 in S2 cells. Expression of Rtnl1 within *Drosophila* S2 cells revealed with anti-Rtnl1 (red). (*a*) Rtnl1 expression displays extensive, but incomplete overlap of with an ER marker, anti-KDEL (green). (*b*) No overlap is seen between Rtnl1 and another ER resident protein, Boca (green), although (*c*) Rtnl1 does show some overlap with a Golgi marker (green). (*d*) The two ER markers, Boca (green) and anti-KDEL (red), only partially overlap suggesting they mark separate compartments of the ER. Rtnl1 appears to be mainly resident in the fraction of the ER identified by anti-KDEL. All scale bars represent 10 μm.

To identify the ER *in vivo*, we made use of a GFP-protein trap insertion (line G198, [31]) in the gene *Protein disulphide isomerase* (*Pdi*). Flies containing this P-element insertion express a Pdi-GFP fusion that is targeted to the ER [44]. The expression of Rtnl1 and GFP was examined in G198 embryos and third instar larvae using a monoclonal antibody against GFP and use of a polyclonal anti-Rtnl1 serum that recognises all isoforms of Rtnl1 provided by W. Chia (National University of Singapore). Embryos with a homozygous deletion at the *Rtnl1* locus show no immunoreactivity, confirming the specificity of this antiserum for Rtnl1 (data not shown). In blastula and gastrula stage embryos, there is a considerable but incomplete co-localisation of Rtnl1 with Pdi-GFP (Fig. 4). In particular, Pdi-GFP localises to the nuclear envelope in the nuclei of the syncytial blastoderm, whereas Rtnl1 is excluded from the nuclear envelope (Fig. 4b–d, arrow). At the gastrula stage, stage 7, Rtnl1 appears to be enriched apically within the invaginating blastoderm cells, in contrast to Pdi-GFP, which is found more uniformly throughout the cell. In third instar larvae, we examined the expression of Pdi-GFP and Rtnl1 within the large cells present in the salivary glands and midgut. These cells are active in secretion and contain an extensive ER. In both gut caeca (Fig. 4j–l) and salivary gland cells (Fig. 4n–p), extensive overlap between Rtnl1 and Pdi-GFP is observed.

We also examined the expression of Rtnl1 in the *Drosophila* S2 tissue culture cell line, in which we compared its expression with several endomembrane markers. As markers for the ER, we used a monoclonal antibody against the KDEL ER retention sequence (anti-KDEL, Stressgen), and antibodies to the low density lipoprotein receptor family chaperone, Boca. Boca has previously been described as an endogenous *Drosophila* molecule found within the ER [37]. We observed extensive co-localisation of Rtnl1 with the ER marker anti-KDEL (Fig. 5a) and a partial overlap with a marker for *Drosophila* Golgi (Calbiochem, [38]) (Fig. 5c). In contrast, the expression of Rtnl1 and Boca appeared to be confined to separate domains and little overlap was observed (Fig. 5b). When we examined the expression of anti-KDEL and Boca, we observed that these markers only showed partial co-localisation (Fig. 5d), thus Rtnl1 appears to be localised to a sub-domain of the ER, which is a subset of the region identified by anti-KDEL and separate from a compartment occupied by Boca. Mammalian reticulons also do not localise with certain ER markers such as calreticulin [25]. Recently, it has been proposed that reticulons preferentially localise to the peripheral tubular ER as opposed to the sheet ER or the nuclear envelope [30]. It is therefore possible that Rtnl1 localisation to a KDEL-positive, Boca-negative sub-compartment of the ER reflects a similar compartmentalisation of reticulons within the ER of insect cells.

A *Rtnl1* loss of function mutant is viable but has a reduced lifespan. To gain a better understanding of reticulon function, we generated a loss-of-function mutation in *Rtnl1*. To generate a mutation that removed all reticulon function, we used a targeted gene deletion strategy to delete the RHD containing common exons. We obtained a P-element insertion, NP7026 (National Institute of Genetics, Japan), located 3Kb upstream of the RHD-encoding exons of *Rtnl1*. Using a combination of P-element-mediated male recombination [45, 46] followed by imprecise P-element excision [47], a deletion in *Rtnl1* was generated. Inverse PCR and PCR mapping were used to confirm that an approximate 4-kb region extending from the original P-insertion site to a point within the 3′UTR of *Rtnl1* had been deleted, removing all the RHD-encoding exons (Fig. 2b, red bar). As a further confirmation that the desired mutation had been obtained, homozygous *Rtnl1* mutant embryos were hybridised to a riboprobe recognising only the RHD-encoding exons. Even after allowing the histochemical reaction to proceed for an extended period of time, no specific *Rtnl1-RHD* mRNA could be visualised (Fig. 6a). In contrast, wild-type embryos hybridised to the same probe under the same conditions exhibited extensive staining reflecting the high levels of *Rtnl1-RHD* mRNA normally present in these animals (Fig. 6b). Similarly, when the *Rtnl1*^{1} embryos were incubated with the polyclonal anti-Rtnl1 serum no immunoreactivity was observed (data not shown).

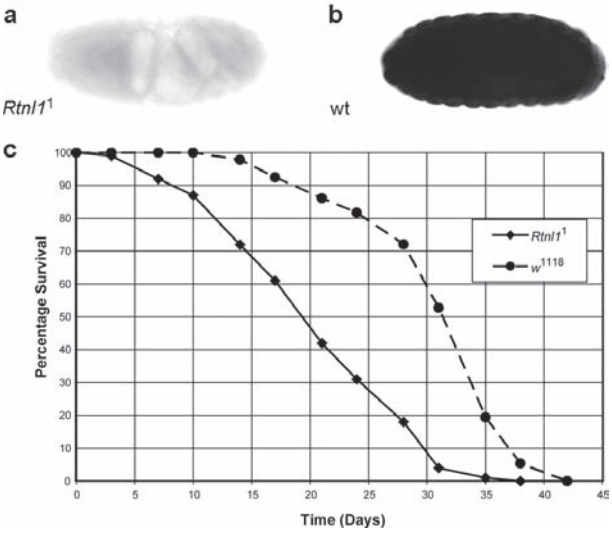

Figure 6. *Rtnl1* mutants show reduced lifespan at 29 °C. A *Rtnl1* knockout mutant, *Rtnl1*^{1}, was generated in which the RHD was deleted (see text and Fig. 2). (*a*, *b*) The absence of RHD-containing transcripts in the homozygous mutant embryos (*b*) was confirmed by *in situ* hybridisation using a riboprobe that recognises the common RHD-encoding exons of *Rtnl1*. After extended periods, no hybridisation was visible when wild-type embryos (*b*) show high levels of *Rtnl1* transcript. (*c*) *Rtnl1*^{1} mutant flies kept at 29 °C show a significant reduction in lifespan when compared with isogenised w^{1118} control flies ($p < 0.001$ using the Log Rank test). For *Rtnl1*^{1} $n = 99$ and for w^{1118} $n = 99$.

Flies homozygous for the *Rtnl1* mutation described above (referred to as *Rtnl1*[1]) are viable, fertile and exhibit no obvious developmental abnormalities. No defects could be observed in the ER morphology of mutant larvae using the anti-KDEL antibody (data not shown). This was particularly surprising considering the high conservation of reticulons across species. Phenotypic analysis of other reticulon gene knockouts has been complicated by potential redundancy due to the existence of paralogues [26, 48–50]. To confirm that loss of *Rtnl1* function removes all reticulon function in *Drosophila*, we confirmed by *in situ* hybridisation that *Rtnl2* is not expressed during embryogenesis and neither is it activated in *Rtnll*[1] animals (data not shown).

To determine whether *Rtnl1*[1] flies nevertheless exhibit subtle defects, we backcrossed the *Rtnl1*[1] mutation into an isogenised background and compared the lifespan of these flies to their isogenic w^{1118} controls. Strikingly, *Rtnl1*[1] flies maintained at 29 °C show a 39% decrease in median lifespan from 31 to 19 days, that is highly significant ($p < 0001$) when the Log Rank test is performed (Fig. 6c). A similar reduction in median lifespan was also observed for *Rtnl1*[1] flies maintained at room temperature (data not shown).

Discussion

The reticulons comprise a family of proteins that are associated with the ER, yet despite the presence of conserved forms of the proteins in a wide variety of eukaryotic organisms their function is poorly understood. One member of the family, RTN4/Nogo, has been implicated as an important regulator of axonal regeneration [5, 11, 12] and possible roles for the reticulons as regulators of ER membrane properties, membrane trafficking or apoptosis are emerging as interacting partners are identified [16, 30]. Here we describe the characterisation of the major *Drosophila* reticulon and provide evidence that this gene shares the mammalian reticulon characteristics of production of multiple isoforms that are incorporated into the ER. In addition, we report that a deletion of the *Rtnl1* locus results in the animal having a shortened lifespan.

In the course of a search for proteins enriched in the embryonic *Drosophila* nervous system we identified the *Drosophila* reticulon, *Rtnl1.* We believe that this is the only reticulon that is normally expressed by *Drosophila.* A second *Drosophila* reticulon, *Rtnl2*, is present within the *Drosophila* genome but this is possibly a retronuon with pseudogene character [3]. *Rtnl2* has a genomic organisation very distinct from other members of the reticulon family as it bears a single intron within its RHD-containing exons. Unlike *Rtnl1*, for which there are greater than 150 ESTs, there are only four ESTs identified for *Rtnl2*, all of which originate from animals that have been challenged with bacteria. We have also been unable to identify any expressed *Rtnl2* transcripts in the wild-type animal using *in situ* hybridisation. In contrast, the Rtnl1 protein is expressed ubiquitously in the embryo and shows increased expression within the nervous system at later stages of embryogenesis. The protein continues to be expressed throughout the animal in post-embryonic stages where it is retained within the nervous system with expression extending throughout axons and at presynaptic specialisations. This enrichment within the nervous system is characteristic of reticulons identified in other species.

We have confirmed that, as in mammals, the *Drosophila Rtnl1* locus generates several mRNA transcripts through alternative promoter usage. A total of seven transcripts are predicted for the *Rtnl1* locus that encode for five different polypeptides. Each of these transcripts includes four C-terminal exons that encode the conserved RHD, again conforming to the mammalian organisation. The largest transcript, *Rtnl1-RF* is mostly restricted to CNS regions with additional expression in vitellophages. The *Rtnl1-RB* and *RE* transcripts encode the same polypeptide and, although we were unable to differentiate the expression of the individual transcripts, it is clear that the encoded polypeptide is also mostly restricted to the nervous system. The *Rtnl1-RD* and *-RG* transcripts encode the same polypeptide and again we were unable to differentiate the expression of the individual transcripts; however, we could show that the encoded polypeptide is restricted to the extraembryonic yolk nuclei and is not expressed within the embryo. The *Rtnl1-RA* transcript that encodes a polypeptide that includes 26 amino acids in addition to the RHD is expressed in the epidermis and the hind- and foregut. It is clear that, like its mammalian counterparts, *Rtnl1* transcripts are expressed in dynamic tissue-specific and developmentally regulated patterns. Interestingly, the expression patterns of *Rtnl1* transcripts exhibit certain features reminiscent of vertebrate reticulons. For example, for vertebrate reticulon genes, it is often the largest transcripts whose promotors are located most 5′ to the gene that show an enrichment in neuronal tissues, while the medium-sized transcripts generally have a wider tissue distribution. Lastly, the shortest transcripts with promoters closest to the RHD-encoding exons are often enriched in muscle. This has been shown to be the case for mammalian Nogo/RTN4 [8], RTN1 [41] and RTN3 [6] as well as *Xenopus* reticulons [42, 51]. It is clear that in *Drosophila* the largest transcripts whose promoters lie closest to the 5′ start of the gene are also predominantly expressed in the nervous system. Thus, reticulon function appears to be required in all cells but the function of the reticulon protein in individual tissues must be modified somehow by the specific additional N-terminal sequences appended to the RHD. The additional N-terminal sequences alternatively spliced to the RHD

in *Drosophila* bear no similarity to the N-terminal sequences found in reticulons in other species. In particular, the N-terminal sequences in the largest isoform bear no resemblance to those found in RTN4/Nogo-A, suggesting that this protein is unlikely to act as an axon outgrowth inhibitor. The variance in N-terminal sequences added to the *Drosophila* RHD compared with those found in other species suggest that these molecules may have different partners from those previously identified for the mammalian reticulons.

We additionally examined the intracellular localisation of the *Drosophila* reticulon *in vivo* and *in vitro*; these experiments demonstrated that Rtnl1 is distributed to the ER. In a recent report, Voeltz et al. [30] suggested that RTN4/Nogo-A is localised to the tubular ER and excluded from sheet ER and the nuclear envelope. Our observations in early embryos reveal that Rtnl1 is also restricted to a peripheral sub-compartment of the ER and is excluded from the nuclear membrane, suggesting it too may be localised to the tubular ER. We also find that Rtnl1 is highly expressed in secretory tissues such as the gut caeca and the salivary glands; whether the ER within these cells has a tubular or sheet conformation is not known. Interestingly, we also find that Rtnl1 expression extends throughout axons and into pre-synaptic specialisations, suggesting that the Rtnl1-positive ER extends considerably beyond the cell body of *Drosophila* neurons.

When we examined the expression of Rtnl1 in *Drosophila* S2 tissue culture cells, we found that it co-localised with an ER marker identified by anti-KDEL but is in a separate domain to that occupied by Boca, another ER targeted protein. Thus our results are in agreement with previous observations that the reticulons are not expressed throughout the ER but may have a role in providing function within a sub-domain of the ER. Recent observations have suggested that RTN4/Nogo-A has a particular role to direct the formation of the tubular ER [30] and that RTN3 is involved in membrane trafficking between the ER and Golgi [52]. However, additional roles for the reticulons have been proposed based on the interaction partners that have been found in two-hybrid or co-immunoprecipitation screens and many of these suggest functions outside the ER. Reticulons have been shown to bind the plasma-membrane protein AP2 [53], SNARES and Rme-1 from endosomes [24, 25], mitochondrial Bcl-2 [21] and the Golgi protein Yip3p [26]. Differing reticulons have been used in these screens and it may be the case that the different reticulons have a common role to regulate membrane traffic within the cell with individual reticulons functioning to regulate trafficking to particular organelles.

It has proven difficult to assign reticulons to particular roles as few investigations have identified a clear phenotype when reticulon function is removed. Over-expression studies have revealed a role to differentiate the tubular ER and a possible role in membrane trafficking between the ER and the Golgi [30, 52]. The absence of loss of function phenotypes may be a consequence of the reticulons acting redundantly with one another or with additional components within the cell. Voeltz et al. [30] have revealed that when reticulon function is removed in yeast in combination with a lack of an interaction partner DP1/Yop1p, which is also normally localised to the tubular ER, this results in the conversion of the tubular ER to form sheet ER, thus one substantiated role for this particular reticulon may be to differentiate particular sub-domains within the ER. When reticulon alone is removed from yeast there is no obvious phenotype, although there is a disruption of the peripheral tubular ER in stress situations. We also found that removal of the major *Drosophila* reticulon, *Rtnl1*, did not result in any significant observable phenotypes, with the animals remaining viable and fertile, and no disruption in ER morphology in salivary glands from $Rtnl1^{1}$ animals could be observed (data not shown). This lack of phenotype is not due to activation of the second *Drosophila* reticulon, *Rtnl2*, as we were unable to detect transcription of *Rtnl2* in animals deficient for *Rtnl1.* It is possible that additional ER proteins may compensate for the loss of reticulon function, or that reticulon function is not necessary for survival under laboratory conditions. A similar lack of phenotypic consequence has been characterised in yeast, where phenotypes only become apparent under conditions of stress or when binding partners, such as Yop1p, are also removed. A *Drosophila* homologue of Yop1p exists within the *Drosophila* genome (CG8331); once mutants in this gene have been generated it would be of interest to examine the effect of removing this gene in combination with a lack of *Rtnl1.* We were, however, able to show that *Drosophila* lacking the *Rtnl1* gene alone had a significantly shorter lifespan. The median lifespan of the *Rtnl1*-deficient flies was 19 days, while their matched controls lived almost 2 weeks longer with a median lifespan of 31 days; similar reductions in life expectancy were observed in flies reared at room temperature. It is as yet unclear why mutations in *Rtnl1* might reduce lifespan. A potential role for reticulons in ER trafficking or in ER sub-compartment specification suggests reduced lifespan could be caused by a reduction in cell survival factors, receptors or other vital cell components reaching their correct target destination, or toxic intracellular accumulations, all of which could result in reduced cell viability. Alternatively, reduced efficacy of ER function may reduce the animals' ability to overcome the accumulated activity of reactive oxygen species [54]. Similarly, loss of Rtnl1 activity may lead to altered apoptotic activity resulting in premature aging. Careful analysis of cell viability and ER morphology in aging animals will be required to determine which of these processes is most compromised in *Rtnl1*-deficient flies.

The *Drosophila* reticulon, *Rtnl1*, shares many characteristics of its mammalian counterparts with a similar genomic organisation and the production of numerous isoforms. These isoforms have specific expression patterns and show an enrichment within the nervous system. Rtnl1 is also found localised to a sub-domain of the ER perhaps co-incident with a role in tubular ER formation. In the absence of *Rtnl1*, the longevity of the animals is reduced, suggesting an important but non-essential role for Rtnl1. The availability of *Rtnl1*-deficient animals will provide the opportunity to identify further components that act with Rtnl1 for the normal function of the ER and in this way perhaps shed more light on the roles of the reticulons in general and their specific roles in particular tissues.

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1 van de Velde, H. J., Roebroek, A. J., Senden, N. H., Ramaekers, F. C. and Van de Ven, W. J. (1994) NSP-encoded reticulons, neuroendocrine proteins of a novel gene family associated with membranes of the endoplasmic reticulum. J. Cell Sci. 107, 2403–2416.
2 Roebroek, A. J., van de Velde, H. J., Van Bokhoven, A., Broers, J. L., Ramaekers, F. C. and Van de Ven, W. J. (1993) Cloning and expression of alternative transcripts of a novel neuroendocrine-specific gene and identification of its 135-kDa translational product. J. Biol. Chem. 268, 13439–13447.
3 Oertle, T., Klinger, M., Stuermer, C. A. and Schwab, M. E. (2003) A reticular rhapsody: phylogenic evolution and nomenclature of the RTN/Nogo gene family. FASEB J. 17, 1238–1247.
4 Oertle, T. and Schwab, M. E. (2003) Nogo and its paRTNers. Trends Cell Biol. 13, 187–194.
5 GrandPre, T., Nakamura, F., Vartanian, T. and Strittmatter, S. M. (2000) Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. Nature 403, 439–444.
6 Di Scala, F., Dupuis, L., Gaiddon, C., De Tapia, M., Jokic, N., Gonzalez de Aguilar, J. L., Raul, J. S., Ludes, B. and Loeffler, J. P. (2005) Tissue specificity and regulation of the N-terminal diversity of reticulon 3. Biochem. J. 385, 125–134.
7 Geisler, J. G., Stubbs, L. J., Wasserman, W. W. and Mucenski, M. L. (1998) Molecular cloning of a novel mouse gene with predominant muscle and neural expression. Mamm. Genome 9, 274–282.
8 Huber, A. B., Weinmann, O., Brosamle, C., Oertle, T. and Schwab, M. E. (2002) Patterns of Nogo mRNA and protein expression in the developing and adult rat and after CNS lesions. J. Neurosci. 22, 3553–3567.
9 Schwab, M. E. (2004) Nogo and axon regeneration. Curr. Opin. Neurobiol. 14, 118–124.
10 Li, S., Liu, B. P., Budel, S., Li, M., Ji, B., Walus, L., Li, W., Jirik, A., Rabacchi, S., Choi, E., Worley, D., Sah, D. W., Pepinsky, B., Lee, D., Relton, J. and Strittmatter, S. M. (2004) Blockade of Nogo-66, myelin-associated glycoprotein, and oligodendrocyte myelin glycoprotein by soluble Nogo-66 receptor promotes axonal sprouting and recovery after spinal injury. J. Neurosci. 24, 10511–10520.
11 Chen, M. S., Huber, A. B., van der Haar, M. E., Frank, M., Schnell, L., Spillmann, A. A., Christ, F. and Schwab, M. E. (2000) Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. Nature 403, 434–439.
12 Prinjha, R., Moore, S. E., Vinson, M., Blake, S., Morrow, R., Christie, G., Michalovich, D., Simmons, D. L. and Walsh, F. S. (2000) Inhibitor of neurite outgrowth in humans. Nature 403, 383–384.
13 Bregman, B. S., Kunkel-Bagden, E., Schnell, L., Dai, H. N., Gao, D. and Schwab, M. E. (1995) Recovery from spinal cord injury mediated by antibodies to neurite growth inhibitors. Nature 378, 498–501.
14 Schnell, L. and Schwab, M. E. (1990) Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. Nature 343, 269–272.
15 Thallmair, M., Metz, G. A., Z'Graggen, W. J., Raineteau, O., Kartje, G. L. and Schwab, M. E. (1998) Neurite growth inhibitors restrict plasticity and functional recovery following corticospinal tract lesions. Nat. Neurosci. 1, 124–131.
16 Yan, R., Shi, Q., Hu, X. and Zhou, X. (2006) Reticulon proteins: emerging players in neurodegenerative diseases. Cell. Mol. Life. Sci. 63, 877–889.
17 He, W., Lu, Y., Qahwash, I., Hu, X. Y., Chang, A. and Yan, R. (2004) Reticulon family members modulate BACE1 activity and amyloid-beta peptide generation. Nat. Med. 10, 959–965.
18 Johnston, J. A., Liu, W. W., Todd, S. A., Coulson, D. T., Murphy, S., Irvine, G. B. and Passmore, A. P. (2005) Expression and activity of beta-site amyloid precursor protein cleaving enzyme in Alzheimer's disease. Biochem. Soc. Trans. 33, 1096–1100.
19 Mannan, A. U., Boehm, J., Sauter, S. M., Rauber, A., Byrne, P. C., Neesen, J. and Engel, W. (2006) Spastin, the most commonly mutated protein in hereditary spastic paraplegia interacts with Reticulon 1 an endoplasmic reticulum protein. Neurogenetics 7, 93–103.
20 Acevedo, L., Yu, J., Erdjument-Bromage, H., Miao, R. Q., Kim, J. E., Fulton, D., Tempst, P., Strittmatter, S. M. and Sessa, W. C. (2004) A new role for Nogo as a regulator of vascular remodeling. Nat. Med. 10, 382–388.
21 Tagami, S., Eguchi, Y., Kinoshita, M., Takeda, M. and Tsujimoto, Y. (2000) A novel protein, RTN-XS, interacts with both Bcl-XL and Bcl-2 on endoplasmic reticulum and reduces their anti-apoptotic activity. Oncogene 19, 5736–5746.
22 Li, Q., Qi, B., Oka, K., Shimakage, M., Yoshioka, N., Inoue, H., Hakura, A., Kodama, K., Stanbridge, E. J. and Yutsudo, M. (2001) Link of a new type of apoptosis-inducing gene ASY/Nogo-B to human cancer. Oncogene 20, 3929–3936.
23 Oertle, T., Merkler, D. and Schwab, M. E. (2003) Do cancer cells die because of Nogo-B? Oncogene 22, 1390–1399.
24 Iwahashi, J., Kawasaki, I., Kohara, Y., Gengyo-Ando, K., Mitani, S., Ohshima, Y., Hamada, N., Hara, K., Kashiwagi, T. and Toyoda, T. (2002) *Caenorhabditis elegans* reticulon interacts with RME-1 during embryogenesis. Biochem. Biophys. Res. Commun. 293, 698–704.
25 Steiner, P., Kulangara, K., Sarria, J. C., Glauser, L., Regazzi, R. and Hirling, H. (2004) Reticulon 1-C/neuroendocrine-specific protein-C interacts with SNARE proteins. J. Neurochem. 89, 569–580.
26 Geng, J., Shin, M. E., Gilbert, P. M., Collins, R. N. and Burd, C. G. (2005) *Saccharomyces cerevisiae* Rab-GDI displacement factor ortholog Yip3p forms distinct complexes with the Ypt1 Rab GTPase and the reticulon Rtn1p. Eukaryot. Cell 4, 1166–1174.
27 Di Sano, F., Fazi, B., Citro, G., Lovat, P. E., Cesareni, G. and Piacentini, M. (2003) Glucosylceramide synthase and its functional interaction with RTN-1C regulate chemotherapeutic-induced apoptosis in neuroepithelioma cells. Cancer Res. 63, 3860–3865.
28 Oertle, T., van der Haar, M. E., Bandtlow, C. E., Robeva, A., Burfeind, P., Buss, A., Huber, A. B., Simonen, M., Schnell, L., Brosamle, C., Kaupmann, K., Vallon, R. and Schwab, M. E.

(2003) Nogo-A inhibits neurite outgrowth and cell spreading with three discrete regions. J. Neurosci. 23, 5393–5406.
29 Dodd, D. A., Niederoest, B., Bloechlinger, S., Dupuis, L., Loeffler, J. P. and Schwab, M. E. (2005) Nogo-A, -B, and -C are found on the cell surface and interact together in many different cell types. J. Biol. Chem. 280, 12494–12502.
30 Voeltz, G. K., Prinz, W. A., Shibata, Y., Rist, J. M. and Rapoport, T. A. (2006) A class of membrane proteins shaping the tubular endoplasmic reticulum. Cell 124, 573–586.
31 Morin, X., Daneman, R., Zavortink, M. and Chia, W. (2001) A protein trap strategy to detect GFP-tagged proteins expressed from their endogenous loci in *Drosophila*. Proc. Natl. Acad. Sci. USA 98, 15050–15055.
32 Campos-Ortega, J. A. and Hartenstein, V. (1985) The embryonic development of *Drosophila melanogaster*. Springer-Verlag, Berlin.
33 Patel, N. H. (1994) Imaging neuronal subsets and other cell types in whole-mount *Drosophila* embryos and larvae using antibody probes. Methods Cell Biol. 44, 445–487.
34 Lin, D. M., Fetter, R. D., Kopczynski, C., Grenningloh, G. and Goodman, C. S. (1994) Genetic analysis of Fasciclin II in *Drosophila*: defasciculation, refasciculation, and altered fasciculation. Neuron 13, 1055–1069.
35 Fujita, S. C., Zipursky, S. L., Benzer, S., Ferrus, A. and Shotwell, S. L. (1982) Monoclonal antibodies against the *Drosophila* nervous system. Proc. Natl. Acad. Sci. USA 79, 7929–7933.
36 Tear, G., Harris, R., Sutaria, S., Kilomanski, K., Goodman, C. S. and Seeger, M. A. (1996) Commissureless controls growth cone guidance across the CNS midline in *Drosophila* and encodes a novel membrane protein. Neuron 16, 501–514.
37 Culi, J. and Mann, R. S. (2003) Boca, an endoplasmic reticulum protein required for wingless signaling and trafficking of LDL receptor family members in *Drosophila*. Cell 112, 343–354.
38 Stanley, H., Botas, J. and Malhotra, V. (1997) The mechanism of Golgi segregation during mitosis is cell type-specific. Proc. Natl. Acad. Sci. USA 94, 14467–14470.
39 Higgins, D. G. (1994) CLUSTAL V: multiple alignment of DNA and protein sequences. Methods Mol. Biol. 25, 307–318.
40 Walker, D. W., Muffat, J., Rundel, C. and Benzer, S. (2006) Overexpression of a *Drosophila* homolog of apolipoprotein d leads to increased stress resistance and extended lifespan. Curr. Biol. 16, 674–679.
41 Senden, N. H., Timmer, E. D., Boers, J. E., van de Velde, H. J., Roebroek, A. J., Van de Ven, W. J., Broers, J. L. and Ramaekers, F. C. (1996) Neuroendocrine-specific protein C (NSP-C): subcellular localization and differential expression in relation to NSP-A. Eur. J. Cell. Biol. 69, 197–213.
42 Klinger, M., Diekmann, H., Heinz, D., Hirsch, C., Hannbeck von Hanwehr, S., Petrausch, B., Oertle, T., Schwab, M. E. and Stuermer, C. A. (2004) Identification of two NOGO/RTN4 genes and analysis of Nogo-A expression in *Xenopus* laevis. Mol. Cell. Neurosci. 25, 205–216.
43 Jackson, M. R., Nilsson, T. and Peterson, P. A. (1990) Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. EMBO J. 9, 3153–3162.
44 Bobinnec, Y., Marcaillou, C., Morin, X. and Debec, A. (2003) Dynamics of the endoplasmic reticulum during early development of *Drosophila melanogaster*. Cell Motil. Cytoskeleton 54, 217–225.
45 Svoboda, Y. H., Robson, M. K. and Sved, J. A. (1995) P-element-induced male recombination can be produced in *Drosophila melanogaster* by combining end-deficient elements in trans. Genetics 139, 1601–1610.
46 Preston, C. R., Sved, J. A. and Engels, W. R. (1996) Flanking duplications and deletions associated with P-induced male recombination in *Drosophila*. Genetics 144, 1623–1638.
47 Salz, H. K., Cline, T. W. and Schedl, P. (1987) Functional changes associated with structural alterations induced by mobilization of a P element inserted in the Sex-lethal gene of *Drosophila*. Genetics 117, 221–231.
48 Kim, J. E., Li, S., GrandPre, T., Qiu, D. and Strittmatter, S. M. (2003) Axon regeneration in young adult mice lacking Nogo-A/B. Neuron 38, 187–199.
49 Zheng, B., Ho, C., Li, S., Keirstead, H., Steward, O. and Tessier-Lavigne, M. (2003) Lack of enhanced spinal regeneration in Nogo-deficient mice. Neuron 38, 213–224.
50 Simonen, M., Pedersen, V., Weinmann, O., Schnell, L., Buss, A., Ledermann, B., Christ, F., Sansig, G., van der Putten, H. and Schwab, M. E. (2003) Systemic deletion of the myelin-associated outgrowth inhibitor Nogo-A improves regenerative and plastic responses after spinal cord injury. Neuron 38, 201–211.
51 Park, E. C., Shim, S. and Han, J. K. (2005) Identification and expression of XRTN2 and XRTN3 during *Xenopus* development. Dev. Dyn. 233, 240–247.
52 Wakana, Y., Koyama, S., Nakajima, K., Hatsuzawa, K., Nagahama, M., Tani, K., Hauri, H. P., Melancon, P. and Tagaya, M. (2005) Reticulon 3 is involved in membrane trafficking between the endoplasmic reticulum and Golgi. Biochem. Biophys. Res. Commun. 334, 1198–1205.
53 Iwahashi, J. and Hamada, N. (2003) Human reticulon 1-A and 1-B interact with a medium chain of the AP-2 adaptor complex. Cell. Mol. Biol. (Noisy-le-grand) 49 Online Pub, OL467–71.
54 van der Vlies, D., Woudenberg, J. and Post, J. A. (2003) Protein oxidation in aging: endoplasmic reticulum as a target. Amino Acids 25, 397–407.