Drosophila Ringmaker Regulates Microtubule Stabilization and Axonal Extension During Embryonic Development

Rosa E. Mino¹, Steve L. Rogers², April L. Risinger³, Cristina Rohena³,⁴, Swati Banerjee¹ and Manzoor A. Bhat¹*

¹Department of Physiology, ³Department of Pharmacology
University of Texas School of Medicine
Health Science Center, San Antonio, TX 78229

²Department of Biology, University of North Carolina, Chapel Hill, NC 27599

⁴Department of Medicine, University of California, San Diego, CA 92093

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¹Address Correspondence to:
Manzoor Bhat, M.S., Ph.D.
Tel: 210-567-4327
Fax: 210-567-4410
Email: bhatm@uthscsa.edu
Summary Statement

We report the characterization of *Drosophila* Ringmaker (Ringer), and demonstrate that Ringer regulates axonal growth through microtubule changes during CNS development.
ABSTRACT

Axonal growth and targeting are fundamental to the organization of the nervous system, and require active engagement of the cytoskeleton. Polymerization and stabilization of axonal microtubules is central to axonal growth and maturation of neuronal connectivity. Studies have suggested that members of the Tubulin Polymerization Promoting Protein (P25α/TPPP) family are involved in cellular process extension. However, no in vivo knockout data exists regarding its role in axonal growth during development. Here we report the characterization of Ringmaker (Ringer), the only Drosophila homolog of long p25α proteins. Immunohistochemical analyses indicate that Ringer expression is dynamically regulated in the embryonic CNS. ringer null mutants show cell misplacement, and errors in axonal extension and targeting. Ultrastructural examination of ringer mutants revealed defective microtubule morphology and organization. Primary neuronal cultures of ringer mutants exhibit defective axonal extension, and Ringer expression in cells induced microtubule stabilization and bundling into rings. In vitro assays showed that Ringer directly affects tubulin, and promotes microtubule bundling and polymerization. Together our studies uncover an essential function of Ringer in axonal extension and targeting through proper microtubule organization.
INTRODUCTION

The polarization of neurons during development gives rise to specialized processes manifested in the form of dendrites and axons. The development of axons involves growth and guidance steps which essentially depend on the neuronal cytoskeleton (Lewis et al., 2013; Goldberg, 2003). A major constituent of the axonal cytoskeleton is the microtubule network, which provides structural support for the growing axon (de Forges et al., 2012; Dent et al., 2011). Microtubules form bundled parallel arrays along the axon, and spread out at the growth cone with their plus end in the direction of growth (Witte and Bradke, 2008; Witte et al., 2008). Microtubule polymerization, bundling and stabilization are critical for correct axonal extension and guidance (Lewis et al., 2013). These processes involve constant catastrophe and rescue events, and a variety of associated factors to maintain its dynamicity (Sept, 2007); (Conde and Caceres, 2009). For instance, microtubule polymerization is aided by +TIPs, and in axons, bundling is accomplished through mediation of proteins like Tau (Prokop, 2013). A large number of microtubule-associated proteins have been identified (Prokop et al., 2013); however, many of those responsible for regulating the neuronal cytoskeleton to mediate axonal growth remain to be characterized.

Tubulin Polymerization Promoting Proteins (TPPPs) are a superfamily of microtubule-associated proteins containing a common C-terminus p-25α domain. Studies on TPPP (TPPP1/p-25α), which is one of three full p-25α containing paralogs in mammals (Orosz, 2012), have linked it to changes in microtubule dynamics such as the induction of double-walled microtubule formations and higher polymerization rates in vitro (Hlavanda et al., 2002). In in vivo settings, changes in postnatal TPPP expression
have been correlated to pathologies like Parkinson’s (Oláh et al., 2006). In patient samples, TPPP colocalizes with α-synuclein aggregates in neurons, and cell studies have shown that increased TPPP stimulates α-synuclein aggregation to inclusions (Lindersson et al., 2005). TPPP loss has also been implicated in developmental disorders, as a high resolution comparative genome hybridization found a cohort of autistic children who exhibited TPPP deletions (Iourov et al. 2010). Further in vitro work showed that TPPP reduction in mammalian oligodendrocytes led to defective differentiation and process extension, suggesting a role in growth of cellular processes during development. These studies, along with the identification of TPPPs as neuron outgrowth modifiers in a Drosophila primary neuron RNAi screen (Sepp et al., 2008), and an in vivo screen in Zebrafish (Aoki et al., 2014; Orosz, 2015) led to the idea that TPPPs are involved in axonal growth. Although these previous studies suggest the involvement of TPPPs in developmental processes, no in vivo long TPPP knockout studies have addressed the endogenous role of these proteins at early stages. In addition, due to potential paralog functional redundancy, developmental studies in mammalian systems may not fully address TPPPs function.

Here we report the identification and functional characterization of the only Drosophila long p-25α homolog, named ringmaker (ringer). Ringer displays a temporally dynamic expression in neurons and later in midline glia during ventral nerve cord (VNC) development. ringer null mutants, generated through imprecise transposable element excision, show misplaced neurons and a variety of axonal phenotypes, including stalling and mistargeting. Ultrastructural analysis of nerve fibers from ringer mutants reveals defective microtubule organization and integrity. In vitro experiments and biochemical
polymerization assays show that Ringer directly affects microtubule stabilization and polymerization, with cells overexpressing Ringer forming rings instead of regularly distributed microtubules. Together, our data demonstrate that Ringer is a major regulator of axonal microtubule organization, which is critically required for proper axonal cytoskeletal architecture and growth during development.
RESULTS

*Drosophila CG45057* Locus encodes Ringmaker (Ringer), a Homolog of Mammalian TPPP/p25α

The *Drosophila CG45057* locus, named *ringmaker (ringer)*, at cytological position 72E2 (Attrill et al., 2015) was uncovered through a deficiency screen aimed at identifying genes involved in VNC development (*R. Mino and M. Bhat, unpublished*). The *ringer* locus has 4 predicted splice variants that encode a polypeptide (Ringer) of 192 amino acids (Attrill et al., 2015; Wilson et al., 2008). To determine any molecular similarities between Ringer and its homologs in other species, we performed protein sequence alignment (Blosum62) (Altschul et al., 1997). These analyses revealed 39% identity and 54% similarity to mouse TPPP, Expectation (E-) value 7e-37, and 37% identity and 56% similarity to human TPPP (E-value 2e-35). As shown in Fig.1A, the most highly conserved region (red text) is the C-terminus, which corresponds to the p25α domain (COBALT-E-value 0.003) (Papadopoulos and Agarwala, 2007) (Fig.1B). The mouse and human orthologs are 24 and 27 amino acids longer than Ringer, respectively (Fig.1A, 1B) (Jensen et al., 2009). To determine the spatio-temporal expression of *ringer* during embryonic development, we performed *in situ* hybridization on stage 17 wild type (*WT*) Canton S (+/+) embryos. Using antisense DIG-labeled probes, we observed prominent *ringer* mRNA expression in the embryonic CNS at the midline of the Ventral Nerve Cord (VNC) (Fig.1C,arrow). The embryonic VNC midline is akin to the mammalian Floor Plate and constitutes a crucial developmental organizing center of the nervous system during development (Jacobs, 2000; Menne et al., 1997). Midline expression of *ringer* was consistent with published data (Fisher et al., 2012), and absent
in sense probe controls (*data not shown*).

To determine Ringer protein expression and subcellular localization, we generated polyclonal antibodies against the full-length Ringer protein (GENBank AY071358) (Benson et al., 2005). Antibody specificity was tested with embryos carrying *Df(3L)BSC649*, which deletes the entire *ringer* locus (Fig.1E, S1A and S2A). Immunohistochemical analyses of *WT* stage 17 embryos revealed that Ringer is prominently expressed in the VNC midline (Fig.1D,arrow), with expression also observed in the surrounding CNS and PNS (Fig.1D,arrowheads; S1C, 1D). Immunoblotting showed that, relative to Tubulin, Ringer has lower expression at embryonic stage 16 and third instar larval stage, but is abundant in adult fly heads (Fig.1E,arrowhead). VNC midline embryonic developmental analyses revealed Ringer is initially detected in a pair of cells at stage 13 (Fig.1Fa,arrowhead), at this stage the pioneer midline axons initiate the formation of intersegmental connections (Kuzina et al., 2011). However at later stages, expression is observed in other midline cells (Fig.1Fb-1Fd,arrowhead), peaking at stage 15 (Fig.1Fc). Further analyses into the identity of these cells determined that at early stages, Ringer surrounds the neuronal nuclear marker ELAV (Fig.1Gb,arrowhead) (Koushika et al., 1996), but not the nuclear lateral glia marker Repo (S1 E-F), suggesting its presence in the neuronal soma (Fig.1Gac,arrowhead). At stages 16 and 17, Ringer neuronal expression is still detected but is stronger in midline glia identified by Wrapper (WRAP), a midline glial marker (Noordermeer et al., 1998; Wheeler et al., 2009) (Fig.1Ha-c,arrows; S1B). Further midline neuronal analysis revealed that at stage 13, Ringer is expressed in MP1 neurons, as evidenced by position and colocalization with the axonal marker FASII.
(Fig.1Ia-c,arrowhead) and the transcription factor Even-Skipped (Eve) (Fig.1Ka-c). At stage 14, in addition to MP1 expression, we observed expression in a subset of Eve-positive neurons (Skeath and Doe, 1998) (Fig. 1Ja-c and 1La-c,arrowheads). The RP2 motoneuron (Fig.1Lb arrowhead), and the aCC/pCC motoneuron/interneuron siblings (Fig.1Lb,arrowhead and inset) robustly express Ringer until stage 16 when expression is reduced. Together these data show that Ringer expression is dynamically regulated, and coincides with stages that correspond with embryonic nervous system cell migration and development.

**Ringer** is a Null Mutant Allele

To determine the effect of Ringer loss in vivo, we carried out transposable-element mutagenesis using line w[1118];Mi-ET1-CG45057[MB04349] and a transposase source in Df(3L)BSC649 background (Fig.2A) (Lin et al., 2014). We screened ~4000 fly lines for imprecise excisions at the *ringer* locus by Polymerase Chain Reaction (PCR), using genomic DNA from each line and primer combinations to identify deletions by shifts in band size (Fig.2B). Lines carrying mutations were sequenced to obtain exact information on deleted regions. We obtained 2 independent lines with deletions in the *ringer* locus. The largest deletion started at the *Minos* element insertion site spanning 3734 base pairs and covered 80% of the *ringer* locus, including the p-25α domain coding region (Fig.2A). Genomic sequences that flank the deletion on the 5’ and the 3’ side are shown here, with the ATG and the termination sites for all predicted polypeptides in the deleted segment (5’AGTTGAGTTTCGACATCGCCAGCATTGCACGTGTCCTAAAGTGATTGGAAAATAAGCCAAGGAATAAAACAAATATTT
TTTGGAAATATATTCAAGAATTTTTGGAACCTTTTTAATAACAAA----GTTTCAC
AAGAATTTCGCTTGAAATAATACACTTTAAAATA-3’). This deletion allele is referred to as \textit{ringer}^{915}. Immunoblot of \textit{ringer}^{915} homozygous adults revealed absence of the 23kDa band corresponding to Ringer, observed in control flies (Fig.2C-D, and S2). Immunostaining of \textit{ringer}^{915} embryos at stage 15 (Fig.2E-F), further confirmed absence of Ringer. Together, immunoblot and immunostaining analyses confirmed that \textit{ringer}^{915} lacks Ringer and represents a null allele.

Next we determined viability at critical developmental stages, as the \textit{ringer}^{915} allele allowed eclosion of homozygous adult flies. To verify at which point \textit{ringer}^{915} mutants are unable to progress through development, we calculated survival percentage within three experimental and control groups with a total of 210 embryos per genotype in three trials. Mean viability data indicates that only 69.9\%(±6.2) of \textit{ringer}^{915} embryos progress to third instar larval stage, compared to \textit{WT}, which has a mean survival of 93.7\%(±2.7) under identical conditions (p=0.0211) (Fig.2G). Mean percent survival to adult stages was calculated by selecting a total of 210 larvae per genotype and following their development until eclosion. Only 34.7\%(±2.9) of mutant larva survived to adulthood whereas 88.8\%(±2.7) \textit{WT} larva eclosed as adults (Fig.2H, p<0.0001). These data suggest that Ringer function affects viability at multiple stages of normal development.

\textit{ringer} Mutants Display Embryonic Nervous System Defects

Since we observed CNS Ringer expression, we next wanted to determine whether Ringer loss caused gross abnormalities in the VNC. \textit{ringer}^{915} mutants were
immunostained with neuronal nuclear marker ELAV (Fig. 2J) and midline glial signaling molecule, SLIT (Rothberg et al., 1988; Rothberg et al., 1990) (Fig. 2L). *ringer* mutants showed misplaced neurons (Fig. 2J, arrowhead) compared to bilaterally symmetric placement in control embryos (Fig. 2I, arrow). Midline glial SLIT levels and distribution did not seem affected in *ringer*<sup>915</sup> (compare Fig. 2L with Fig. 2K). Quantification of neuronal misplacement in three independent experiments with a total of ~100 embryos, show that an average 71.7% (± 3.62) mutant embryos exhibited at least 2 segments with errors in bilateral symmetry of neurons at stage 16, in comparison to only 7.02% (±2.92) observed in WT (p<0.0001) (Fig. 2M). These data suggest that loss of Ringer may affect final placement of embryonic neuronal cells in the VNC midline.

Given the neuronal misplacement phenotype observed in *ringer* mutants, we wanted to determine whether loss of Ringer had any consequences on the CNS neuropile, and more specifically, in axonal development. To analyze stereotypical organization of CNS axonal tracks, we used Fasciclin II (FASII), an axon marker (Lin et al., 1994), and the neuronal membrane marker, horse radish peroxidase (HRP) (Jan and Jan, 1982). Starting at stage 13, the stage at which Ringer is first observed, FASII-positive axons exhibit an overall collapse toward the midline (Fig. 3Ba, asterisk, compare with Fig. 3Aa) with instances of mistargeting (Fig. 3Ba, arrows), sometimes preventing axons from reaching the next segment (Fig. 3Ba, arrowhead). This trend continues in *ringer*<sup>915</sup> through stages 14, 15 and 16 (Fig. 3Da, 3Fa and 3Ha, compare with control, Fig. 3Ca, 3Ea, and 3Ga). At stage 16, we observed more pronounced guidance defects in which axons cross the midline and extend posteriorly along FASII tracts (Fig. 3Ha, arrow, compared to 3Ga). To determine the phenotypic variability, we scored a
total of 200 stage 16 embryos for presence of FASII phenotypes in three individual experiments per genotype. We found 74%(±2.8) of ringer915 embryos had severe phenotypes (Fig.3I), including 53%(±4.5) of them presenting axonal collapse toward the midline, and 11.3%(±2.4) exhibiting intersegmental axon breaks (Fig.3J). Both phenotypes were observed in 9.7%(±3.2) of embryos (Fig.3J). Additionally, 20%(±3.4) of ringer915 showed mild axonal stalling scored by inability of the lateral FASII tract to reach the next segment by stage 16 (Fig.3I). In contrast, 94.9%(±0.5) control embryos did not present any of these phenotypes and only 0.5%(±0.3) presented stalling (Fig.3I). The overall CNS structure as highlighted by HRP, also revealed defects in organization similar to those observed in FASII analysis (Fig.3Bb, 3Db, 3Fb, 3Hb, arrowheads, compare to Fig.3Ab, 3Cb, 3Eb, 3Gb). Additionally, to rule out any contribution by possible second site mutations created during mutagenesis, we quantified severe FASII phenotypes observed in ringer915/+ and ringer915/Df(3L)BSC649 embryos. Comparable to the wild type population which showed 5.133%(±0.55) of embryos with a phenotype, 9.59%(±2.7) of ringer915/+ embryos exhibited severe FASII disruption (p=5931). Conversely, 65.67%(±2.33) of ringer915 homozygous mutants exhibited a phenotype similar to a 59.81%(±3.3) observed in the ringer915/Df(3L)BSC649 population (p=0.3857) (S.1 I-J), suggesting neuronal phenotypes originate due to the disruption of the ringer locus. Together, these studies revealed that Ringer is required for proper axonal tract formation and overall architecture of the CNS midline during embryonic development.

To determine whether nervous system re-expression of Ringer was sufficient to rescue axonal defects in ringer mutants, we generated UAS-ringer strains and expressed Ringer using elav-GAL4 in ringer mutant background (Fig.3Ka-b) (Lin and
FASII axons in *elav-GAL4, UAS-ringer, ringer<sup>915</sup>* embryos showed significant rescue compared to *ringer* mutants (Fig. 3Kb, arrows). For rescue quantification, 300 embryos were analyzed in 3 independent experiments per genotype. By stage 16, only 23.9% (±0.5) of rescue embryos presented severe phenotypes compared to 74% (±2.8) in *ringer* mutants (p<0.0001) and 5.1% (±0.6) in WT (p ≤ 0.05) (Fig. 3L and 3M). Since Ringer is expressed in both midline neurons and glia in the embryonic VNC, we addressed whether the observed neuronal defects were a result of loss of neuronal Ringer or lack Ringer in midline glia. To circumvent the contribution of midline glia, we analyzed the level of rescue of severe phenotypes at stage 14, prior to midline glia Ringer. Phenotypic analysis of stage 14 *elav-GAL4; UAS-ringer, ringer<sup>915</sup>* embryos noted a significant rescue (p<0.0001) from 70.3% (±1.3) *ringer<sup>915</sup>* exhibiting a severe phenotype to 21.75% (±4) (Fig. 3L). Only 6.1% (±1.3) control embryos showed a severe phenotype at stage 14 (Fig. 3L). There was no significant difference between rescue levels at stage 14 and 16, suggesting a minimal contribution of midline glia to the neuronal phenotype in *ringer<sup>915</sup>* (Fig. 3L, p=0.7). These data suggest that Ringer may function cell autonomously in midline neurons, and that midline glial Ringer is not essential for its axonal embryonic developmental function.

In addition to suggestions that TPPP absence may lead to developmental disorders (Iourov et al., 2010; Lehotzky et al., 2010), reported cases of postnatal TPPP disruption leading to pathology are associated to *in vivo* increased protein expression (Oláh et al., 2011). To determine the effect of changes in Ringer protein levels in only a subpopulation of Ringer-expressing embryonic neurons, we used the available line *eve-GAL4; UAS-tau-LacZ*, which allowed to us to observe Eve-positive neuron soma and
projections as well as drive other UAS transgenes under the same promoter. β-Galactosidase immunostaining of eve-GAL4:UAS-tau-LacZ lines (Fig.4Ab) show the proper arrangement of early stage 16 Ringer expressing RP2, aCC, and pCC neurons (Fig.4Ab,arrowhead and 4Ac, circles) with their corresponding projections (Fig.4Ab, asterisk). Knockdown of Ringer with UAS-ringer-RNAi line TRiP.HMS01740}attP40 (Ni et al., 2010) under eve-GAL4, lead to a minimum of two segments with Eve-neuron placement defects in 67.25%(±0.58) of the embryonic population (Fig.4Bb-d and 4D), compared to 5%(±3.8) in eve-WT (Fig.4A and 4D) (p<0.0001). In the case of Ringer overexpression, we also observed neuronal misplacement (Fig.4Cb-d,arrowhead) in 21.83%(±1.94) of embryos (Fig.4D, p=0.0082 to eve control) as well as axonal defects (Fig.4Cb, asterisk, S3). Interestingly, in both knockdown and overexpression cases, phenotypes were stronger in RP2 neurons. The phenotypes observed in Eve-positive neurons are reflected in FASII immunostaining which shows disruption of axonal bundles (Fig.4 Aa, Ba, Ca,arrowhead) and cell outline position (Fig.4 Aa, Ba, Ca,arrows). Rescue experiments in which Ringer was reintroduced in Eve-positive cells alone, exhibited a modest reduction of neuronal misplacement phenotypes to 44.29%(±2.98) (Fig.4D, p=0.0012 to RNAi, and p<0.0001 to wild type, for each experiment, n=100). Together, these experiments suggest that reduction or increase in Ringer in neurons causes neuron-specific deficits in the CNS midline, and that optimal levels of Ringer are required for proper neural development.

**Changes in Ringer Expression Affect Neuronal Process Extension**

Studies on mammalian TPPP have shown that p-25α proteins have the ability to affect microtubule dynamics (DeBonis et al., 2015; Otzen et al., 2005; Tirián et al.,
2003; Tőkési et al., 2010), as well as cellular projection extension (Lehotzky et al., 2010). We wanted to determine the consequences of Ringer changes on microtubules and how that affected axons. Since in vivo tubulin distribution and single axon identification is complex due to the presence of numerous neurons in a relatively small area, we addressed axonal extension aspects in primary neuronal cultures from control (Fig 4G), ringer<sup>915</sup> (Fig 4H) and Ringer-overexpressing larval brains (Fig.4I). We determined neuronal cell identity by immunostaining with neuronal nuclear marker ELAV (Fig.4Eb, 4Fb) or the membrane marker HRP (Fig.4Gb, 4Hb). Analysis of WT CNS neurons showed that Ringer is expressed in the neuronal soma, and within axonal projections (Fig.4Ea,arrow) (Fig.4Ec, see merged Ringer and ELAV). As expected, ringer mutant primary neurons lacked Ringer immunoreactivity (Fig.4Fa) (Fig.4Fc, Ringer and ELAV merged image). Next, we immunostained control and ringer<sup>915</sup> primary neurons with: anti-acetylated tubulin (Fig.4Ga, 4Gc, 4Ha, 4Hc, 4la, 4lc) to highlight axonal microtubules, and anti- HRP (Fig.4Gb, 4Gc; 4Hb, 4Hc, blue) to label neuronal membranes. In WT neurons, acetylated microtubules exhibit uniform distribution along the axon and in the cell body it forms a prominent ring around the soma periphery (Fig.4Ga, 4Gc,arrows). In contrast, ringer mutant neurons have irregular acetylated tubulin distribution within axons, and asymmetric distribution at the soma (Fig.4Ha,c,arrows), with tubulin more prominent toward the side proximal to the axon (Fig.4Ha, asterisk). Conversely, increased Ringer expression in actin-GAL4>UAS-ringer neurons, which where identified using a combination of FASII, mCherry or Ringer, and acetylated Tubulin antibodies, appears to intensify distribution of acetylated microtubules throughout the cell (Fig.4Ia). In addition, acetylated Tubulin accumulated
at the growth cone area (Fig.4lb, arrowhead, Fig.4lc). These data suggest that changes in Ringer expression alter the distribution of acetylated microtubules in the axons.

As shown in Fig.3A-H, loss of Ringer affects axonal development, we investigated if lack of Ringer leads to defects in axonal extension in cultured neurons. We carried out axon length measurements specifically in FASII-positive neurons, which express Ringer, using acetylated tubulin and HRP as markers for axon limits. We generated percent frequency distribution curves for axonal lengths measured in 500-700 WT control (Fig.4J, blue), ringer$^{915}$ (Fig.4J, teal), and Ringer overexpression groups (Fig.4J, elav-GAL4>UAS-ringer, magenta). Three independent experiments were carried out under the same conditions 24 hours after plating, and although we observed the formation of longer processes in these cultures, only FASII-axon lengths up to 100μm were used for analysis. Wild type axons had a normal (p 0.06, D'Agostino & Pearson test) distribution with most of the population within 0-50μm in axon length and a maximum distribution at around 8μm. ringer$^{915}$ neurons had maximum distribution at shorter lengths, reaching 3 to 4μm (Fig.4J). The majority of elav-GAL4>UAS-ringer axons lingered at 4-5μm, with no significant difference between ringer mutants and Ringer-overexpressing neurons. Mean axonal length measured across experiments for wild type neuron population was 13μm(±1.4), whereas in ringer mutants mean axonal length is 4.3μm(±0.4) (p 0.0041, Fig 4K). Ringer-overexpressing neurons exhibited a mean axonal length of 5.3μm(±2.7). In elav-GAL4>UAS-ringer; ringer$^{915}$ neurons, mean axonal length increased to 6.1μm(±0.4) (p 0.0335), but axons did not reach full phenotypic rescue (Fig.4K). In agreement with the primary culture studies, axonal stalling phenotypes were also observed in in vivo embryonic overexpression under
actin-GAL4. We analyzed stages 13 through 16 ringer mutants using HRP and FASII (S3) which revealed that FASII-axons extend at a slower rate (S3 compare B,D,F to A,C,E). This is more evident at stage 16, with the formation of 3 FASII tracts, when overexpression lead to lateral tract delay in reaching the next segment. This stalling was quantified in three separate experiments following this phenotype at stage 16 in a total 150 embryos. Compared to background controls, which only saw delay in 18.8%(±2.3) of embryos, 81%(±1.6) Ringer overexpressing embryos exhibit axonal stalling (p<0.0001). Together, our cultured primary neuron and in vivo experiments suggest that too little or too much Ringer may prove detrimental to normal axonal outgrowth during development.

Ringer Induces Abnormal Microtubule Organization, and Promotes Microtubule Bundling and Polymerization in vitro

Phenotypic analysis of ringer mutants combined with primary culture analyses suggested that Ringer is involved in microtubule organization. We wanted to test whether Ringer expression in insect S2 cells affects their microtubule architecture. We generated UAS-ringer-mCherry, UAS-ringer, and mCherry control constructs and expressed each under pMT-GAL4 in cultured S2 cells. Transfected cells were immunostained using anti-Ringer (Fig.5A-B, asterisk), anti-mCherry (Fig.5C, red) and anti-α-tubulin (Fig.5A-C, green). At lower exogenous Ringer levels (Fig.5A, asterisk), microtubules were sparse, failed to extend to the cell periphery, and exhibited an unusually high degree of curvature and looping (Fig.5A,arrows) not observed in controls which have normal microtubule architecture (Fig.5A-C, S4). At higher levels of Ringer
expression (Fig. 5B, asterisks), microtubules were bundled into compact rings that encircled the central cytoplasm and never extended into the cell periphery (Fig. 5B, arrowhead). We carried out quantification of microtubule phenotypes observed in mCherry-Ringer or mCherry control expressing cells. Three replicates with n>=100 cells per treatment were then binned into Wild type, intermediate (sparse and curved), and strong (bundled rings) microtubule phenotypes. An average of 5.94% (±0.55) mCherry-Ringer cells exhibited wild type microtubules compared to 90% (±8.507) in control samples (p<0.0001). In 29.85% (±2.99) of mCherry-Ringer cells, microtubules were thinly dispersed and curved compared to 10% (±8.507) control (p=0.1757). Due to variability in the intermediate group, statistics involved data transformation. In the case of strong microtubule phenotypes 64% (±3.089) mCherry-Ringer cells were identified with ring structures whereas no cells with strong phenotype were seen in controls (p<0.0001). These experiments show that Ringer expression affects microtubule distribution in single cells, and that increased levels of Ringer may have more deleterious consequences on microtubule organization.

To further determine whether these ring structures are stabilized against microtubule depolymerization, we treated transfected cells with colchicine and examined its effect on Ringer-induced microtubule bundles. Consistent with a microtubule stabilizing activity, rings were resistant to high doses of colchicine (50 µM) (Fig. 5E, asterisk, arrowheads), a concentration that induced microtubule fragmentation and depolymerization in untransfected cells (Fig. 5Eb, arrow). We made further inquiries on the nature of Ringer-induced microtubule rings (Fig. 5Fa, 5Ga, blue) with antibodies against K40A acetylated tubulin (T7451, Sigma) (Fig. 5Fb, 5Gb, red) and α-tubulin.
This triple-immunostaining revealed that these rings are extensively acetylated (Fig.5Fd, 5Gd, arrows) (mCherry control in S4), a post-translational modification widely observed in axonal microtubules. These effects of ectopic Ringer were also observed in HEK293 cells (S4) where tyrosinated microtubules remained unaffected. To examine Ringer localization in living S2 cells, we co-expressed mCherry-Ringer with GFP-α-tubulin (Fig 5Ha-d) or GFP-actin (S4) to perform live imaging. We only imaged cells with low Ringer expression as high expression caused saturation and impaired imaging. As shown in Fig.5H, a still at 10 seconds reveals colocalization between Ringer (Fig.5Hb, red) and microtubules (Fig.5Ha, green, merged image Fig.5Hc and full cell image Fig.5Hd, arrow). In contrast, S2 cells coexpressing mCherry-Ringer and GFP-actin showed no colocalization (S4). Taken together, these analyses indicate that Ringer regulates microtubule architecture and dynamics by inducing microtubule stabilization.

Since Ringer is able to affect microtubule organization in vivo and stabilize microtubules in cells, we wanted to know if purified Ringer is sufficient to induce changes in Tubulin dynamics. To test this, we expressed and purified recombinant GST-Ringer (Fig.6A-B) and performed in vitro microtubule polymerization assays (Risinger et al., 2013; Shelanski et al., 1973) (Fig.6C). When purified Ringer was added in equimolar (18μM) concentration to purified tubulin, optical density at 340nm reached a maximum value of 0.40 with half maximum reached in 18 minutes. In contrast, buffer and GST alone controls had maximum values of 0.06 and 0.04, respectively, and took 30 minutes to reach half maximum. As a positive control, we replicated these experiments in the presence of Taxol (Sigma) (Arnal and Wade, 1995; Schiff and...
Horwitz, 1980). Taxol was added at 10μM and tubulin polymerization reached a density of 0.11 with half maximum after 2 minutes. Addition of GST-Ringer at 36μM decreased the time for half maximum density to 11 minutes, though maximum density remained at ~0.4, higher than that of Taxol. These biochemical data indicate that Ringer is sufficient to promote tubulin polymerization in vitro.

The pronounced change in maximum optical density and half maximum time after addition of Ringer during in vitro assays, lead us to use electron microscopy to directly evaluate the nature and extent of Tubulin changes. Buffer (Fig.6Da-b) and GST alone (Fig.6Ea-b) samples, exhibited background level of polymerized microtubule density. Samples containing Taxol, showed higher microtubule density (Fig.6Fa-b), with some lateral aggregation of microtubules (Fig.6Fb). GST-Ringer samples, both at 36μM (Fig.6Ga-b) and 18μM (Fig.6H), showed a dramatic increase in polymerized microtubule density compared to controls (Fig.6D-E). Addition of GST-Ringer to microtubules also promoted microtubule bundling, with aggregates reaching sizes of 820 nm in width (Fig.6Gb compare with Fig.6Fb,arrowheads). These data demonstrate that Ringer is sufficient to induce microtubule polymerization, bundling and stabilization, and suggest that in vivo Ringer has the potential to regulate proper microtubule assembly, architecture and distribution necessary for axonal growth during neuronal development.

**Ringer is Required for Proper Axonal Microtubule Organization in vivo**

Our cell culture and in vitro experiments suggested that the phenotypes observed in vivo are the result of changes in axonal microtubules. To directly investigate whether Ringer loss contributes to structural changes in axonal microtubules, we analyzed larval
segmental nerves. We used this system due to their larger size compared to embryonic nerves. Immunohistochemical analysis showed that these neurons also express Ringer along their axon (Fig. 7 Aa), which is absent in ringer mutants (Fig. 7Ba). Co-immunostaining against α-tubulin showed that ringer\textsuperscript{915} nerves have disorganized microtubules within the axon (Fig. 7Bb, arrows). These wavy microtubule profiles were not observed in control axons (Fig. 7Ab, +/+). Next we carried out ultrastructural analysis of axons from control and ringer\textsuperscript{915} to observe direct changes in microtubules. Imaging of transverse sections revealed that axonal microtubule cross-section profile is compromised in ringer mutants (Fig. 7E, F, G, arrowheads). In WT axons, microtubules are seen as circular hollow rings with regular distribution within the axon (Fig. 7C, arrows, higher magnification Fig. 7D, arrows). In ringer mutants, circular microtubule cross-sections appeared abnormally structured (Fig. 7F, G, arrowheads). Normal microtubule profiles were quantified in 6 wild type and 8 ringer\textsuperscript{915} mutant nerves from independent larvae and expressed as a percentage of total microtubule population per axon. A minimum of 100 axons per genotype were counted. ringer mutants exhibited an average of 26.84\%\,(±2.8) normal microtubule profiles compared to the 84.74\%\,(±2.69) observed in WT axons (Fig. 6H; p<0.0001). Ultrastructural analysis also revealed changes in microtubule distribution (Fig. 6G, arrowheads) consistent with those observed in immunostaining (Fig. 7A-B). Wild type axons exhibit characteristic uniform microtubule distribution, whereas ringer mutants have microtubule accumulations and are unevenly spaced (Fig. 7G, arrowheads). Together, these data demonstrate the Ringer is required for normal axonal microtubule assembly and organization during neuronal development.
DISCUSSION

Precise axon growth and guidance rely on microtubule polymerization, stabilization and bundling. These processes are central in establishing neuronal connectivity. Various proteins affecting microtubule dynamics have been characterized in the context of process extension (Lewis et al., 2013; Sánchez-Soriano et al., 2007). Proteins containing p25-α domains are expressed in embryonic and postnatal brain (Skjoerringe, 2006), and are known to alter microtubule dynamics (Hlavanda et al., 2002). However, the majority of studies do not address their relevance during early development (Orosz, 2012). Using in vivo and in vitro studies, we address the previously uncharacterized function of Drosophila TPPP (Ringmaker), the only long p25-α containing protein in Drosophila, and its importance in neuronal development.

Neural Development and Ringer Expression

Through mRNA and protein localization, our work uncovers that Ringer is present in the nervous system, and that its expression is variable and tightly modulated in the embryonic CNS midline. We also provide evidence that Ringer is necessary for correct nervous system development. Loss of Ringer results in soma misplacement and defects in axonal extension and guidance in agreement with neuron-specific knockdown experiments showing similar defects. That loss of Ringer results in axonal disruption is strengthened by knockdown studies in vitro (Lehotzky et al., 2010) and in zebrafish (Aoki et al., 2014) which have shown TPPPs to have an effect on process extension. Similarly, ringer was identified as a neuronal outgrowth modifier candidate (Sepp et al., 2008).
ringer mutants exhibit phenotypic variability. Initially, we supposed these differences were due to maternal Ringer contribution, a suspicion arising from experiments involving deficiency lines (S11-J). However, all ringer mutant embryos analyzed were from homozygous stocks, which rules out this possibility. Phenotypic variance may also arise due to compensation by other proteins. For instance, TPPP has been suggested to bundle microtubules similar to Tau (DeBonis et al., 2015). In Drosophila neurons, dTau knockdown only shows exacerbated neuronal degeneration when combined with futsch mutations. (Bolkan and Kretzschmar, 2014; da Cruz et al., 2005). We hypothesize that Ringer may act in a manner similar to Tau. Additionally, ringer null mutants exhibit decreased organism viability. Lack of Ringer, as in the case of Tau, leads to reduced viability but not complete lethality (Bolkan and Kretzschmar, 2014).

Ringer and Microtubule Stabilization

Our studies determined that Ringer, like mammalian TPPPs, is able to regulate microtubule dynamics. This is evidenced in vivo by microtubule disruption at segmental nerves in ringer mutants, and supported by our primary culture studies in which changes in Ringer translate into changes in acetylated tubulin. Ringer likely has a conserved stabilizing and bundling function similar to mammalian TPPPs (Hlavanda et al., 2002) Our cell culture experiments too suggest this, as they show Ringer can protect microtubules from depolymerization in addition to altering microtubule architecture, further underscoring a stabilizing function. Furthermore, our purified Ringer data show that no other external factors are necessary to induce microtubule dynamics.
changes. Thus, our work demonstrates that Ringer alone is sufficient to induce higher rates of microtubule polymerization as well as bundling and stabilization.

**Ringer-Dependent Microtubule Changes in Axonal Extension and Guidance**

Our work provides evidence that Ringer regulates microtubule changes necessary for axonal development. Ringer is expressed along the axon in primary neurons, and at cellular margins and membrane ruffle areas in S2 cells, a localization concomitant with process growth (Ayala et al., 2007). Moreover, axon extension and growth cone advancement rely on microtubules (McCaig, 1989). Consequently, in *ringer* mutants exhibiting axonal stalling and breaks, phenotypes may be representative of lower microtubule polymerization rates resulting from lack of Ringer’s function. This is supported by evidence that Ringer is necessary endogenously for proper axonal extension, and micrographs showing axonal microtubule disruption in *ringer* mutants.

Surprisingly, both *ringer* mutant and overexpressing neurons exhibit delayed axonal extension. Though defects observed in Ringer overexpression *in vivo* may be explained by Ringer contribution from surrounding cells, overexpression in primary neurons and *in vivo* Eve-neurons also result in soma placement and axonal phenotypes, revealing that there is a cell autonomous Ringer function.

The similar phenotypes produced by Ringer loss- and gain of function appear counterintuitive. However, *in vitro* data show that Ringer has the ability to promote microtubule polymerization and bundling. Studies revealed that modest microtubule overstabilization leads to an overall decrease in dynamics (Yvon et al., 1999). It is possible that Ringer overexpression stabilizes microtubules sufficiently to prevent
axonal advancement, whereas in *ringer* mutants, axons delay advancement due to lower tubulin polymerization. Additionally, Ringer loss may lead to depolymerization due to higher susceptibility to severing agents (McNally and Vale, 1993; Qiang et al., 2006). Perhaps there are Ringer concentration thresholds (Olah and Ovadi, 2014), post-translational modifications (Kleinnijenhuis et al., 2008), or other factors (Takahashi et al., 1991; Tőkési et al., 2010), which decide in favor of a specific function.

Conversely, guidance phenotypes in *ringer* mutants may be an indirect result of delays in axonal extension. During embryogenesis, VNC midline neurons extend their axons as they migrate ventrally (Araujo and Tear, 2003). In mutants, axons from misplaced neurons may not extend at a normal rate causing them to miss cues resulting in guidance defects. Additionally, axon guidance defects are repeatedly accompanied by severe neuronal misplacement, suggesting these two phenotypes are linked to migration errors. Alternatively guidance phenotypes may result from a Ringer function in growth cone directional movement through differential microtubule stabilization (Buck and Zheng, 2002). Thus, we postulate that during axonal development, Ringer regulates microtubule stabilization necessary for correct spatial distribution and polymerization to direct growth.

Interestingly, none of the phenotypic rescue experiments yielded a full recovery. Besides FASII embryonic phenotypic rescue, other attempts proved modest at best. We think that these differences are not due to changes in transgene expression, but from diverging protein level requirements between systems. Moreover, our FASII rescue measurements were performed relative to the integrity of the combined neuronal connections, whereas single-cell measurements were made unhindered by
environmental cues. Another possibility is that Ringer may be necessary in surrounding cells like lateral glia, and our antibodies are not robust enough to detect their Ringer expression. If this is the case, *elav-GAL4* rescue which expresses Ringer in all neurons and lateral glia at early stages (Berger et al., 2007), would be the only driver able to rescue phenotypes. Though our observations do not discard a Ringer function in other cells that may influence development, they support the idea of an endogenous cell autonomous Ringer function in neurons.

In summary, our work has demonstrated that Ringer contributes to development in the regulation of axonal extension. We have shown that Ringer is sufficient to promote microtubule stabilization, bundling and polymerization; and that its absence likely affects axonal microtubule dynamics leading to extension delays, mistargeting and consequently abnormal neural development.
MATERIALS AND METHODS

Molecular Constructs and *in situ* Hybridization

*ri*ger constructs were generated using cDNA (*RE39465, GenBank*) from DGRC. For generation of Ringer antibodies and GST-Ringer, primers with flanking enzyme sites, and a 582bp-cDNA *ri*ger fragment containing start and stop codons were subcloned into *pET28(a+)* and *pGEX-4T-1*, and expressed in *E. coli* (*BL21*, Invitrogen). *UAS-ri*ger expression constructs were generated by insertion of 1.2Kb of *ri*ger sequence including 150bp of 5’UTR into *pUASTattB* vector. In *UAS-mCherry-ri*ger, mCherry was inserted at the 5’ end, 4bp upstream the *ri*ger start codon. Insect S2 cells and HEK293 cells were used to confirm expression of recombinant constructs prior experimentation.

*In situ* hybridization was performed as described in Kearney et al (Kearney et al., 2004). *In vitro* transcription was carried out with PCR product from the first 400 base pairs of *ri*ger locus as a template (1277073, Roche). Sense probe prepared under identical experimental conditions served as a negative control.

Generation of *ri*ger Mutants

To generate mutations in the *CG45057* locus, *Mi[ET1]CG45057MB04349* males were crossed to females carrying the transposase *P[hsLMiT]2.4*, both in *Df(3L)BSC649* background. Resulting progeny was heat-shocked at 37°C for 2hrs until pupation. Mosaic F1 flies were crossed to *yw;D/TM6,Tb* to obtain males with single deletions. Progeny were tested for deletion size using PCR primers flanking the insertion site.
All stock lines were obtained from Bloomington Stock Center unless otherwise specified. Lines used: \textit{elav}^{C155}\text{-GAL4}, \textit{eve-GAL4:UAS-tau-LACZ}, and \textit{actin}^{5c}\text{-GAL4}, balanced over combinations of \textit{CyO,Twi-GFP; TM3,Ser,Twi-GFP} or \textit{FM7,Kr-GFP} or \textit{TM3,Ser,Kr-GFP}. \textit{UAS-ringer} and \textit{UAS-mCherry-ringer} fly strains were generated via \textit{PhiC31} integrase-mediated transgenesis (GenetiVision, TX). Knockdown experiments with the corresponding controls were performed at 25\textdegree C using the available TRIP line TRiP.HMS01740\text{attP40}. \textit{Canton S} and \textit{WCS} lines were used as wild type controls.

\textbf{Ringer Antibody Generation, immunostaining and Immunoblotting}

Primary antibodies against Ringer were generated in guinea pig and rat at Cocalico Biologicals, PA. His-tagged Ringer was expressed in \textit{E. coli BL21} for 3.5hrs at 25\textdegree C after induction with 0.1mM IPTG. His-Ringer Protein was purified with Talon Metal Affinity Resin beads (Clontech) using standard procedures. Antibodies were preabsorbed over \textit{ringer} mutant or deficiency embryos prior to their use in immunostaining procedures.

For immunoblotting and immunostaining standard procedures were used (Banerjee et al., 2011). Primary antibodies used were: guinea pig anti-Ringer (1:3000), anti-FasII (DSHB,1:500), anti-ELAV (DSHB,1:500), anti-Wrap (Wheeler et al., 2009), anti-Slit (DSHB,1:250), anti-GFP (Life Technologies,1:500), anti-\textit{β}-Gal (Promega,1:500), Acetylated Tubulin (Sigma T7451 and T6793,1:2000) and \textit{α} Tubulin (DSHB,1:50000). To visualize HRP, we used Dylight 488, 568, and 648 conjugated antibodies (Jackson ImmunoResearch, 1:400). Infrared conjugated (LI-COR,1:10000) secondaries, and fluorescent Alexa Fluor 488, 568 and 647 (Life Technologies, 1:400) secondaries were
Generation of Larval Primary Neuronal Cultures

Primary cultures from third instar larval brains from wild type, homozygous ring mutant and mCherry-Ringer overexpressing stocks were generated and immunostained as described (Egger et al., 2013). Cultures were plated on Polylysine-D coated glass bottom dishes (Corning, Biocoat) until fixation. Antibodies were used as listed above.

Insect S2 Cell Culture, Transfection, and Immunofluorescence

S2 cells were cultured in Schneider's medium (Invitrogen) supplemented with 10% heat-inactivated calf serum (HyClone Laboratories) and penicillin-streptomycin (Invitrogen). Cells were grown in 6-well plates and transfected with pMT-GAL4 and UAS-ringer (1µg each) using FugeneHD (Promega) according to manufacturer's protocol. On day 2, cells were induced with 100µM copper sulfate for 24 hours. On day 3, transfected cells were plated on concanavalin A-treated coverslips (MP Biologicals), washed for 2 minutes with PEM buffer (100 mM Pipes, pH 6.9; 1mM EGTA; 1mM MgCl2), and fixed for 20 minutes with 5% paraformaldehyde (EM Sciences) in PEM buffer. Some cells were treated with 50µM colchicine (Sigma) for 2 hours prior to fixation. After permeabilization with 0.1% Triton X-100 in PBS, cells were blocked with 5% normal goat serum and stained with anti-Ringer and anti-α-tubulin (DMA1α, Sigma). After washing, Cy2 and Cy3 fluorescent secondaries (Jackson Immunologicals) were used. Cells were imaged on an inverted Nikon Eclipse Ti using a CoolSnap HQ CCD.
camera (Roeper Scientific) run by Nikon Elements.

For live imaging experiments, S2 cells were transfected with UAS-mCherry-
ringer, pMT-GAL4, and pMT-GFP-α-tubulin 48 hours prior to observation. Expression
was induced with 100μM copper sulfate 16-24 hours prior to experiment. Cells were
seeded onto glass-bottom dishes (MatTek) in serum-free Schneider’s Drosophila
medium supplemented with 100x antibiotic-antimycotic (Invitrogen) and allowed to
attach for 1hr. Time-lapse imaging was performed on a motorized total internal
reflection fluorescence (TIRF) system (Nikon) mounted on an inverted microscope (Ti;
Nikon) equipped with a 100x/1.49 objective lens, driven by NIS Elements. Images were
captured with an Andor-Clara Interline camera (Andor Technology).

Microtubule Polymerization and electron microscopy

For polymerization assays, GST and GST-tagged Ringer were expressed in E.
coli (BL21) at 30oC. After centrifugation, bacteria were resuspended in TETN buffer
(20mM Tris, pH 8.0, 100mM NaCl, 1mM EDTA, and 0.5% of Triton-X-100), and
sonicated. For purification, GST proteins were bound to glutathione beads (GE
Healthcare) at 4°C overnight. Subsequent washes were done using high salt TETN
buffer. Elution was performed in of 20mM glutathione and 50mM Tris-HCL pH 8.0. The
quality of purified proteins was assessed by Coomassie Blue, Silver stain and
immunoblotting (S2).

In vitro polymerization assays were carried out as described (Risinger et al.,
2013) using 18μM 99% porcine Tubulin (BK006P,Cytoskeleton). A spectrophotometer
running Softmax Pro was used to measure density. Experiments included GST-Ringer
at 18μM and 36μM, GST at 18μM and 36μM, GST elution buffer only, Tubulin buffer only or 10μM Paclitaxel. Immediately following assays, samples were processed for ultrastructure analysis as described (Risinger et al., 2013).

Electron microscopy of larval nerves was performed as previously described (Banerjee et al., 2006). Electron micrographs were obtained using a digital camera JEOL 1400 microscope system.

**Imaging Processing, Quantification and Statistical analysis**

Imaging was performed with a Zeiss LSM710 confocal. Identical parameters were set for control and mutant samples. Representative images are maximum intensity projections from 40x confocal Z-stacks with 0.4μm interval and 1X zoom for embryos, and 0.5 μm with 0.9X zoom for primary cultures. For embryonic phenotype quantification, a Zeiss Axioscop2 plus fluorescence microscope and AxioCam camera were used. ImageJ was used for axon length measurements. Images were processed with ImageJ and Adobe Photoshop.

All data are presented as the mean ± Standard Error of the Mean (SEM). Statistically significant differences were determined using Student T-test or one-way ANOVA. In cases in which variances were significantly different, data was transformed to $y=\log(y)$ for analysis. All statistical tests were performed using GraphPad Prism7. For significance: n.s. indicates $P>0.05$, * indicates a $p$ value of $\leq0.05$, ** indicates a $p$ value of $\leq0.01$, and *** indicates a $p$ value of $\leq0.001$, and **** is $p<0.0001$
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Competing Interests

The authors declare no competing or financial interests

Author Contributions:

Mino, R designed, performed and interpreted experiments, prepared the manuscript.
Rogers, S and Risinger, A performed and interpreted experiments.
Rohena, C performed experiments.
Banerjee, S: provided reagents and analysis.
Bhat, M Designed and interpreted experiments, edited the manuscript.
References


Figure 1. **CG45057 (ringmaker) is Dynamically Expressed in the Embryonic CNS.**

*Drosophila* CG45057, now *ringmaker* (*ringer*) encodes a 192aa polypeptide. (A) Cobalt alignment (E-value 0.003) of *Drosophila* Ringer (Dm), mouse TPPP1 (Mm) and human TPPP1 (Hs) shows Ringer has 54% and 56% similarity to mouse and human, respectively. Conserved areas are shown in red. (B) To-scale representation of p-25α domains (purple). LCR are Low Complexity Regions. (C) *ringer* mRNA expression in stage 17 wild type (+/+) embryo CNS (arrow). (D) Ringer protein expression in the CNS midline (arrow), lateral CNS, and PNS (arrowheads) (Bar=20μm). (E) Immunoblots showing Ringer in embryo, larva and adult (23kDa,arrowheads). (F) Midline Ringer expression is first observed in stage 13 neurons (F,arrowheads) and later other neurons (a-e,arrowheads) and midline glia (Fc-e,arrows). (G) Immunostaining of stage 15 +/- embryos with Ringer and ELAV showing Ringer in neurons (b,arrowhead). (H) Colocalization at stage 17 of Ringer and WRAP in midline glia (c,arrow). (I, K) At stage 13, Ringer colocalizes with FASII, but not Even-skipped-positive neurons (arrowhead). (J) At Stage 14 other FASII neurons express Ringer (arrowheads). (L) Stage 15 EVE-positive RP2 motoneuron (arrowhead), and the aCC/pCC motoneuron/interneuron siblings (inset) also express Ringer. (Bar=10μm).
Figure 2. *ringer* null mutants exhibit embryonic nervous system defects.

(A) *ringer* null mutant generation using *Mi-ET1-CG45057[MB04349]* and the extent of genomic deletion *ringer*915 (red line). Exons are in purple. (B) Deletion size and location confirmation by PCR. (C-D) Immunoblots showing loss of 23kDa Ringer in *ringer*915 flies (arrow). (E-F) Wild type midline Ringer is absent in *ringer*915 embryos. (G, H) Viability analyses on *ringer*915 compared to +/+ indicate decrease in mean survival at transitions
from embryo to larva (K, p=0.0211) and larvae to adult (L, p<0.0001) (n=210). (I, J) ELAV immunostaining at stage 15 in +/+ (I) and ringer<sup>915</sup> embryos (J) shows neuronal misplacement and bilateral symmetry errors in mutants (arrow). (K, L) SLIT immunostaining in +/+ (K) and ringer<sup>915</sup> (L) shows unaffected midline glia in ringer mutants. (M) Quantification of embryos with neuronal misplacement phenotype as a percent of the population. 71.7%(± 3.62) ringer<sup>915</sup> embryos showed changes at stage 16, compared to 7.02%(±2.92) in WT (n=100, p<0.0001). (Bar=10 μm).
Figure 3. Lack of *ringer* results in embryonic axonal growth and targeting defects

(A-H) Analysis of CNS axons at stages 13 through 16 using HRP (Bb, Db, Fb, Hb
compare to Ab, Cb, Eb, Gb ) and FASII reveal changes in the neuropil. Axons exhibit overall collapse toward the midline (Ba, Da, Fa, Ha, asterisk) with instances of mistargeting (Ba, Da, Fa, Ha -arrows) and intersegmental breaks (Ba, Da, Fa, Ha – arrowheads). (I) Phenotypic quantification at stage 16 (n=200) of \textit{ringer}^{915} and +/+ found severe phenotypes in 74\%(±2.8) \textit{ringer}^{915} embryos, and 20\%(±3.4) mild axonal stalling (p<0.0001). (J) Breakdown of severe phenotype in comparison to +/+. \textit{ringer}^{915} exhibited 53\%(±4.5) axonal collapse, 11.3\%(±2.4) axon breaks and 9.7\%(±2.92) both phenotypes. (K) Severe phenotypes were rescued by introduction of \textit{UAS-Ringer} under \textit{elav-GAL4} in mutant background (Bar=10μm). (L) Quantification of phenotypic rescue (n=300). At stage 14 phenotypes were observed in 21.75\%(±4) rescue embryos (p<0.0001). By stage 16, 23.9\%(±0.5) rescue embryos presented phenotypes compared to \textit{ringer}^{915} (p<0.0001) and +/+ (p<0.05). There was no significant difference between rescue at stage 14 and 16 (p=0.7). (M) Breakdown of phenotypes observed in stage 16 rescue animals.
Figure 4. Changes in Ringer levels affect neuronal position and process extension.
(A-C) FASII (a), β-Galactosidase (b), and Ringer (c) immunostaining of eve-GAL4:UAS-
tau-LacZ embryos show arrangement of early stage 16 RP2, aCC, and pCC neurons
and their projections. (B) Ringer changes in Eve-neurons lead to axonal (A-C, arrow
asterisk) and soma defects (A-C, b-arrowheads). For each genotype, neuron soma
placement is shown by circles in c, and midline glia are MG. FASII immunostaining
shows axonal bundle disruption (Aa-Ca). (D) Quantification of neuronal misplacement
(n=~100). 67.25% (±0.58) of knockdown (p<0.0001) and 21.83% (±1.94) overexpressing
(p=0.0082) embryos exhibited misplacement compared to 5% (±3.8) in WT. Rescue
experiments exhibited a modest reduction to 44.29% (±2.98) (p=0.0012-knockdown, and
p<0.0001-WT). (E-F) Primary neuronal cultures from larval CNS show that Ringer is
also expressed in axons (arrow), and absent in mutant cultures. (E-I) Neuron identity
was determined by ELAV (Db, Eb) or HRP (Fb, Gb) (Bar=1μm). (G-I) Immunostaining
for acetylated tubulin. In ringer915 (H) and overexpressing embryos (I) microtubules
appear disorganized (arrowheads) compared to +/+ (G). Arrowhead in Ringer
overexpression (I) shows Ringer accumulation at the axon tip (Bar=5μm). (J) Percent
frequency distribution of FASII primary neurons up to 30μm. Quantification included
neurons with axons up to 100μm that were positive for FASII, HRP and acetylated
tubulin (K) After 24hrs, mean axon length for +/+ was 13μm (±1.4) compared to
4.3μm (±0.4) in mutants (p 0.0041). Overexpression and elav-GAL4>UAS-ringer in
mutant background showed mean axonal lengths of 5.3 (±2.7) and 6.1μm (±0.4)
(p=0.0335), respectively.
Figure 5. Ringer affects morphology and stability of microtubules in cells

(A-C) mCherry-tagged and Untagged Ringer was expressed in S2 cells and immunostained for tubulin. Low Ringer expression (asterisk) leads to abnormal microtubule curvature and failure to grow into cell periphery (A, arrow). (B) At high Ringer expression (asterisks), microtubules are incorporated into a central ring-like bundle (arrowheads) not seen in m-Cherry controls (C, arrow). (D) Quantification of microtubule phenotypes (n>=100). Individual cells were scored and binned into Wild type, intermediate, and strong microtubule phenotypes. 5.94%±0.55% of mCherry-Ringer cells had wild type microtubules compared to 90%±8.507% in control (p<0.0001). Intermediate phenotypes were observed in 29.85%±2.99% of mCherry-Ringer cells and 10%±8.507% in control (p=0.1757). Strong phenotypes were observed in 64%±3.089% mCherry-Ringer cells whereas no cells with strong phenotype were seen in mCherry controls (p<0.0001). (E) Ringer-induced bundles are stabilized against depolymerization by colchicine (50µM) (arrowhead) compared to controls (arrow). (F) Immunostaining in high Ringer (Fa, Ga) with Tubulin (Fc, Gc) and Acetylated Tubulin (Fb, Gb) show that induced rings are formed of acetylated microtubules. (H) Live-imaging stills at 10 seconds of S2 cells co-expressing GFP-Tubulin and mCherry-Ringer under pMT-GAL4 show partial colocalization of Ringer to microtubules (arrow). Panels Hb-d are magnified from a section in Ha. (Bar=5µm).
Figure 6. Ringer directly affects microtubule dynamics
(A-B) Representative Silver stain 12% gel (A) and anti-GST immunoblot (B) showing purified proteins after second round of purification for GST-Ringer (~50KDa, 2μg) and GST alone (~25KDa, 1μg) used for in vitro experiments. (C) in vitro tubulin polymerization assays measured at 340nm and 37°C show purified GST-Ringer addition is sufficient to promote changes in microtubule polymerization rate and magnitude. (D-H) Representative images showing the ultrastructure of polymerization assay samples. (D-E) Buffer and GST-only controls show unaided microtubule polymerization density. Single microtubules are marked with arrows. (F) Addition of Taxol increases microtubule density and promotes some bundling (arrowheads). (G-H) Addition of GST-Ringer to microtubules resulted in an increase of polymerization, and had a microtubule bundling effect (Ga, Gb and H arrowheads). (Da, Ea, Fa and Ga, bar=10 microns; Db, Eb, Fb, Gb and H, bar=100nm)
Figure 7. Lack of Ringer results in axonal microtubule defects.

(A-B) Staining for α-Tubulin and Ringer in +/- and $\text{ringer}^{915}$ 3rd instar larval segmental nerves. Microtubules appear wavy and disorganized in mutants (Bb, arrows) (Bar=5μm).

(C-G) Representative transmission electron micrographs of +/- (C-D) and $\text{ringer}^{915}$ mutant segmental nerves (E-G). (C-D) In +/-, axonal microtubules are normally observed as circular structures spread throughout the transverse face of the axon (Bar=800nm). (F-G) In $\text{ringer}^{915}$ nerves, microtubule appearance (F-G, arrows) and distribution (F-G, arrowheads) is compromised (F-Bar=400nm). G shows a different example of microtubule accumulation in mutant nerves (Bar=800nm). (H) Quantification of average normal microtubule profiles per genotype as a percentage of the total per nerve shows that 84.74%(±2.69) of +/- (n=6) nerves exhibit a higher proportion of normal microtubules than the 26.84%(±2.8) seen in $\text{ringer}$ mutants (n=8) (p<0.0001).
Supplementary 1. RINGER is expressed in the nervous system of *Drosophila* embryos. Stage 16 embryos were stained with guinea pig RINGER antibody generated by our lab against the whole protein. (A) Embryos carrying Deficiency Df(3L)BSC649 (a) which deletes the *ringer* locus does not exhibit the nervous system expression observed in WCS (b). (B) Co-localization between Ringer and the midline glia marker Wrapper. (C) Notice Ringer CNS expression outside the midline (Ca). (C-D) Co-localization of RINGER embryonic staining in the Stage 17 PNS (Da) with ELAV (Db), a neuron nuclear marker. Notice RINGER expression surrounding neuronal nuclei indicating RINGER is expressed in PNS neurons (Bc). (E-F) Co-localization of Ringer with the lateral glia marker Repo at two developmental stages. Though Ringer does not appear to be present in lateral glia, we observe glia distribution defects in all mutants (G), possibly a result of neuropil disruption. (H) Quantification of glia in three abdominal segments per embryo shows Ringer absence does not affect glia cell number (n=10). (I) Immunostaining of *Ringer*915 transheterozygous over WCS and Deficiency (arrow). (J) Quantification of observed Strong FASII phenotypes in transheterozygous. Averages form left to right: 5.13±0.55%, 9.59±2.7%, 65.67±2.33% and 59.81±3.3%. The authors would like to indicate that preliminary experimentation showed changes in phenotype incidence based on parental stock genotypes; to avoid possible maternal contribution effects the parental female genotype for *ringer*915/WT was WCS, whereas the parental female for *ringer*915/DF was *ringer*915.
**Supplementary 2: Biochemistry controls.**

(A) Western blot of Stage 16 wild type, Deficiency BSC649 homozygous and ringer^915^ mutant embryos probed with GP83 Ringer antibody at 1:3000. Shown are also bands lower than expected 25kDa. (B) Full western blot showing background bands, and Ringer level differences between ringer^915^ mutant, Wild Type and Act-GAL4>UAS-Ringer overexpressing 0-16hr embryos using Tubulin as a loading control. (C) Western blot showing Ringer expression in S2 cells transfected with 10μg of UAS-Ringer and UAS-mCherry-Ringer at expected sizes. (D) Coomassie staining of gels after secondary purification of GST-Ringer and GST. Estimated loaded for GST-Ringer: 5μg, 2μg, 500ng and for GST alone: 3μg, 1μg, and 300ng. Lys is 1μL of lysate after the first purification used as a control.
Supplementary 3. In vivo Overexpression of Ringer results in axonal stalling.

(A-H) Ringer overexpression in Drosophila embryos using Act-GAL4 in wild type background leads to FASII axon defects. Representative images of immunostaining using HRP (Ab, Bb, Cb, Db, Eb, Fb, Gb) and FASII HRP (Aa, Ba, Ca, Da, Ea, Fa, Ga) to look at neuronal membranes and FASII axons, respectively. Analysis was performed at stages 13 through 16 to observe overall CNS changes resulting from Ringer overexpression. Axons in the third tract at stage 16 have trouble reaching the next segment (Ha-c arrow, compare to Ga-c). (I) Quantification of stage 16 FASII phenotype in overexpressing embryos compared to Act5c-GAL4 and UAS-ringer control lines shows that while control exhibited lateral tract disruption in 18.8% ±2.3 of embryos. In Ringer overexpressing embryos, 81% ±1.6 axons had a problem reaching the next segment (p<0.0001). (J) When Ringer overexpressing embryos are analyzed at late stage 17 prior to L1, some 3rd FASII tracts have reached their target (Ja arrow). (K-L) Comparison of neuronal soma and axonal placement using B-Galactosidase staining at stage 14 in eve-GAL4>UAS-tauLacZ (K), eve-GAL4>UAS-tauLacZ in Ringer615 mutant background (L), and eve-GAL4>UAS-tauLacZ knockdown (M). Notice milder segmental disorganization in knockdown compared to mutant.
Supplementary 4. RINGER directly affects morphology of microtubules in HEK293 cells

RINGER tagged with mCherry on its C-terminus was expressed in cultured HEK293 cells. Constructs were generated through NEBuilder (New England Biolabs, CAT#E2621S). pcDNA3 mCherry LIC cloning vector (6B) sequences were used to create an mCherry backbone and fuse it with ringer cDNA starting at its ATG site, and including a Kozak sequence at the 5' end. Cells were transfected using X-fect (CAT#) with 5μg of DNA in 6-well plates. Fixation with 4% paraformaldehyde and immunostaining were done 48hr after transfection. High RINGER expression (Aa, Ca) lead to acetylated microtubule ring-shaped bundles as those observed in S2 cells (Ac, arrows). Immunostaining to observe Tyrosinated Tubulin did not exhibit the same ring-like structures (Cc, arrows). Images of untransfected controls are included (B, D). (E-F) Immunostaining of transfected S2 cells with Acetylated tubulin and mCherry antibodies. Notice Acetylated tubulin retraction from the periphery in Ringer-transfected cells (E) compared to mCherry-transfected control (F). (G) Live imaging on S2 cells co-expressing GFP-Actin (green) and mCherry-RINGER (red) under pMT-GAL4 for colocalization analysis. Notice tubular-shaped RINGER expression (F) Live imaging on S2 cells co-expressing GFP-Tubulin (green) and mCherry-RINGER (red) under pMT-GAL4. RINGER colocalizes with Tubulin more clearly in peripheral areas.