The Drosophila dynamin, shibire, contributes to blood cell division and differentiation, but may not be required for host defence against parasitic wasps

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The *Drosophila* dynamin, *shibire*, contributes to blood cell division and differentiation, but may not be required for host defense against parasitic wasps

Master’s Thesis
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Abstract

The Hymenopteran parasites of Drosophila melanogaster have developed powerful and complex methods for overpowering the defenses of their host species. Key to the parasitic success of some species has been the directed deployment of specialized molecular carrier packages designed to deliver the means of host defense destruction. These specialized molecular carriers take the form of virus-like particles, or VLPs. VLPs are thought to contribute to the suppression of the host immune response, both cellular and humoral, that is witnessed in many parasitoid infections.

Precisely how VLPs gain entry into host cells, as well as the exact nature of the cargo they carry, is not understood. This project aimed to explore endocytosis/phagocytosis as a candidate means of VLP entry, by examining the role of shibire, the Drosophila dynamin homolog, in the context of endoparasitoid infection. Hemocyte-specific RNAi-mediated knockdown of shibire expression revealed interesting, previously undescribed, cellular phenotypes. However, in the context parasitic infection, loss of shibire expression did not lead to a blockage of VLP cell entry, or a rescue of host viability. This result suggests that VLP entry may be mediated through a shibire-independent process. Future experiments aimed at elucidating potential mechanisms are proposed.
**Introduction**

In the wild, *Drosophila* species act as hosts to a number of parasites and pathogens (Govind, 2008). As a response to the various immune challenges presented by their natural enemies, they have developed several highly specific cellular and humoral innate immune responses (Lemaitre and Hoffman, 2007; Govind, 2008). One such response is the sequestration and cellular encapsulation of invading foreign bodies, such as the eggs that are deposited into the bodies of *D. melanogaster* larvae by several families of parasitoid wasp (Sorrentino et al., 2002; Schlenke et al., 2007; Heavner et al., 2014). This encapsulation reaction is an acute-phase inflammatory immune response that is highly spatially and temporally regulated. The proliferation, differentiation, and recruitment of larval blood cells to the site of parasitic infection, for the purposes of sequestration and elimination of the invading wasp egg, is tightly orchestrated, and the activation and resolution of these processes are under the control of a number of highly conserved immune signaling pathways (Sorrentino et al., 2002; Sorrentino et al., 2004; Agaisse and Perrimon, 2004; Wertheim et al., 2005; Schlenke et al., 2007; Lemaitre and Hoffman, 2007; Heavner et al., 2014).

Although *D. melanogaster* larvae can mount, or attempt to mount, a robust immune response to parasitic wasp infection, several species of wasp have developed strategies for compromising the effectiveness of this response (Carton et al., 1986; Chiu et al., 2001, 2002; Morales et al., 2005; Schlenke et al., 2007; Heavner et al., 2014). One particularly virulent species is *Leptopilina heterotoma*. Upon parasitic infection by *L. heterotoma*, VLPs, or virus-like particles, are injected into the hemolymph of *D. melanogaster* larvae. The subsequent activity of the molecular cargo carried by the VLPs is believed to be responsible for the incapacitation of the larval immune system witnessed in *L. heterotoma* infections (Morales et al., 2005; Rizki and
In *L. heterotoma* infections, cellular encapsulation and degradation of the implanted wasp egg by agents of the larval immune system is prevented, allowing the wasp progeny to develop unhindered. This process, in part, is a consequence of the activity of the injected VLPs, which quickly become bound to lamellocytes, specialized immune cells of *D. melanogaster* that are induced upon infection, and are necessary for a proper egg encapsulation response (Sorrentino et al., 2002; Rizki and Rizki, 1992). After internalization of the VLPs, the lamellocytes assume a bipolar morphology, and are subsequently lysed (Morales et al., 2005; Rizki and Rizki, 1984, 1990, 1992, 1994). In addition, plasmatocytes, macrophage-like hemocytes of *D. melanogaster*, have been shown to take up VLPs through phagocytosis (Rizki and Rizki, 1990), ultimately resulting in apoptotic cell death (Chiu and Govind, 2002; Govind lab unpublished results).

This lysis and apoptotic death of the effector cells of larval immunity, a consequence of VLP molecular cargo activity, speaks to the effectiveness of *L. heterotoma* virulence factors, but begs the question of how the VLPs are able to gain entry into host hemocytes. Although *L. heterotoma* VLPs have been shown to be present in phagocytic vacuoles in plasmatocytes (Rizki and Rizki, 1990), if, and how, they escape lysosomal degradation is unknown. In addition, lamellocytes are known to be phagocytically incompetent (Kurucz et al., 2007), leaving VLP entry into that cell type a completely open question.

One possible means by which *L. heterotoma* VLPs might gain entry into host hemocytes is through one of the many types of endocytic pathways known to exist in *Drosophila* (Guha, et al., 2003). In *Drosophila*, an essential mediator of many endocytic pathways is the *shibire* gene product *Drosophila* dynamin (dDyn), a large GTPase, and the only *Drosophila* homolog to vertebrate dynamin, a mechanochemical enzyme involved in many membrane-remodeling events.
Temperature-sensitive shibire mutants, presenting a paralysis phenotype at non-permissive temperatures (>29°C), were initially described as defective in synaptic vesicle recycling, (Kosaka and Ikeda, 1983) but dDyn activity is not restricted to the nervous system, and has been shown to mediate endocytosis and in a variety of tissues, including larval hemocytes (Tsuruhara et al., 1990; Chen et al., 1992; Dornan et al., 1997; Guha et al., 2003). In addition to dynamin’s role as a key mediator of many endocytic pathways, it has pleiotropic effects on cell biology, and has been shown to have important roles in actin reorganization (Schafer, 2004), the dynamic instability of microtubules (Tanabe and Takei, 2009), and centrosome cohesion (Thompson et al., 2004). It is also known to be required for cytokinesis (Thompson et al., 2002), and phagocytosis in macrophages (Gold et al., 1999; Huynh and Grinstein, 2008).

The creation of various transgenic shibire strains (Kitamoto, 2001; Moline et al., 1999), has allowed researchers to explore differential requirements for dynamin activity in the endocytic pathways of various tissues. Much of this previous work (Tsuruhara et al., 1990; Chen et al., 1992; Dornan et al., 1997; Kitamoto, 2001; Guha et al., 2003) utilized the classical temperature-sensitive alleles of shibire, and relied on the dominant-negative nature of these alleles to inhibit dynamin function. A problem with this approach is that although the classical temperature-sensitive alleles of shibire exhibit disrupted GTPase activity at the restrictive temperatures, dominant-negative isoforms of dynamin have been shown to retain the ability to polymerize and interact with various proteins via their other, non GTPase, functional domains (Thompson et al, 2004; Tanabe and Takei, 2009; Ferguson and Camilli, 2012). For this reason more recent studies have utilized various methods - complete genetic knockout, RNAi, photoinactivation – in an
effort to more comprehensively eliminate any dynamin functionality (Raimondi et al, 2011; Park et al, 2013; Kasprowicz et al 2014).

With the desire to uncover as much meaningful information as possible, and for the above reasons, we decided to eliminate shibire function through RNAi-mediated knockdown. Our goal was to determine if eliminating shibire function could disrupt cell entry of the L. heterotoma VLP, prevent host immune suppression, and ultimately rescue the encapsulation response of infected D. melanogaster larvae. In order to achieve this goal a targeted shutdown of endocytosis/phagocytosis in specific immune cell types was effected though the use of the UAS/GAL4 system of directed expression (Brand and Perrimon, 1993).

Utilizing a UAS-shiRNAi transgene, coupled with hemocyte-specific GAL4 driver lines, knockdown of endogenous shibire transcription was targeted to the immune cells of parasitized larvae. The reasoning behind this approach was - if VLP cargo activity is the primary cause of host immune suppression, and endocytosis/phagocytosis is the means of entry for the VLP, then disruption of the endocytic/phagocytic pathway in VLP-targeted host immune cells should prevent the shutdown of host immune responses, and a proper egg encapsulation response should proceed. In order to control and examine this process with greater flexibility, this study utilized three different experimental workflows, which are described in the methods.

What this study found, however, was that while hemocyte-specific RNAi-mediated knockdown of shibire expression did reveal some interesting, previously undescribed, cellular phenotypes, in the context parasitic infection, loss of shibire expression did not lead to a blockage of VLP cell entry, or a rescue of host viability. This result then suggests that VLP entry may be mediated through a shibire-independent process.
Methods and Materials

Insect Stocks:

*D. melanogaster* stocks: *Canton S* or *yw* were used as wild type. *eyeless-GAL4 (*, *UAS-shi^{RNAi} (Bloomington # 28513), *hop^{Tum-1} msn-GAL4; UAS-mcd8-GFP* (recombinant 15.2), *w^{1118}; Pxn-GAL4, UAS-GFP* (Stramer et al., 2005). Parasitoid wasps: *L. boulardi* strain G486 (Y. Carton), *L. heterotoma* strain Lh14 (described in Schlenke et al., 2007).

Hemocyte smears, cell counts and imaging:

Wandering third instar larvae were bled onto slides, fixed, and stained with Hoescht 33342 and rhodamine-conjugated phalloidin using standard protocols (Sullivan et al., 2000). anti-p40 (Chiu et al., 2006) antibody staining was carried out as described in (Paddibhatla et al., 2010). Cell counts were performed with a hemocytometer as previously described (Qiu et al., 1998). Images were obtained on either a Zeiss Axioplan 2, or Zeiss LSM710 confocal microscope, and editing of images for publication was done in Adobe Photoshop CS5.

Wasp infection of *Drosophila* larvae:

After approximately 48 h of development, fly larvae were exposed to 8-12 *L. boulardi* (G486) or *L. heterotoma* (Lh14) females for 24 hrs. at 25°C. Parasitized third-instar larvae were washed in PBS and examined under a stereomicroscope for the presence of melanotic capsules. If any melanotic capsules were found, the animal was dissected to determine if infection or superinfection had taken place. All larvae scoring positive for infection were imaged with a digital CCD camera mounted on a Leica fluorescent dissection stereomicroscope. Hemocyte smears and imaging were performed as above.
Adult viability and tumor penetrance/expressivity screens:

To assess adult viability, progeny were screened the day of eclosion. In the context of the $hop^{Tum-1}$ mutation, the number of eclosed FM7/Y males, in both the control and experimental groups, was set to 1, or 100%. The viability of the eclosed $hop^{Tum-1}/Y$ males of both groups was calculated relative to their FM7 brothers. In the context of $Pxn-GAL4$ driven $UAS-shi^{RNAi}$ expression, the number of eclosed TM6B/+ adults, in both the control and experimental groups, was set to 1, or 100%. The viability of the eclosed $UAS-shi^{RNAi}$ adults of both groups was calculated relative to their TM6B siblings. For tumor penetrance/expressivity, wandering third instar male larvae were scored for both the presence and size of tumors. Tumors of at least one body segment or larger were scored as large tumors, while those tumors that were smaller than one body segment were scored as small tumors. Statistical analysis of all data was performed by two-tailed Student’s $t$-tests.

Phagocytosis assay

Thirty third instar larvae from each experimental group were surface sterilized with 70% ethanol, and hemolymph was collected by puncturing the integument using dissection forceps into 200 µl of 7% BSA/PBS in 22-mm coverslip-bottom dishes. Cells were allowed to adhere for 1 hr., and then incubated with 5 µl of 1 mg/ml rhodamine-conjugated $E. coli$ BioParticles® (Life Technologies) for 2 hrs. As a positive control for phagocytic inhibition, some cells were additionally treated with 5 µl of 1 mg/ml of the actin polymerization inhibitor cytochalasin D (Life Technologies) (data not shown). Cells were then fixed, stained, mounted and imaged as above.
Validation of the $UAS\text{-}shi^{RNAi}$ construct

Previous research demonstrated that dysregulation of endocytosis, through the ectopic expression of $UAS\text{-}shi^{ts}$ constructs in the developing eye tissue of $Drosophila$ embryos, results in the formation of aberrant adult eye tissue (Kitamoto, 2001). In order to validate the functionality of the $UAS\text{-}shi^{RNAi}$ construct, crosses were performed between eyeless-$GAL4$ males and $UAS\text{-}shi^{RNAi}$, and $Canton\ S$ females. The resulting progeny were raised from egglay to adulthood at 27°C. Ectopic expression of $UAS\text{-}shi^{RNAi}$ at 27°C resulted in a phenotype similar, although stronger, to that seen with UAS-shi$^{ts}$ expression, resulting in the formation of grossly aberrant anterior cephalic structures, and death at the pupal stage (Fig. S1).

Experimental Workflows

Three main experimental workflows were used in this study: $in\ vivo$ only; $in\ vitro$ only; and a combination of both. For the $in\ vivo$ studies, infections of control and experimental animals were carried out as described above, followed by either encapsulation assay screens, or the collection of hemolymph that was then stained, mounted, and imaged as above. For the $in\ vitro$ studies, hemolymph from control and experimental animals were collected and cultured as described in the phagocytosis assay, but were then incubated with venom gland extract from Lh14 wasps, which was collected as described in (Morales et al., 2005). Cells were then stained, mounted and imaged as above. For the combination studies, infections of control and experimental animals were carried out as described above, followed by hemolymph collection and culturing as described in the phagocytosis assay. Cells were then stained, mounted, and imaged as above.
**Results**

*shibire* knockdown in \(hop^{Tum-1}\) mutant lamellocytes exacerbates tumorigenesis

Before exploring the functional consequences of *shibire* knockdown on cellular encapsulation, or parasitic success, the phenotypic consequences of loss of *shibire* expression in the hemocytes of unparasitized *D. melanogaster* larvae had to first be addressed. Attention was first given to *shibire* knockdown in lamellocytes. In order to drive expression of the \(UAS-shi^{RNAi}\) transgene specifically in lamellocytes, a lamellocyte specific \(GAL4\) driver, \(misshapen-GAL4\), is required (Tokusumi et al., 2002). However, in a wild-type genetic background, in the absence of parasitic infection, lamellocytes make up only <1% of the total hemocyte population (Williams 2007). So, in order to acquire a sufficient number of lamellocytes, transgene expression was driven in a highly immune-competent genetic context – the \(hopscotch^{Tum-1}\) mutant background.

\(hop^{Tum-1}\) is a constitutively active JAK kinase mutation that results in stimulation of the JAK/STAT signaling pathway even in the absence of immune challenge (Luo et al., 1997). This ectopic signaling leads to an overproliferation of larval hemocytes, and the differentiation of the specialized effector cell type of cellular encapsulation, the lamellocyte. The chronic inflammation created by the unneeded and uncontrolled production of lamellocytes results in hemocyte aggregation, invasion of self-tissue, and the eventual formation of melanotic hematopoietic neoplasias (Harrison et al., 1995; Luo et al., 1995).

For the purposes of this study, a previously unpublished recombinant lamellocyte-specific \(GAL4\) driver line known as 15.2 was utilized. Two versions of this line were used – one that is homozygous for the \(hop^{Tum-1}\) mutation, the \(misshapen-GAL4\) driver, and also carries a
membrane-bound GFP marker - \textit{UAS-mCD8-GFP}; and one that carries the FM7 balancer chromosome, and is heterozygous for the \textit{hop}^{Tum-1} mutation.

In order to first assess the cellular phenotypic effects of lamellocyte-specific RNAi-mediated knockdown of endogenous \textit{shibire} expression in \textit{hop}^{Tum-1} mutants, hemolymph smears from experimental and control animals were collected and examined by confocal microscopy. The gross morphology of \textit{shibire}-depleted lamellocytes was significantly changed with respect to control samples, taking on a highly twisted and tortured appearance (Fig. 1 - A, B), although the ability to adhere and form melanotic neoplasias was not compromised (Fig. 1 - C, D). Adult males were screened for viability (Fig. 1 - E), and larval males were scored for tumor penetrance and expressivity (Fig. 1 - F). RNAi-mediated knockdown of endogenous \textit{shibire} expression in \textit{hop}^{Tum-1} mutant lamellocytes did not result in a change in adult male viability, relative to controls (Fig. 1 - E). Tumor penetrance and expressivity, however, were affected significantly (Fig. 1 - F). The percentage of male larvae expressing the \textit{UAS-shi}^{RNAi} transgene that did not score positively for tumor formation dropped by a wide margin, relative to controls, while the percentage scoring positively for large tumorous masses, relative to controls, increased significantly. Male larvae scoring positively for small tumorous masses did not experience a significant change, relative to controls. Even upon gross visual examination, the difference in phenotype is readily apparent (Fig. 1 - C, D).

\textit{shibire}-depleted \textit{hop}^{Tum-1} mutant lamellocytes exhibit an increased encapsulation capacity against avirulent Lb G486 attack, but cannot protect against Lh14 attack

After establishing that \textit{shibire}-depleted \textit{hop}^{Tum-1} mutant lamellocytes exhibited gross morphological changes, and exacerbated tumorigenesis, the following goal was to determine if
lamellocyte-specific *shibire* knockdown affected proper cellular functionality in the context of parasitic infection. *hop<sup>Tum-l</sup>* mutant animals have been shown previously to be greatly immune-competent, with an increased ability to encapsulate deposited eggs and larvae from the wasp species *Leptopilina boulardi* (Morales et al., 2005).

We first examined if *shibire*-depleted *hop<sup>Tum-l</sup>* mutant lamellocytes retained this increased encapsulation capacity in the face of avirulent wasp infection. *hop<sup>Tum-l</sup>* mutants, expressing lamellocyte-specific *UAS-shi<sup>RNAi</sup>*, were infected by the relatively avirulent *L. boulardi* strain G486 (Schlenke et al., 2007). Animals were scored 24 hours post-infection for the presence of melanized encapsulated wasp progeny. Compared to control animals, the experimental group displayed a statistically significant higher rate of encapsulation (Fig. 2 - A). Melanized eggs and larvae were then mounted, fixed, counterstained, and imaged by fluorescent confocal microscopy in order to validate that the melanized capsule was indeed formed through the actions of the experimentally manipulated cells (Fig. 2 - B). Lamellocytes in circulation that were not part of a capsule appeared normal and non-bipolar in morphology (data not shown).

Our next goal was to see if *hop<sup>Tum-l</sup>* mutants, expressing lamellocyte-specific *UAS-shi<sup>RNAi</sup>*, would be subject to immune suppression if infected by the very virulent *L. heterotoma* strain Lh14 (Schlenke et al., 2007). Evidence of immune suppression would consist of the host’s lamellocytes assuming a bipolar morphology, along with a lack of ability to encapsulate deposited Lh14 eggs or larvae (Morales et al., 2005; Rizki and Rizki, 1984, 1990, 1992, 1994). Subsequent to Lh14 infection, *hop<sup>Tum-l</sup>* mutants, expressing lamellocyte-specific *UAS-shi<sup>RNAi</sup>*, showed a complete lack of ability to encapsulate deposited Lh14 wasp eggs or larvae (Fig. 2 - C), and displayed bipolar lamellocytes (Fig. 3 – bottom panels). 
In addition to destroying the encapsulation capabilities of *D. melanogaster* larvae, Lh14 infections have also been previously shown to greatly decrease the tumorous phenotype of *hop<sup>Tum-1</sup>* mutants (Morales et al., 2005). With this in mind, tumor penetrance and expressivity of Lh14-infected male larvae were scored, with the experimental group showing significant differences relative to controls (Fig. 2 - D). The percentage of male larvae expressing the *UAS-shi<sup>RNAi</sup>* transgene that did not score positively for tumor formation dropped significantly, relative to controls, while the percentage scoring positively for small tumorous masses, relative to controls, increased by a large margin. Male larvae scoring positively for large tumorous masses did not experience a significant change, relative to controls. However, although a greater number of *UAS-shi<sup>RNAi</sup>* expressing larvae showed the presence of small tumorous masses than did controls (Fig. 2 – D), all small tumorous masses observed displayed a ‘broken-up’ phenotype, as if a larger pre-existing mass had been broken into smaller pieces (Fig. 2 - E, F). Altogether, these observations suggested that knockdown of *shibire* in lamellocytes may not prevent the cellular entry of Lh14 VLPs. With these results in mind, it was now necessary to examine this question in a more direct manner, via examination of the localization of the Lh14 VLP-specific protein p40.

**p40 localization in lamellocytes**

Functional analysis of *shibire*-depleted lamellocytes had established that while they had an increased ability to encapsulate the developing wasp progeny of a relatively avirulent wasp species (2 – A), when faced with the attack of the more virulent Lh14, no change from control animals was observed (2 – C).
In order to more directly examine what was happening on the level of the individual cells, hemocyte smears were collected from Lh14-infected larvae, and $UAS-shi^{RNAi}$ expressing lamellocytes exhibiting a bipolar morphology were analyzed for the intracellular presence of anti-p40, an Lh14 VLP protein-specific antibody (Chiu et al., 2006). As Lh14 VLPs are much too small (~300 nm) to visualize unless electron microscopy is used (Rizki and Rizki, 1990, 1994), p40 antibody signal localization is used as a proxy for VLP localization (Chiu et al, 2006). Localization of anti-p40 signal within lamellocytes of infected control animals was specifically solely nuclear in distribution (Fig. 3 – middle panels), with no change in this localization observed in the $UAS-shi^{RNAi}$ expressing lamellocytes of infected animals in the experimental class (Fig. 3 – bottom panels). From these observations it appears that lamellocyte-specific knockdown of shibire expression does not prevent cellular entry of Lh14 VLPs, or at the very least does not prevent the entry of the Lh14 VLP-specific protein, p40.

*shibire* knockdown in plasmocytes leads to the formation of giant, multinucleate cells in a small fraction of cells

After first focusing on the phenotypic and functional consequences of *shibire* knockdown on lamellocytes, experiments were subsequently aimed at examining the consequences of *shibire* depletion in plasmocytes. In order to drive expression of the $UAS-shi^{RNAi}$ transgene specifically in plasmocytes, a plasmocyte specific GAL4 driver, *Peroxidasin-GAL4*, was utilized (Stramer et al., 2005). This driver line additionally carries a $UAS-GFP$ marker transgene.

To assess the cellular consequences of *shibire* knockdown in *Pxn-GAL4* plasmocytes, hemolymph smears were examined by confocal microscopy. Confocal micrographs revealed that a subset of the *shibire*-depleted plasmocytes had undergone a drastic phenotypic change,
enlarging in both nuclear and cell volume, and that some of these abnormally large cells had additionally become bi-, tri-, or multi- (up to 9) nucleate (Fig. 4 - A, B). As dynamin had previously been shown to be required for proper cytokinesis (Thompson et al., 2002), the number of giant/multinucleate cells were counted and the ratio of giant/multinucleate cells to normal plasmatocytes was determined, to see if it differed at all from the larval circulating hemocyte mitotic index that would be expected in a normal, non-immune challenged context (Fig. 4 - C). With a mean value of 2.2 %, the proportion of giant/multinucleate cells to normal cells did not substantially differ from the normal larval circulating hemocyte mitotic index of 1-2 % that has previously been reported (Qiu et al., 1998), suggesting that the giant/multinucleate phenotype is likely due to a defect in cytokinesis brought about by a lack of dynamin in the affected cells. In addition, total circulating hemocyte counts showed no significant difference in comparison to controls (Fig. 4 - D).

To assess the adult organismal phenotypic consequences of such an extreme larval cellular phenotype, albeit manifested in a minority of cells, adult flies were screened for viability. The viability screen revealed that all animals that expressed UAS-\textit{shi}^{RNAi} through the Pxn-GAL4 plasmatocyte-specific driver died as pupae, with a 0% eclosion rate (Fig. 4 - E). This result was unexpected, although potential non-plasmatocyte-specific \textit{peroxidasin} expression during mid-pupal stages might provide an explanation (Graveley et al., 2011).

\textit{shibire}-depleted plasmatocytes retain the ability to internalize foreign particles

The next goal was to validate the phagocytic incompetence of \textit{shibire}-depleted plasmatocytes by assaying their ability to internalize rhodamine-conjugated \textit{E. coli} BioParticles. As the stated experimental approach of the study was the targeted shutdown of
endocytosis/phagocytosis in *Drosophila* larval hemocytes, and dynamin has been shown to be required for phagocytosis (Gold et al., 1999; Huynh and Grinstein, 2008), it was assumed that by expressing \textit{UAS-shi^{RNAi}} in plasmatocytes, phagocytic competence would be eliminated. Surprisingly, however, after 2 hours of incubation with the \textit{E. coli} BioParticles, \textit{shibire}-depleted plasmatocytes still retained the ability to internalize the particles (Fig. 6 – bottom panels), although the intracellular distribution differed from control cells (Fig. 6 – middle panels). As a positive control, after treatment with the actin polymerization inhibitor cytochalasin D, plasmatocytes assayed for bead uptake did not prove to be phagocytically competent (data not shown).

\textit{shibire}-depleted plasmatocytes retain normal encapsulation capacity against the avirulent Lb G486, but cannot protect against Lh14 attack

Next, in order to determine if knockdown of \textit{shibire} expression in plasmatocytes might compromise the encapsulation capacity of \textit{UAS-shi^{RNAi}} expressing \textit{Pxn-GAL4} larvae, control and experimental animals were infected by the relatively avirulent \textit{L. boulardi} strain G486. Wild-type \textit{D. melanogaster} larvae exhibit a normally low to medium encapsulation rate against Lb G86, which was confirmed by the infected control group (Fig. 5 – A), with the experimental class showing no statistically significant difference.

In comparison, Lh14-infected \textit{Pxn-GAL4; UAS-shi^{RNAi}} animals displayed a definite lack of encapsulation capacity, and did not differ from infected controls (Fig. 5 – B). It appeared that, much like lamellocyte-specific \textit{shibire} knockdown, plasmatocyte-specific \textit{shibire} knockdown may not prevent the cellular entry of Lh14 VLPs. However, just like in the context of
lamellocyte-specific *shibire* knockdown, it was now necessary to examine this question in a more direct manner in *Pxn-GAL4; UAS-shi^{RNAi}* plasmatocytes.

**p40 localization in plasmatocytes**

In order to more directly examine the consequences of Lh14 infection on the level of the individual *shibire*-depleted plasmatocytes, hemocyte smears were collected from Lh14-infected larvae, and the *UAS-shi^{RNAi}* expressing plasmatocytes were analyzed for the intracellular presence of anti-p40. Localization of anti-p40 signal within plasmatocytes of infected control animals differs from that seen in lamellocytes, being cytoplasmic rather that nuclear, as well as being punctate in appearance, rather than diffuse (Fig. 7 – middle panels). No change in this localization was observed in the *UAS-shi^{RNAi}* expressing plasmatocytes of infected animals in the experimental class (Fig. 7 – bottom panels). Just as with the lamellocyte-specific knockdown of *shibire* expression, this observation leads to the conclusion that plasmatocyte-specific knockdown of *shibire* expression does not prevent cellular entry of Lh14 VLPs, or at the very least does not prevent the entry of the Lh14 VLP-specific protein, p40.

**Discussion**

Overall, the functional consequences of knocking down *shibire* expression in *Drosophila* larval hemocytes were inconsistent with the hypothesized experimental outcomes. However, while loss of *shibire* expression may not have lead to a blockage of VLP cell entry, or a rescue of host viability, hemocyte-specific RNAi-mediated knockdown of *shibire* expression did reveal some interesting, previously undescribed, phenotypes.

By knocking down *shibire* expression in *hop^{Tum-1}* mutant lamellocytes, perturbing *shibire*-dependent endocytosis appeared to exacerbate tumorigenesis. One interpretation for the
unexpected exacerbation of the $hop^{Tum-l}$ phenotype is that knockdown of $shibire$ expression is causing novel/ectopic signaling due to potential alterations in membrane organization within the cell. Previous studies have shown that cellular signaling in general, and immune signaling in particular, is modulated through endocytic pathways (Lund et al., 2010; Dobrowolski and De Robertis, 2012). How the endocytic modulation of immune signaling is subverted, in the context of chronic inflammation, has not been characterized in *Drosophila*.

In microarray data collected from $hop^{Tum-l}$ mutant hemocytes (Irving et al., 2005), almost every gene implicated in endocytic processes exhibited significantly upregulated expression. It may be that the increased expression of endocytic pathway genes shown by the microarray data is due to an attempt by the cell to reestablish signaling homeostasis by increasing receptor complex degradation. If inhibition of endocytosis, due to $shibire$ knockdown, is preventing the cell’s mostly feeble attempts at reestablishing signaling homeostasis, then this might possibly result in changes in cell morphology, cell membrane properties, altered adhesion and migration abilities, and immodulated immune signaling. All of which could in turn be contributing to the $hop^{Tum-l}$ melanotic tumor phenotype. This explanation would also support work that has shown that JAK/STAT signaling is competent from both the cell membrane and endosomal compartments, and that endocytosis acts as a negative regulator of JAK/STAT signaling (Vidal et al., 2010).

The highly melanized hemocyte aggregate phenotype observed in $UAS-shi^{RNAi}$ expressing larvae is interesting, as it might imply that tumorous hemocyte aggregate formation can occur as a result of cell-autonomous immodulation of growth, differentiation, adhesion, or immune signaling within lamellocytes, and would further support research that has demonstrated a tumor-suppressive role for $shibire$ (Vaccari & Bilder, 2009). Studies such as (Vidal et al., 2010), which
argue for endocytosis as a negative regulator of certain signaling pathways would also lend credence to such a role.

Depleting plasmatocytes of *shibire* expression gave rise to the multinucleate cell phenotype, which at first appeared quite strange. However, this phenotype might be easily explained as result of defective cytokinesis, which requires dynamin for its proper resolution (Thompson et al., 2002). This notion is further supported by the fact that the small proportion of circulating hemocytes in the *Pxn-GAL4; UAS-shiRNAi* animals that display this phenotype is comparable in magnitude to the proportion of total circulating hemocytes normally undergoing mitosis (Qiu et al., 1998). However, post-wasp infection there is a limited hematopoietic mitotic burst that occurs as the host immune system responds to parasitic attack (Sorrentino et al., 2002). Wasp infected *Pxn-GAL4; UAS-shiRNAi* animals appeared to display a larger proportion of cells showing the multinucleate phenotype than non-parasitized *Pxn-GAL4; UAS-shiRNAi* animals (preliminary observations), which might be a explained by an infection-induced mitotic burst, but requires further analysis. In future experiments, *UAS* constructs expressing mutant alleles for Toll signaling components could be made to induce hematopoietic hyperproliferation in *Pxn-GAL4* plasmatocytes. This mimic of the post-infection mitotic burst might be utilized in conjunction with *UAS-shiRNAi* expression as a means to further examine this phenomenon. The fact that the multinucleate phenotype is not observed when *UAS-shiRNAi* is expressed in lamellocytes could be explained by the fact that lamellocytes are a terminally differentiated post-mitotic cell type (Krzemien et al., 2010), thus obviating any need for dynamin-dependent cytokinesis.

More interesting is the apparent ability of *shibire*-depleted plasmatocytes to still retain the capacity for foreign object internalization. This result runs counter to the expected
experimental outcome, but might be explained by results described in a recent study (Kasprowicz et al., 2014), in which expression of $UAS$-$shi^{RNAi}$ in neuronal cells led to bulk retrieval of cell membrane, and the creation of large membranous cisternae within the cells. If a similar phenomenon is happening here, then normal phagocytosis might indeed have been blocked in the $shibire$-depleted plasmatocytes, but if the $E. coli$ BioParticles had been attached to the cell surface during some form of bulk membrane uptake, then that might have led to their circumstantial internalization. This idea is partially supported by the differences in the intracellular distribution of the $E. coli$ BioParticles observed between the $shibire$-depleted and control plasmatocytes.

When looking at the consequences that $shibire$ knockdown has on immune function, in the context of wasp infection, it appears that at a global level there is no effect. In the case of both $hop^{Tum-1}$ mutants, expressing lamellocyte-specific $UAS$-$shi^{RNAi}$, and $Pxn$-$GAL4$; $UAS$-$shi^{RNAi}$ animals, when infected with the virulent wasp strain Lh14 there was no observed change to encapsulation capacity, relative to controls. In addition, no difference in anti-p40 localization, relative to controls, was witnessed. One may draw the conclusion from these results that knockdown of $shibire$ expression in these cell types does not prevent entry of the VLP, and thus host immune function is suppressed. However, p40 localization is not the same as VLP localization, and can only be suggestive of the potential mechanisms at play.

Future experiments would benefit from the use of an anti-dynamin antibody, both to examine the normal intracellular distributions of dynamin in both plasmatocytes and lamellocytes, and as a means of further validating the knockdown of endogenous expression. It would also be informative to examine how the expression of the different GFP-marker constructs used in both cell types correlates with anti-dynamin staining, and ultimately, with the different
phenotypes observed in both. Electron microscopy studies will more precisely correlate p40 localization with VLP localization, both in the wild-type context and in the context of UAS-
\textit{shi}^{RNAi} expression, and these experiments are ongoing in our lab.

It might actually be the case that expression of hemocyte-specific \textit{shibire} knockdown does in fact block p40 entry, and by proxy, VLP entry, but that an alternative uptake mechanism, such as the bulk endocytosis mentioned above (Kasprowicz et al., 2014) becomes active in the absence of \textit{shibire} activity. Further work in this area, including the additional studies planned above will shed much needed light on the processes at work here. Alternatively, it may indeed be true that VLP entry requires the other, \textit{shibire}-independent arm of endocytosis (Guha et al., 2003; Gupta et al., 2014), and through the experimental perturbation of other endocytic players, the mechanism of VLP entry will eventually be worked out.

This work resulted in some very curious and unexpected experimental outcomes, and a good deal of additional research is needed to begin to understand the mechanisms and processes at play. \textit{Drosophila melanogaster}, and its \textit{Hymenopteran} parasites, are an excellent model system for exploring the molecular and cellular dynamics of host-pathogen interactions. By seeking a better understanding of the details of the immunological evolutionary arms-race carried out between these organisms, we hope to gain important insights into the evolution and conservation of mechanisms of immunity, cellular functions such as endocytosis, and the signaling pathways involved in both. Any insights made in these areas can have a direct impact on human health, as evidenced by the promising recent research into utilizing designer VLPs to deliver proteins or other macromolecules into diseased or disabled cells (Kaczmarczyk et al., 2011).
References


Figure 1 shibire knockdown in hop^{Tum-1} mutant lamellocytes exacerbates tumorigenesis. (A) Confocal micrograph of hop^{Tum-1}/Y lamellocytes (B) Confocal micrograph of hop^{Tum-1}/Y; UAS-shi^{RNAi} lamellocytes (C) Brightfield image of third instar hop^{Tum-1}/Y larvae (D) Brightfield image of third instar hop^{Tum-1}/Y; UAS-shi^{RNAi} larvae (E) Percent viability of hop^{Tum-1}/Y and hop^{Tum-1}/Y; UAS-shi^{RNAi} adults. Student’s t-test, P>0.05; n>200 in both classes. (F) Tumor penetrance of hop^{Tum-1}/Y and hop^{Tum-1}/Y; UAS-shi^{RNAi}; shi^{RNAi} third instar larvae. Double asterisk indicates significant difference, student’s t-test, P<0.01; control n>300, experimental n>400.
Figure 2  
**shibire**-depleted hop^Tum-l/1 mutant lamellocytes exhibit an increased encapsulation capacity against avirulent Lb G486 attack, but cannot protect against Lh14 attack. (A) Percentage of Lb G486 wasp eggs encapsulated by hop^Tum-l/1; UAS-shi RNAi third instar larvae. Singe asterisk indicates significant difference, student’s t-test, P<0.05 control n>100, experimental n>100 (B) Confocal micrograph of a Lb G486 wasp larvae encapsulated by hop^Tum-l/1; UAS-shi RNAi lamellocytes. (C) Percentage of Lh14 wasp eggs encapsulated by hop^Tum-l/1; UAS-shi RNAi third instar larvae. (D) Tumor penetrance of Lh14-infected hop^Tum-l/1 and hop^Tum-l/1; UAS-shi RNAi third instar larvae. Double asterisk indicates significant difference, student’s t-test, P<0.01 control n>100, experimental n>200.
Figure 3  p40 localization in lamellocytes (Top panels) Confocal micrographs of anti-p40 stained uninfected hop$^{Tum-1}$ lamellocytes. (Middle panels) Confocal micrographs of anti-p40 stained Lh14-infected hop$^{Tum-1}$ lamellocytes. (Bottom panels) Confocal micrographs of anti-p40 stained Lh14-infected hop$^{Tum-1/Y; UAS-shi^{RNAi}}$ lamellocytes.
Figure 4 *shibire* knockdown in plasmatocytes leads to the formation of giant, multinucleate cells in a small fraction of cells. (A) Confocal micrograph of *Pxn-GAL4* plasmatocytes. (B) Confocal micrograph of *Pxn-GAL4; UAS-sh*RNAi plasmatocytes. (C) Percentage of total *Pxn-GAL4* and *Pxn-GAL4; UAS-sh*RNAi plasmatocytes displaying giant/multinucleate phenotype. (D) Total hemocytes per mm³ for *Pxn-GAL4* and *Pxn-GAL4; UAS-sh*RNAi third instar larvae. Student’s t-test, *P* > 0.05; n>50 in both classes. (E) Percent viability of *Pxn-GAL4* and *Pxn-GAL4; UAS-sh*RNAi adults. Student’s t-test, *P* > 0.01; n>800 in both classes.

Figure 5 *shibire*-depleted plasmatocytes retain normal encapsulation capacity against the avirulent Lb G486, but cannot protect against Lh14 attack. (A) Percentage of Lb G486 wasp eggs encapsulated by *Pxn-GAL4* and *Pxn-GAL4; UAS-sh*RNAi third instar larvae. Student’s t-test, *P* > 0.05; n>75 in both classes. (B) Percentage of Lh14 wasp eggs encapsulated by *Pxn-GAL4* and *Pxn-GAL4; UAS-sh*RNAi third instar larvae. Student’s t-test, *P* > 0.05; n>100 in both classes.
**Figure 6** *shibire*-depleted plasmatocytes retain the ability to internalize foreign particles. (Top panels) Confocal micrograph of *Pxn-GAL4* plasmatocytes incubated with rhodamine-conjugated *E. coli* BioParticles for 2 hrs. (Bottom panels) Confocal micrograph of *Pxn-GAL4; UAS-shi<sup>RNAi</sup>* plasmatocytes incubated with rhodamine-conjugated *E. coli* BioParticles for 2 hrs.
Figure 7  p40 localization in plasmatocytes (Top panels) Confocal micrographs of anti-p40 stained uninfected Pxn-GAL4 plasmatocytes. (Middle panels) Confocal micrographs of anti-p40 stained Lh14-infected Pxn-GAL4 plasmatocytes. (Bottom panels) Confocal micrographs of anti-p40 stained Lh14-infected Pxn-GAL4; UAS-shiRNAi plasmatocytes.
Figure S1  Ectopic embryonic expression of UAS-shi^{RNAi} by eyeless-GAL4 results in the formation of aberrant adult eye and cephalic tissue.